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Rho GTPases in Neuronal Apoptosis and Neurodegeneration

Trisha Stankiewicz

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Rho GTPases in Neuronal Apoptosis and Neurodegeneration

A Dissertation

Presented to

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In Partial Fulfillment

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Doctor of Philosophy

by

Trisha R. Stankiewicz

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Advisor: Dr. Daniel A. Linseman
Several studies have identified Rho family GTPases (i.e. Rho, Rac, Cdc42) as mediators of diverse critical cellular processes, such as actin cytoskeleton remodeling, gene transcription, cell-cell adhesion, and cell cycle progression. However, more recent data highlight an essential role for Rho GTPases as regulators of neuronal morphology and neuronal survival. In particular, Rac GTPase generally induces neurite outgrowth and promotes neuronal survival while Rho GTPase typically provokes neurite retraction and induces neuronal apoptosis. However, the precise signaling pathways that regulate neuronal survival downstream of Rho GTPases and the potential involvement of dysregulated activity of Rho GTPases as a causative factor in the progression of neurodegenerative diseases remains to be elucidated.

Consistent with a pro-survival function for Rac in neurons, inhibition of Rac with *Clostridium difficile* toxin B (ToxB) or expression of a dominant negative Rac1 mutant significantly induces activation of the “death executioner” caspase-3 and subsequent apoptosis in primary cerebellar granule neuron (CGN) cultures. We have previously shown that Rac inhibition evokes apoptosis via inactivation of a pro-survival mitogen activated protein kinase kinase (MEK)1/2/ extracellular signal regulated-kinase (ERK)1/2 cascade which functions in healthy CGNs to promote the degradation of the pro-apoptotic Bcl-2 homology-3 domain (BH3)-only protein Bim while also repressing a pro-apoptotic Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling
cascade. Here, we reveal that ToxB induces the JAK-dependent phosphorylation (activation) of STAT5, which is sufficient to induce apoptosis through transcriptional repression of pro-survival Bcl-xL. Collectively, these data implicate Rac GTPase as a critical mediator of CGN survival.

Given that ToxB inhibits Rho, Rac, and Cdc42, we aimed to elucidate the effects of more targeted Rac inhibition in CGNs. NSC23766 specifically inhibits Rac GTPase activation by the Rac-specific GEFs Tiam1 and Trio. Targeted inhibition of Rac GTPase via NSC23766 treatment in CGNs evoked apoptosis via repression of unique MEK5/ERK5, p90Rsk, and Akt-dependent pro-survival pathways. Furthermore, selective inactivation of Rac induced the activation and translocation of the pro-apoptotic BH3-only protein Bad to the mitochondria, where it has been demonstrated to induce apoptosis. Intriguingly, adenoviral expression of a constitutively active MEK5 protected CGNs from apoptosis induced by NSC23766, but not ToxB. These data demonstrate that selective inhibition of Rac GTPase with NSC23766 versus global inhibition of Rho GTPases with ToxB induces cell death via repression of distinct MAP kinase signaling pathways.

We also report that treatment with either ToxB or Clostridium sordelli lethal toxin, which also inhibits Rac GTPase, induces caspase-dependent downregulation of the pro-survival transcriptional corepressor, C-terminal binding proteins (CtBPs), in CGNs. Interestingly, we demonstrate that downregulation of CtBPs undergoing apoptosis does not appear to occur via enhanced degradation or reduced transcription. Instead, our data suggest that caspases indirectly regulate the expression of CtBPs in CGNs undergoing apoptosis in a manner that occurs post-transcriptionally and is dependent on intact
miRNA machinery. Finally, inhibition of CtBPs with 4-methylthio-2-oxobutyric acid (MTOB) provoked CGN apoptosis that was associated with induction of the CtBP target Noxa. These data indicate a novel function for CtBPs in regulating survival of primary CGNs.

Finally, we examined the involvement of Rho GTPases in regulating the survival of motor neurons derived from embryonic stem cells (ESCs) as selective degeneration of motor neurons in the spinal cord and motor cortex underlies the pathology of amyotrophic lateral sclerosis (ALS). Consistent with our previous findings in CGNs, inhibition of Rac GTPase via NSC23766 treatment resulted in apoptotic cell death of ESC-derived motor neurons that was coincident with deactivation of pro-survival ERK5 and Akt signaling pathways. Surprisingly, we report that the BH3-only protein Bad localized exclusively to the nucleus in ESC-derived motor neurons treated with the Rac inhibitor. In agreement with an antagonistic relationship exerted between Rac GTPase and Rho GTPase as a determinant of neuronal survival, we also report that constitutive activation of Rho GTPase via CN03 treatment induced Rho kinase (ROCK)-dependent apoptosis of ESC-derived motor neurons. Next, we investigated Rac and Rho GTPase activity and localization in motor neurons in vivo. In the G93A mutant Cu, Zn superoxide dismutase (mSOD1) transgenic mouse model of ALS, we demonstrate that active Rac1-GTP is reduced within spinal cord motor neurons when compared to age-matched wild type (WT) littermates. Furthermore, we report that total RhoB is expressed in the soma and nucleus of WT motor neurons; however, RhoB shows a marked redistribution to neuronal processes in end-stage mice harboring the G93A SOD1 mutation. These data underscore the importance of Rho family GTPases in maintaining
motor neuronal survival and suggest that *diminished* Rac GTPase activity or *enhanced* Rho GTPase activity may each contribute to selective loss of motor neurons during the progression of ALS.
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CHAPTER ONE: INTRODUCTION

Rho family GTPases (i.e. RhoA, Rac1, Cdc42) are best known for their role in regulating actin cytoskeleton dynamics. In neurons, Rho GTPases have an important and conserved function in guiding dendritic arborization, growth cone development, and axon guidance. In addition, more recent efforts have underscored an important function for Rho GTPases in regulating neuronal survival and death. In the regulation of both neuronal cytoskeletal dynamics and neuronal survival, Rho GTPase and Rac GTPase typically exhibit antagonistic functions. For example, Rho GTPase generally stimulates axonal retraction and neuronal death while Rac GTPase typically promotes axonal growth and neuronal survival. In addition to \textit{in vitro} and \textit{in vivo} animal models of neurodegenerative disease, recent studies in humans implicate that dysregulation of Rho family GTPases may potentially underscore the etiology of some forms of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS). Nonetheless, the precise signaling pathways that are disrupted in neurons exposed to dysregulated Rho family GTPase activity remain incompletely understood. Thus, given the potential role of Rho GTPases in regulating neuronal development and survival, a major focus of this thesis is to elucidate the pro-survival and pro-apoptotic signals transmitted by members of the Rho GTPase family in models of neuronal demise.
1.1 Apoptosis as a mechanism of neuronal death

Although neuronal cell death can occur through many diverse mechanisms, accumulating evidence suggests that neuronal apoptosis appears to be a major contributing factor common to several devastating neurodegenerative diseases and acute neuronal injury. Apoptosis is a conserved process of programmed cell death that is crucial to the proper development of organisms. While inadequate apoptosis within an organism can lead to cancer, excessive apoptosis can underlie the progression of neurodegenerative diseases. In healthy cells, members of the B cell lymphoma (Bcl)-2 family of proteins tightly regulate the two principal pathways that transmit apoptotic signals in neuronal cells, the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway (Fig. 1.2).

1.1.1 Overview of the Bcl-2 family members as regulators of apoptosis

Abundant evidence demonstrates that signals of cell survival and cell death are critically regulated by the Bcl-2 family of proteins (Fig. 1.1). This family is classified into three functionally and structurally distinct categories. The first subfamily includes the pro-survival family members, most notable Bcl-2, Bcl-xL, and Mcl-1. These proteins are critical constituents of the cell, functioning to negatively regulate pro-apoptotic proteins. The second subfamily includes multi-domain pro-apoptotic Bax and Bak which, when activated, act at the mitochondria to induce permeabilization of the outer mitochondrial membrane and release of apoptogenic molecules (e.g. cytochrome C). The final subgroup of the Bcl-2 family comprises the Bcl-2 homology-3 domain (BH3)-only pro-apoptotic proteins. This family includes Bid, Bim, Bad, and Puma, amongst several others. Numerous reports have demonstrated that the balance between pro-survival and
pro-apoptotic Bcl-2 family members represents a vital checkpoint in whether or not a cell commits to apoptosis (Danial and Korsmeyer, 2004; Youle and Strasser, 2008).

1.1.2 Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is aptly named as it requires an extracellular signal to induce apoptosis (Fig. 1.2). This cascade is activated when a death ligand (e.g. Fas ligand) binds to a transmembrane death receptor (e.g. Fas) and leads to receptor oligomerization and recruitment of the Fas-associated death domain (FADD) adaptor protein. The death domain of the adaptor protein recruits pro-caspase-8 and forms the death inducing signaling complex (DISC). DISC formation triggers proteolysis of pro-caspase-8 to its active form, caspase-8, which then cleaves and activates two critical executioners of apoptosis, pro-caspase-3 and pro-caspase-7. An alternate function of caspase-8 is cleavage of the BH3-only protein Bid to an active form termed truncated Bid (tBid). Truncated Bid translocates to the mitochondria and initiates the intrinsic apoptotic pathway by inducing the oligomerization of Bax and Bak, discussed below. Thus, tBid is an important link between the extrinsic and intrinsic apoptotic cascades (Li et al., 1998; Yin, 2006).

1.1.3 Intrinsic apoptotic cascade

Central to the intrinsic apoptotic pathway is the mitochondria whose outer membrane’s permeabilization is a critical determinant in executing the death program (Fig. 1.2). The permeability of the mitochondria is tightly regulated by the balance of pro-survival and pro-apoptotic Bcl-2 family members. Under normal conditions, pro-survival proteins such as Bcl-2 and Bcl-xL function to neutralize the inherent pro-death activity of multi-domain pro-apoptotic family members. Intrinsic apoptosis is provoked
by apoptotic signals that originate within the cell, such as DNA damage or oxidative stress. Following apoptotic stimulation, BH3-only proteins respond by either inactivating pro-survival Bcl-2 members or by directly activating Bax and Bak to form homo- or hetero-oligomeric pores in the outer mitochondrial membrane (Kuwana et al., 2005; Kim et al., 2006). This allows subsequent release of apoptogenic factors such as cytochrome C (cytC) and Smac/Diablo. CytC binds monomeric apoptotic protease-activating factor-1 (APAF-1), inducing a conformational change that permits heptamerization and recruitment of pro-caspase-9 into a large complex known as the apoptosome (Zou et al., 1999). Once activated, pro-caspase-9 activates the death executioners caspase-3 and caspase-7 (Hakem et al., 1998).
Figure 1.1 Members of Bcl-2 family. A. Pro-survival members of the Bcl-2 family. B. Multi-domain, pro-apoptotic members of the Bcl-2 family. C. BH3-only, pro-apoptotic members of the Bcl-2 family.
Figure 1.2 Overview of apoptotic pathways. The extrinsic pathway is activated when a death ligand (e.g. Fas ligand) binds to a transmembrane receptor (e.g. Fas) to induce recruitment of the Fas-associated death domain (FADD) adaptor protein. Pro-caspase-8 is recruited and forms the death inducing signaling complex (DISC), which triggers the activation of caspase-8 and the subsequent activation of pro-caspase-3 and pro-caspase-7. An additional function of caspase-8 is the cleavage of Bid to its active form, truncated Bid (tBid). tBid translocates to the mitochondria and initiates the intrinsic apoptotic pathway by inducing the oligomerization of Bax and Bak. Bak and Bak form homo- or hetero-oligomeric pores in the outer mitochondrial membrane, allowing for the release of apoptogenic factors such as cytochrome C. CytC binds monomeric apoptotic protease-activating factor-1 (APAF-1), inducing a conformation change that permits heptadimerization and recruitment of pro-caspase-9 into a large complex known as the apoptosome. Once activated, pro-caspase-9 activates pro-caspase-3 and pro-caspase-7.
1.1.4 Evidence supporting a role for apoptosis as a contributing factor to the etiology of human neurodegenerative disorders

Although neuronal cell death can occur through many diverse mechanisms, accumulating evidence suggests that neuronal apoptosis appears to be a major contributing factor common to several devastating neurodegenerative diseases. For example, Alzheimer’s disease (AD) is the most common form of dementia and is characterized by extensive loss of neurons in the hippocampus. Although the involvement of apoptosis in the progression of AD remains controversial, increased DNA fragmentation consistent with apoptotic cell death has been reported in AD brain tissue samples when compared to aged control brains (Su et al., 1994; Troncoso et al., 1996). In further agreement with apoptotic cell death, Masliah et al. (1998) reported that AD patient brain samples demonstrated increased immunoreactivity of the death executioner of apoptosis, caspase-3.

In addition to AD, Huntington’s disease (HD) is a fatal autosomal dominant disorder characterized by loss of cells in the striatum and cortex, resulting in involuntary body movements (chorea). Early studies identified that DNA fragmentation consistent with apoptotic cell death may occur in HD striatal sections as Tdt-mediated dUTP–biotin nick end labeling (TUNEL) staining was increased when compared to neurologically normal postmortem brain tissue (Dragunow et al., 1995; Thomas et al., 1995; Butterworth et al., 1998). In addition, mitochondrial dysfunction has been reported in HD brains and studies examining lymphoblasts from HD patients revealed increased stress-induced apoptosis and enhanced activation of caspase-3 (Sawa et al., 1999). Furthermore, both
activation of caspase-1 and caspase-8 was observed in HD patient brain samples when compared to neurologically unaffected controls (Ona et al., 1999; Sánchez et al., 1999).

As a final example, amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder characterized by loss of motor neurons from the motor cortex, brain stem, and upper and lower spinal cord, resulting in loss of motor function and eventually death due to respiratory failure. Increased DNA fragmentation consistent with apoptotic cell death has been reported in the motor cortex, brain stem, cervical cord, thoracic cord, and lumbar cord of ALS patients when compared to neurologically normal control samples (Ekegren et al., 1999; Martin, 1999). Furthermore, an analysis of mRNA expression in spinal motor neurons from postmortem ALS tissue revealed decreased expression of pro-survival Bcl-2 mRNA and enhanced expression of pro-apoptotic Bax mRNA (Mu et al., 1996). In agreement with disruption of the balance of Bcl-2 family members as a causative factor underlying mitochondrial-dependent neuronal apoptosis, Bcl-2 protein expression is diminished and Bax and Bak expression is enhanced in mitochondrial fractions from ALS patients when compared to age-matched controls (Ekegren et al., 1999; Martin, 1999). Collectively, these studies highlight the potential involvement of apoptotic cell death as a common factor underlying the etiology of several diverse neurodegenerative diseases.

1.2 Introduction to C-terminal Binding Proteins (CtBPs)

CtBPs were initially identified as binding partners for the E1A transforming proteins (Schaeper et al., 1995). Although the invertebrate genome encodes one CtBP protein, two CtBPs (CtBP1 and CtBP2) are encoded by the vertebrate genome and perform both unique and duplicative functions (Chinnadurai, 2002). CtBP1 and CtBP2
are closely related and act as transcriptional corepressors when activated by nicotinamide adenine dinucleotide (NADH) binding to their dehydrogenase domains (Kumar et al., 2002). Binding of NADH induces a conformational change within CtBPs that promotes association with DNA-binding transcriptional repressors, such as the E-box repressor ZEB (Postigo and Dean, 1999). Experimental evidence suggests that the majority of DNA-binding transcriptional repressors appear to recruit CtBPs through a PxDLS sequence (Chinnadurai, 2002). More recent studies have demonstrated that CtBPs exert transcriptional repression primarily via recruitment of a corepressor complex to DNA that consists of histone deacetylases and histone methyltransferases (Shi et al., 2003). Nonetheless, CtBPs can also repress transcription through an HDAC-independent mechanism (Meloni et al., 1999; Koipally et al., 2000).

More recent studies have demonstrated a critical function for CtBPs in the transcriptional repression of pro-apoptotic genes. For instance, murine embryonic fibroblasts (MEFs) deficient in CtBP1 and CtBP2 demonstrated enhanced sensitivity to apoptosis and showed increased expression of the pro-apoptotic proteins Bax, Puma, and Noxa (Grootecaes et al., 2003; Bergman and Blaydes, 2006). Furthermore, human colorectal cancer HCT116 cells deficient in CtBP2 demonstrated increased expression of the BH3-only pro-apoptotic protein Bik (Kovi et al., 2010). In addition to a critical role for CtBPs in transcriptional co-repression of pro-apoptotic genes, a recent study by Paliwal et al. (2012) demonstrated that CtBP2 can also bind to the promoter region and enhance the transcriptional activation of Tiam1 to promote a pro-oncogenic function of CtBP2 in cancer cell migration. Nonetheless, while recent efforts have characterized the
involvement of CtBPs in promoting cellular survival in non-neuronal cell types, the involvement of CtBPs in regulation of neuronal survival remains to be elucidated.

1.3 Introduction to Rho Family GTPases

The Rho family of GTPases belongs to the Ras superfamily of GTPases of low molecular weight (~21 kDa) guanine nucleotide binding proteins. Although the Rho GTPase family is further divided into seven subfamilies (Rho, Rac, Cdc42, Rnd, RhoD, RhoBTB, and RhoH), the most extensively studied members are RhoA, Rac1, and Cdc42. In the last few decades, studies have demonstrated that Rho family GTPases are important regulatory molecules that link surface receptors to the organization of the actin cytoskeleton. However, the involvement of Rho GTPases in maintaining other essential cellular functions has also been described, such as gene transcription (Hill et al., 1995), cell cycle progression (Olson et al., 1995), and regulation of cellular survival and death (Heasman and Ridley, 2008; Linseman and Loucks, 2008).

Rho family GTPases are molecular switches that are activated by a variety of cell-surface receptors, including tyrosine kinase and adhesion receptors, cytokine receptors, and G-protein coupled receptors (Kjoller and Hall, 1999; Sah et al., 2000). While the N-terminal domain of Rho GTPases is highly conserved, the C-terminal domain contains more variation and may confer diverse functions for members of the Rho GTPase family (Didsbury et al., 1990). Rho GTPases contain both nucleotide-and Mg$^{2+}$-binding pockets. While nucleotide (GDP or GTP)-binding pockets within Rho GTPases regulate the conformation of the switch regions, Mg$^{2+}$ is important for high-affinity binding of guanine nucleotides (Rossman et al., 2005). In addition, Rho family GTPases also contain a ~200 residue Dbl homology (DH) domain and an adjacent, C-terminal, Pleckstrin
homology (PH) domain. The DH domains of Rho GTPases facilitate the exchange of GDP for GTP while the PH domains bind phosphoinositides to localize Rho GTPases to plasma membranes (Rossman et al., 2005).

Similar to Ras family GTPases, activation of Rho GTPases is regulated by cycling between an inactive GDP-bound state and an active GTP-bound state (Fig. 1.3). The binding of GTP elicits a conformational change within Rho GTPases which promotes their interaction with various downstream effectors. Rho family GTPase activation is facilitated by guanine nucleotide exchange factors (GEFs) that catalyze the conversion of GDP to GTP while inactivation of Rho GTPases is induced by GTPase activating proteins (GAPs) that stimulate the intrinsic ability of Rho GTPases to hydrolyze GTP to GDP. The third class of molecules that regulate Rho GTPase activity, guanine dissociation inhibitors (GDIs), sequester Rho GTPases in a GDP-bound state in the cytosol and the dissociation of GDIs is required for membrane localization and subsequent activation of Rho family GTPases.
Figure 1.3 Activation of Rho family GTPases. Guanine nucleotide exchange factors (GEFs) catalyze the conversion of GDP to GTP to enhance Rho GTPase activation. GTPase activating proteins (GAPs) inactivate Rho GTPases by stimulating the ability of Rho GTPases to hydrolyze GTP to GDP. Finally, guanine dissociation inhibitors (GDI) regulate Rho GTPase activation by sequestering Rho GTPases in a GDP-bound state in the cytosol.
1.4 Functions of Rho GTPases in regulating cytoskeletal dynamics

1.4.1 Functions of Rho family GTPases in regulating the actin cytoskeleton

Classic studies in serum-starved Swiss 3T3 fibroblasts underscored a crucial function for Rho family GTPases in regulating actin cytoskeletal dynamics (Hall, 1998). For example, addition of the Rho GTPase-activator lysophosphatidic acid (LPA) led to the formation of stress fibers and focal adhesion complexes while microinjection of a constitutively active mutant of Rac induced the formation of lamellipodia and the associated adhesion complexes. Finally, microinjection of a GEF for Cdc42 enhanced the formation of filopodia and the associated adhesion complexes (Hall, 1998). More recent efforts have identified the key signaling pathways that link Rho GTPases to modulation of the actin cytoskeleton (Fig. 1.4).

The p21-activated kinase 1-6 (PAK1-6) family of serine/threonine kinases can be activated by both Rac and Cdc42 via the Cdc42/Rac-interactive binding (CRIB) motif. Although six isoforms of PAK have been identified, PAK1-3 are the most extensively characterized and it has been demonstrated that their kinase activity is stimulated upon interaction with Rac or Cdc42 (Wells and Jones, 2010). When the crystal structure of PAK1 was revealed by Lei et al. (2000), it became apparent that an autoinhibitory switch domain within the N-terminus of PAK1 prevents the activation of the C-terminal kinase domain. Binding of Rac or Cdc42 stimulates activation of PAK via an autophosphorylation event which disrupts the interaction between the autoinhibitory domain and the kinase domain (Buchwald et al., 2001). Once activated, PAKs can directly phosphorylate and inhibit the activity of myosin light chain kinase (MLCK), effectively reducing the phosphorylation of the downstream effector myosin II regulatory
chain (MLC) and ultimately leading to the formation of lamellipodia while promoting the disassembly of stress fibers (Sanders et al., 1999; Bokoch, 2003; Watanabe et al., 2007). In addition to regulation of MLCK, PAK activity has also been shown to positively regulate LIM kinase via phosphorylation. Phosphorylation of LIM kinase stimulates its ability to phosphorylate and inhibit the actin capping protein, cofillin (Yang et al., 1998; Edwards and Gill, 1999; Dan et al., 2001). Cofilin functions as a destabilizing factor for actin filaments; therefore its inhibition enhances the actin polymerization that is necessary for the Rac-dependent formation of lamellipodia and membrane ruffles (Yang et al., 1998; Bamburg, 1999). Finally, recent studies suggest that Rac and Cdc42 transmit signals to the actin cytoskeleton via Wiskott-Aldrich-syndrome (WASP) family of proteins. In particular, activated Cdc42 directly binds both WASP and neuronal WASP (NWASP), inducing the activation of actin related protein 2/3 (Arp2/3) to promote the formation of new actin filaments (Machesky and Gould, 1999; Kim et al., 2000). Conversely, Rac indirectly activates Arp2/3 via an interaction with additional members of the WASP family, WAVE1-3 (Eden et al. 2002; Kunda et al., 2003; Schenck et al., 2003). Thus, Rac and Cdc42 signal through many diverse pathways to induce cytoskeletal rearrangements.

Research has identified two major downstream effectors that are involved in Rho GTPase-mediated cytoskeletal rearrangements, Rho Kinase I and II (ROCKI/II) and the Diaphanous formin subfamily (Dia). Experimental evidence has established that Rho/ROCK signaling can directly oppose Rac/PAK signaling to influence cytoskeletal dynamics. For instance, while activation of Rac/PAK reduces the phosphorylation of MLC via inhibition of MLCK, activation of Rho/ROCK leads to the inhibition of myosin
light chain phosphatase (MLCPase), increasing the phosphorylation and activation of MLC to induce the subsequent formation of actin stress fibers (Kimura et al., 1996). Nonetheless, Rho/ROCK signaling can also act in a manner parallel to Rac to induce cytoskeletal rearrangements. For example, Rho-dependent activation of ROCK can increase the phosphorylation and activation of LIMK, ultimately resulting in inhibition of the downstream effector cofilin to promote actin polymerization (Maekawa et al., 1999; Ohashi et al., 2000). In addition to ROCK activation, Rho GTPase can also disrupt an autoinhibitory interaction between the N- and C-terminal domain of Dia proteins, stimulating their regulation of actin and microtubule cytoskeleton dynamics (Alberts, 2001). Thus, Rho GTPase regulates the actin cytoskeleton through activation of ROCK or Dia.

The antagonistic relationship established between Rac/Pak and Rho/ROCK signaling underscores the importance of maintaining a delicate balance between Rho family GTPase signaling in order to properly regulate actin cytoskeletal dynamics. Furthermore, the potential functions and importance of Rho GTPases in maintaining neuronal morphology are underscored by their crucial regulation of actin cytoskeletal dynamics.
Figure 1.4 Rho GTPase signaling pathways regulating actin cytoskeletal dynamics. Rho activates its downstream effector ROCK to increase MLC and coflin phosphorylation and induce the subsequent formation of actin stress fibers. Rac signals to PAK to decrease MLC phosphorylation, leading to the generation of lamellipodia. Rac and Cdc42 both activate Arp2/3 to induce actin polymerization and the formation of filopodia.
1.4.2 Functions of Rho family GTPases in regulating of neuronal morphology

In accordance with a conserved function in regulation of actin cytoskeleton dynamics, Rho family GTPases have been highlighted as key modulators of neuronal morphology. Similar to regulation of the actin cytoskeleton, Rho and Rac/Cdc42 exhibit an antagonistic relationship to determine neuronal morphology (Fig. 1.5). Indeed, an early study by Kozma et al. (1997) demonstrated that microinjection of Rac or Cdc42 in N1E-115 neuroblastoma cells enhanced growth cone development and neurite outgrowth, whereas the Rho inhibitory cytotoxin Clostridium botulinum C3 coenzyme abolished Rho-dependent growth cone collapse and neurite retraction. In the same cell line, axonal outgrowth induced by the guidance molecule netrin-1 required both Rac and Cdc42, while downregulation of either Rho or ROCK was sufficient to induce axonal outgrowth (Li et al., 2002). Further, both neurite retraction and F-actin disassembly induced by LPA in N1E-115 neuroblastoma cells were mimicked by constitutively activated RhoA and blocked by a dominant negative mutant of RhoA (Kranenburg et al., 1997). Similarly, in PC12 cells, constitutive activation of Rho or microinjection of the downstream effector of Rho, Rho kinase (ROCK), induced neurite retraction while a kinase dead mutant of ROCK was unable to stimulate neurite retraction (Katoh et al., 1998a,b; Sebök et al., 1999). Further illustrating the antagonistic relationship between Rac and Rho in determining neuronal morphology, PC12 cells exposed to nerve growth factor (NGF)-induced neurite outgrowth in a Rac-dependent manner that was inhibited by activation of either RhoA or ROCK (Yamaguchi et al., 2001). Collectively, these studies highlight that the intricate balance between Rho family GTPases is a critical determinant of neuronal morphology.
A major downstream target of Rac and Cdc42, PAK, has been shown to mediate growth cone development and neurite outgrowth (Fig. 1.5). For instance, membrane targeting of PAK1 has been demonstrated to induce neurite outgrowth of PC12 cells while expression of dominant negative PAK1 construct inhibits this effect (Daniels et al., 1998). Further highlighting the antagonistic signaling characteristic of Rac and Rho GTPases, PAK5-induced neurite outgrowth in PC12 cells occurred in a kinase-dependent mechanism and was abolished by expression of constitutively active RhoA (Dan et al., 2002). Intriguingly, early evidence provided in cortical neurons suggests that Rac may regulate the length of PAK activation as Rac-dependent hyperphosphorylation of PAK1 was shown to decrease its kinase activity (Nikolic et al., 1998). In addition to neurite outgrowth, Rac signaling to PAK has also been implicated in axonal guidance and pathfinding. For example, Rac and PAK have been demonstrated to interact with the SH2/SH3 adaptor protein DOCK to control the direction of axon extension in Drosophila photoreceptors and olfactory neurons (Hing et al., 1999; Newsome et al., 2000; Ang et al., 2003).

In opposition to Rac/Cdc42 signaling to PAK, Rho kinase (ROCK) has been identified as a major downstream target of Rho-dependent neurite retraction (Fig. 1.5). In retinal ganglion cell cultures, expression of the repulsive guidance molecule ephrin-A5 induced collapse in a mechanism consistent with activation ROCK as inhibition utilizing the putative ROCK inhibitor, Y-27632, diminished the collapse of growth cones (Wahl et al., 2000). In N1E-115 neuroblastoma cells, expression of a constitutively active mutant of ROCK was sufficient to induce neurite retraction whereas expression of a dominant negative ROCK mutant attenuated LPA-induced neurite retraction concurrent with
enhanced phosphorylation of MLC and remodeling of intermediate filaments and microtubules (Amano et al., 1998, Hirose et al., 1998). Interestingly, while activation of ROCK alone is sufficient to induce neurite retraction and cell rounding in N1E-115 cells, this effect is blocked by coexpression of dominant negative Rac or Cdc42, suggesting that ROCK-dependent neurite retraction may be mediated, in part, through deactivation of Rac and/or Cdc42 activity (Hirose et al., 1998).

Many studies in neurons support the general model that Rac/Cdc42 elicit neurite outgrowth and growth cone development while Rho induces neurite retraction and growth cone collapse. However, it is important to note that Rho family GTPases can have opposing effects on neuronal morphology. For example, expression of a dominant negative mutant of Rac1 has been demonstrated to promote neurite outgrowth in dorsal root ganglion (DRG) cells (Fournier et al., 2003) and constitutively active Rac1 increases the proportion of collapsed growth cones in DRG neurons (Jin and Strittmatter, 1997; Vastrik et al., 1999). Similarly, constitutive activation of Rac1 was shown to decrease the length of the longest neurite in mouse cortical neurons (Kubo et al., 2002). Furthermore, in some studies, Rho GTPase has been demonstrated to induce neurite formation. Indeed, expression of dominant negative RhoA inhibited axon growth in hippocampal neurons (Ahnert-Hilger et al, 2004). While these discrepancies in Rho family GTPase functions may reflect differences in either organism specific- or cell type-specific regulation of neuronal morphology, they also further underscore the importance of tight regulation of Rho GTPase activation in order for proper nervous system development.
1.5 Rho family GTPases in regulation of neuronal survival and apoptosis

The essential functions of Rho family GTPases in regulating neuronal growth cone formation, neurite outgrowth, and neuronal development suggest that Rho GTPases have an important and conserved function in mediating neuronal survival and death. Indeed, similar to the opposing functions exerted between members of the Rho GTPase family in maintenance of neurite outgrowth and growth cone formation, Rac activation typically promotes neuronal survival while Rho activation elicits neuronal death. In further support of an antagonistic relationship, recent efforts have identified a key signaling network that maintains neuronal survival downstream of Rho family GTPases, particularly between Rac GTPase and Rho GTPase (Fig. 1.5).

1.5.1 Principal Rac-dependent pro-survival signaling pathways

In accordance with a conserved function in regulating neurite outgrowth and growth cone formation, previous studies have demonstrated an important role for Rac GTPase in transmitting pro-survival signals in neurons (Fig. 1.5). Rac GTPase signals to the downstream effector PAK1 to promote survival of cerebellar granule neurons (CGNs) (Johnson and D’Mello, 2005) and inhibition of Rac via the large cytotoxin *Clostridium difficile* Toxin B induces CGN apoptosis via deactivation of PAK (Le et al., 2005; Loucks et al., 2006). In neurons and other cells, PAK is known to promote cellular survival through activation of mitogen activated protein kinase (MAPK) pathways. In CGNs in particular, PAK activates a pro-survival MEK1/2/ERK1/2 signaling cascade which functions in healthy CGNs to enhance degradation of the pro-apoptotic BH3-only protein Bim, as well as, repress a pro-apoptotic JAK/STAT signaling cascade (Loucks et al., 2006). In addition to transmission of pro-survival signals, PAK can also promote
cellular survival through phosphorylation of the BH3-only protein Bad, which enhances the association of Bad with cytosolic 14-3-3 scaffolding proteins, effectively inactivating the BH3-only protein by targeting it away from the mitochondria where it functions to induce apoptosis (Zha et al., 1996; Schürmann et al., 2000). In addition to PAK, Rac often exerts pro-survival signaling through activation of the downstream effector phosphatidylinositol 3-kinase (PI3K). Once activated, PI3K promotes cellular survival by activating Akt or p90rsk to enhance the phosphorylation of Bad (Datta et al., 1997; del Peso et al., 1997; Tan et al., 1999).

1.5.2 Principal Rho-dependent pro-apoptotic signaling pathway

In further support of the antagonistic relationship between members of the Rho GTPase family, Rho GTPase signaling characteristically antagonizes Rac-dependent pro-survival signaling pathways (Fig. 1.5). The major downstream effector of Rho, ROCK, activates phosphatase and tension homolog (PTEN) which can indirectly antagonize the activation of Akt by Rac. Indeed, siRNA against PTEN diminished the ability of constitutively active RhoA to reduce the phosphorylation (activation) of Akt in human embryonic kidney cells (Li et al., 2005). Consistent with a pro-survival function for Akt in phosphorylating the pro-apoptotic protein Bad, inhibition of ROCK prevented the dephosphorylation (activation) of Bad in the penumbra area of rats subjected to ischemia (Wu et al., 2012). The inhibition of Akt by the Rho/ROCK/PTEN pathway occurs indirectly as PTEN dephosphorylates phosphatidylinositol (3, 4, 5)-triphosphate into phosphatidylinositol (4, 5)-biphosphate to terminate Akt activation (Lai et al., 2014). These previous studies have served to highlight that balanced signaling between Rho GTPase and Rac GTPase is a critical determinant of neuronal survival.
Figure 1.5 Rho GTPase signaling pathways regulating neuronal survival. Rho activates a downstream ROCK/PTEN pathway that indirectly inactivates the pro-survival protein Akt to suppress neurite outgrowth and neuronal survival. Rac signals to PI3K to activate Akt to enhance neurite outgrowth and neuronal survival. Rac also activates PAK/MEK/ERK signaling to promote neuronal survival.
1.6 Evidence of dysregulated Rho family GTPase activity underlying the selective motor neuron degeneration observed in ALS

ALS is a devastating neurodegenerative disorder characterized by loss of motor neurons of the motor cortex, brain stem, and upper and lower spinal cord. Patients that receive a diagnosis of ALS typically succumb to the disorder within 2-5 years due to respiratory failure. Approximately 10% of ALS cases appear to be caused by a genetic component, while the remaining 90% are considered sporadic in nature. In 1993, the glycine to alanine substitution at position 93 in Cu, Zn-superoxide dismutase (SOD1) was one of eleven mutations initially identified to be causative in familial ALS. Although the G93A SOD1 mutation remains the best characterized to date, mutations in additional genes such as Alsin (ALS2), TDP43, and FUS have also been linked to other familial forms of ALS. Currently, the only drug that is approved for the treatment of ALS, Riluzole, is an anti-glutamateric compound that extends the lifespan of patients a mere 3 months (Miller et al., 2007). Thus, it is imperative that new molecular targets are identified for the treatment of ALS. Recent evidence suggests that a loss of Rac GTPase activity or gain of Rho GTPase activity may underlie the disease progression in ALS.

1.6.1 Loss of Rac and ALS

Recessive mutations in a Rac guanine nucleotide exchange factor (GEF), alsin (ALS2), have been reported in juvenile forms of ALS, primary lateral sclerosis, and infantile-onset ascending hereditary spastic paralysis (Hadano et al., 2007). When overexpressed in cultured human cells, alsin mutants are rapidly degraded, suggesting disease progression occurs through a mechanism consistent with alsin loss-of-function (Yamanaka et al., 2003). Although alsin is a GEF for both Rab5 and Rac, alsin
colocalizes with Rac in the growth cones of neurons and regulates Rac signaling and neurite outgrowth through its intrinsic GEF activity, thus suggesting a link between Rac disruption and neurodegenerative disease (Topp et al., 2004; Kanekura et al., 2005; Tudor et al., 2005). In addition to juvenile onset ALS, a recent study identified a mutation in the PH domain of alsin that underlies the development of infantile-onset ascending hereditary spastic paraplegia (Wakil et al., 2014). Given the specificity of the DH/PH tandem repeat for activation of Rac GTPase, but not Rab5, (Zheng, 2001; Topp et al., 2004; Tudor et al., 2005), this study further highlights that a loss of Rac activity may underlie the progression of early onset forms of ALS.

The critical role of Rac in maintaining neuronal survival is evidenced by the fact that disruptions in Rac signaling have been shown to induce apoptosis in a variety of diverse neuronal cell types, including primary motor neurons (Kanekura et al., 2005; Jacquier et al., 2006; Loucks et al., 2006; Stankiewicz et al., 2012). In spinal motor neurons isolated from embryonic rats, siRNA knockdown of alsin induces neurite retraction and cell death that is abrogated by expression of constitutively active Rac (Jacquier et al., 2006). Furthermore, alsin antagonizes NSC34 motor neuronal death induced by the expression of ALS-causing SOD1 mutants (mSOD1) via activation of a pro-survival Rac/PI3K/Akt signaling cascade (Kanekura et al., 2005). Intriguingly, accumulating evidence suggests that SOD1 may regulate the activity of Rac GTPase. In particular, neuronal apoptosis induced via expression of either G93A or H80R mSOD1 in SH-SY5Y dopaminergic cells correlated with a decrease in Rac GTPase activity. Indeed, expression of a constitutively active Rac GTPase mutant abrogated mSOD1-induced
neuronal death while expression of dominant negative Rac GTPase alone induced apoptosis (Pesaresi et al., 2011).

In addition to mutations that code for alsin and SOD1, recent evidence also suggests that ARHGEF16, a GEF for Rac GTPase, is hypermethylated and downregulated in postmortem sporadic ALS spinal cord samples when compared to samples from neurologically normal controls (Figueroa-Romero et al., 2012). Thus, dysregulation of Rac GTPase may be a common factor underlying both familial and sporadic forms of ALS. While accumulating evidence has highlighted a critical function for Rac GTPase in maintaining neuronal survival, the involvement of Rac in the etiology of ALS has only been examined recently and the exact mechanism by which disruptions in Rac activity may induce neuronal apoptosis remains poorly understood. Alterations in Rac activity in motor neurons and mouse models of ALS warrants further investigation as it has significant potential to elucidate insights into neurodegenerative disease and delineate novel therapeutic targets.

1.6.2 Gain of Rho GTPase activity in ALS

Although recent efforts are only beginning to elucidate a role for enhanced Rho GTPase activation in the disease progression of ALS, hyperactivation of Rho GTPase has been underscored as a contributing factor in many other models of neurodegenerative disease and neuronal trauma. For example, while total RhoA expression was unaltered following lateral fluid percussion brain injury in rats, RhoA activity was increased in both the cortex and hippocampus ipsilateral and contralateral to the injury (Dubreuil et al., 2006). Furthermore, in an in vitro model of Phenylketonuria brain injury, phenylalanine induced mitochondrial-dependent apoptosis of cortical neurons occurred via a mechanism
consistent with activation of RhoA (Zhang et al., 2010). A recent study has also suggested a role for RhoA in calcium-dependent excitotoxic cell death in rat neurons (Semenova et al., 2007). In addition to RhoA, RhoB has also been implicated in neuronal apoptosis following ischemia as increases in RhoB expression occur following ischemic injury and precede DNA fragmentation indicative of apoptosis (Trapp et al., 2001). Moreover, corticohippocampal neurons deficient in RhoB are resistant to staurosporine-induced apoptosis and RhoB knockout mice display diminished basal levels of caspase-3 activity in the adult brain (Barberan et al., 2011). Collectively, these data implicate that aberrant activation of RhoA and/or RhoB may contribute to neuronal apoptosis in a variety of in vitro and in vivo models of neurodegenerative demise.

In addition to the above studies demonstrating the involvement of Rho GTPase in various models of neurodegeneration, recent efforts have begun to highlight that aberrant Rho GTPase activity may also underlie the pathology of ALS. For example, although the significance remains unknown, the Rho guanine nucleotide exchange factor (RGNEF) displays enhanced cytoplasmic inclusions in spinal motor neurons from both sporadic and familial ALS patients (Keller et al., 2012; Droppelmann et al., 2013). However, future studies will be required to identify the importance of RGNEF-positive spinal motor neuron cytoplasmic inclusions. Nonetheless, in accordance with enhanced Rho GTPase activity as a causative factor to motor neuronal death in ALS disease progression, ROCK activity is increased in the G93A mSOD1 mouse model of ALS, as well as, sporadic ALS patients (Hu et al., 2003; Capitanio et al., 2012; Conti et al., 2014). While the above studies indicate that a gain of Rho GTPase activity may underlie the progression of ALS,
relatively few studies have examined the precise mechanism by which unopposed activation of Rho GTPase may contribute to the motor neuronal death observed in ALS.

1.7 Models to study neuronal apoptosis

1.7.1 Cerebellar granule neurons

The relatively simple process of isolating rat cerebellar granule neurons has made their use in studies of neuronal apoptosis widespread. These primary neuronal cultures are extremely homogeneous and have been used extensively to examine the molecular mechanisms involved in neuronal apoptosis. Early studies conducted in CGNs exposed to low potassium determined that CGNs undergo apoptotic cell death as evidenced by DNA fragmentation (D’Mello et al., 1993). Further, it was previously demonstrated that CGNs subjected to low potassium conditions undergo extrinsic apoptosis as evidenced by increased expression of Fas ligand and caspase-3 (Ginham et al., 2001; Fig. 1.6). In addition, CGNs also appear to be sensitive to intrinsic (mitochondrial) apoptosis as CGNs derived from mice overexpressing the pro-apoptotic protein Bax or deficient in the pro-survival protein Bcl-2 displayed enhanced sensitivity to apoptotic cell death when compared to CGNs isolated from wild type mice (Tanabe et al., 1997). In addition to these prior studies demonstrating that CGNs undergo both intrinsic and extrinsic apoptosis, we have shown that inhibition of Rac GTPase utilizing large Clostridial cytotoxins or expression of a dominant negative Rac1 mutant induces mitochondrial-dependent apoptosis of CGNs. Cell death occurred in a mechanism consistent with DNA fragmentation and activation of the death executioner of apoptosis, caspase-3 (Linseman et al., 2001; Le et al., 2005; Loucks et al., 2006). Thus, primary CGN cultures provide an
excellent *in vitro* model that can be used to study the Rac-dependent signaling pathways that regulate neuronal survival.
Figure 1.6 CGNs undergo caspase-dependent apoptosis. CGNs were incubated in either control medium or 25k- medium deprived of potassium for 24 h. Following incubation, CGNs were fixed with 4% paraformaldehyde and stained for DAPI (blue), β-tubulin (green), and active caspase-3 (red). Note that CGNs deprived of trophic support display condensed/fragmented nuclei and enhanced activation of caspase-3 indicative of apoptosis.
1.7.2 Embryonic Stem Cell-Derived Motor Neurons

Traditionally, ALS has been a difficult disease to model in vitro due to the fact that it is characterized by the loss of upper and lower spinal motor neurons. Motor neurons isolated from embryonic mice and rats do not yield high numbers, making it difficult to perform experiments that may require high cellular content. Furthermore, it is unknown if motor neurons isolated from embryonic rodents are mature enough to mimic those that are lost in aging-related disorders such as ALS. To overcome this issue, Wichterle and colleagues (2002) developed a protocol to terminally differentiate mouse ESCs into motor neurons which express GFP under control of the motor neuron-specific HB9 homeobox promoter. Following a one week differentiation protocol, approximately 30% of the cultured cells are identified as GFP-expressing motor neurons (Fig. 1.7). ESC-derived motor neurons can populate the embryonic spinal cord, project axons, and form synapses with muscle cells (Wichterle et al., 2002). Thus, ESC-derived motor neurons represent a novel in vitro model that can be utilized to study the involvement of Rho family GTPases in regulating the survival of motor neurons.
Figure 1.7 Generation and differentiation of ESC-derived motor neurons. ESCs harboring an enhanced GFP (eGFP) transgene driven by the motor neuron-specific Hb9 promoter were cultured on a layer of mouse embryonic fibroblasts. After detachment with trypsin, embryoid bodies (EBs) were cultured in suspension in differentiation medium containing retinoic acid to induce the formation of motor neuron progenitors. After 24 h, sonic hedgehog and glial cell line-derived neurotrophic factor were added to further promote the differentiation of neural precursor cells into spinal motor neurons. After a 48 h incubation, EBs were collected and digested with papain to produce a single cell suspension. Cells were then plated onto matrigel-coated culture dishes (top panel). After 24 h on matrigel, EGFP+ motor neurons appear healthy and extend long processes as well as multiple shorter neurites (bottom panel). Consistent with Wichterle et al. (2002), approximately 30% of cells terminally differentiated into spinal motor neurons.
CHAPTER TWO: SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT)-5 MEDIATES NEURONAL APOPTOSIS INDUCED BY INHIBITION OF RAC GTPASE ACTIVITY

2.1 Abstract

In several neuronal cell types, the small GTPase Rac is essential for survival. We have shown previously that the Rho family GTPase inhibitor *Clostridium difficile* toxin B (ToxB) induces apoptosis in primary rat cerebellar granule neurons (CGNs) principally via inhibition of Rac GTPase function. In the present study, incubation with ToxB activated a proapoptotic Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, and a pan-JAK inhibitor protected CGNs from Rac inhibition. STAT1 expression was induced by ToxB; however, CGNs from STAT1 knock-out mice succumbed to ToxB-induced apoptosis as readily as wild-type CGNs. STAT3 displayed enhanced tyrosine phosphorylation following treatment with ToxB, and a reputed inhibitor of STAT3, cucurbitacin (JSI-124), reduced CGN apoptosis. Unexpectedly, JSI-124 failed to block STAT3 phosphorylation, and CGNs were not protected from ToxB by other known STAT3 inhibitors. In contrast, STAT5A tyrosine phosphorylation induced by ToxB was suppressed by JSI-124. In addition, roscovitine similarly inhibited STAT5A phosphorylation and protected CGNs from ToxB-induced apoptosis. Consistent with these results, adenoviral infection with a dominant negative STAT5 mutant, but not wild-type STAT5, significantly decreased ToxB-induced apoptosis of CGNs. Finally, chromatin immunoprecipitation with a STAT5 antibody
revealed increased STAT5 binding to the promoter region of prosurvival Bcl-xL. STAT5 was recruited to the Bcl-xL promoter region in a ToxB-dependent manner, and this DNA binding preceded Bcl-xL down-regulation, suggesting transcriptional repression. These data indicate that a novel JAK/STAT5 proapoptotic pathway significantly contributes to neuronal apoptosis induced by the inhibition of Rac GTPase.

### 2.2 Introduction

Rho family GTPases are important mediators of cellular development, survival, and death. The most well characterized members of the family are RhoA, Rac1, and Cdc42. Although best known for regulating actin cytoskeletal dynamics, Rho GTPases also play important roles in cell cycle progression (Olson et al., 1995), gene transcription (Hill et al., 1995), and cell-cell or cell-matrix adhesion (Clark et al., 1998; Fukata et al., 1999). In recent years, the role of Rho GTPases in neuronal survival has begun to be investigated. For example, inhibitors of 3-HMG-CoA reductase (statins) decrease the localization of Rho GTPases to the plasma membrane and induce apoptosis in rat cortical neurons (Tanaka et al., 2000). We have shown previously that the function of Rac is essential for the survival of cerebellar granule neurons (CGNs) as the inhibition of Rac with either large Clostridial cytotoxins or overexpression of a dominant negative Rac mutant induces mitochondrially dependent apoptosis of these cells (Linseman et al., 2001). In a similar manner, use of either dominant negative Rac or siRNA against the Rac guanine nucleotide exchange factor alsin (ALS2) results in apoptosis of primary cultured spinal motor neurons (Jacquier et al., 2006). The critical role of Rac in neuronal survival is further evidenced by the finding that ALS2 is mutated in juvenile onset amyotrophic lateral sclerosis. Although changes in Rac activity in patients harboring
disease-causing ALS2 mutations have not been directly evaluated, disruption of Rac function as a possible underlying cause of neurodegenerative disease is suggested by the fact that alsin mediates Rac-dependent prosurvival signaling in primary motor neurons (Jacquier et al., 2006). Collectively, these findings implicate Rac as a crucial mediator of neuronal survival and suggest that disruption of Rac activity may contribute to the progression of neurodegenerative disorders.

We have reported previously that inhibition of Rho GTPases with *Clostridium difficile* toxin B (ToxB) and in particular inhibition of Rac lead to the derepression of an as yet undefined proapoptotic JAK/STAT pathway (Loucks et al., 2006). The JAK/STAT pathway has been shown to play a critical role in cytokine signaling, and JAK activation can turn on an array of downstream effects including cell proliferation, differentiation, and apoptosis (Igaz et al., 2001). An important feature of the JAK/STAT signaling cascade is that it can exert either a prosurvival or proapoptotic effect depending upon the stimulus and cell type. For example, cytoprotective signals are transmitted from the gp130 receptor to a prosurvival JAK/STAT3 pathway in cardiac myocytes (Yamauchi-Takahara and Kishimoto, 2000). Moreover, data implicate constitutive activation of STAT1 and STAT3 proteins in breast cancer cells (Watson and Miller, 1995). Conversely, more recent data have emerged to suggest that the JAK/STAT pathway may also induce apoptosis under certain cellular conditions. For instance, STAT1 has been shown to mediate IFN-γ-induced apoptosis in liver cells treated with the hepatotoxic compound galactosamine (Kim et al., 2003). In addition, chromatin immunoprecipitation experiments performed in thymocytes suggest that glucocorticoids induce apoptosis through repression of prosurvival Bcl-xL in a STAT5-dependent manner (Rocha-Viegas
et al., 2006). Although it is clear that JAK/STAT activation can induce apoptosis in diverse non-neuronal cell types, the significant involvement of this signaling pathway in neuronal apoptosis has only recently been recognized.

In a previous study, we showed that inhibition of Rac induces CGN apoptosis by inactivating a prosurvival p21-activated kinase PAK/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade. Although we have demonstrated that disruption of this pathway results in the derepression of a proapoptotic JAK/STAT pathway, we have yet to identify which particular STAT family members mediate neuronal apoptosis in response to ToxB (Loucks et al., 2006). Thus, the current study focuses on identifying the STAT family members involved and the consequences of STAT activation downstream of Rac inhibition in CGNs. These primary neuronal cultures are extremely homogeneous and have been used extensively to examine molecular mechanisms involved in neuronal apoptosis (Linseman et al., 2001; D’Mello et al., 1993; Contestabile, 2002; Vaudry et al., 2003). Although we show that Rac inhibition leads to the up-regulation of STAT1 expression and enhanced tyrosine phosphorylation of STAT3, we report that these transcription factors are not responsible for inducing apoptosis in ToxB-treated CGNs. Instead, we demonstrate that STAT5 is activated and subsequently translocates into the nucleus to transcriptionally repress prosurvival Bcl-xL in Rac-inhibited CGNs. To our knowledge, these results are the first to identify a proapoptotic function for STAT5 in primary neurons.
2.3 Materials and Methods

2.3.1 Reagents

*C. difficile* toxin B was isolated or prepared as a recombinant protein as described previously (Just et al., 1995). The polyclonal antibodies used for immunoblotting STAT1, STAT3, and phosphorylated STAT5 (pSTAT5) were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-linked secondary antibodies and reagents for enhanced chemiluminescence detection were from Amersham Biosciences. The polyclonal antibody used to detect active caspase-3 by immunocytochemistry was from Promega (Madison, WI). For Western blotting, active caspase-3 was detected with a polyclonal antibody from Abcam (Cambridge, MA). 4, 6-Diamidino-2-phenylindole (DAPI), Hoechst dye 33258, and a monoclonal antibody against β-tubulin were from Sigma. Anti-rat and anti-mouse Cy3- or FITC-conjugated secondary antibodies for immunofluorescence were from Jackson ImmunoResearch Laboratories (West Grove, PA). The monoclonal antibody against LAP-2 and the polyclonal total STAT1 and total STAT5 antibodies used for Western blotting were from BD Biosciences. Purvalanol A, JSI-124, roscovitine, mifepristone, JAK3 inhibitor, and the small molecule JAK inhibitor I (2-(1, 1-dimethyl) 9-fluro-3, 6-dihydro-7H-benz[h]imidaz [4, 5-f] isoquinolin-7-one; pan-JAK inhibitor (pan-JI)) were from Calbiochem. The specific JAK1/2 inhibitor ruxolitinib was purchased from ChemieTek (Indianapolis, IN), and the JAK3 inhibitor tofacitinib was from Selleck Chemicals (Houston, TX).

2.3.2 CGN culture

Rat CGNs were isolated and cultured from 7-day-old Sprague-Dawley rat pups of both sexes (15–19 g) as described previously (Linseman et al., 2001). Briefly, CGNs
were plated on 35-mm-diameter plastic dishes coated with poly-l-lysine at a density of 2.0 \times 10^6 cells/ml in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mm KCl, 2 mm l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cytosine arabinoside (10 µm) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. With this protocol, cultures were ~95% pure for granule neurons. In general, experiments were performed after 6–7 days in culture.

2.3.3 CGN culture from STAT1 knock-out (KO) mice

STAT1 KO mice and their wild-type littermates were obtained commercially from Taconic (Hudson, NY). CGNs from these mice were isolated and cultured essentially as described above.

2.3.4 Cell lysis and immunoblotting

After treatment as described under “Results,” CGN whole cell lysates or immune complexes of STAT5A or STAT5B were prepared for Western blotting essentially as described previously (Loucks et al., 2006). Protein concentrations were determined by a commercially available protein assay kit (BCA, Thermo Scientific), and SDS-polyacrylamide gel electrophoresis was performed using equal amounts of protein followed by transfer to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Nonspecific binding sites were blocked in phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% Tween 20 (PBS-T), 1% bovine serum albumin (BSA), and 0.01% sodium azide for 1 h at room temperature (25 °C). Membranes were incubated for 1 h in primary antibody diluted in blocking solution. Excess primary antibody was removed by washing the membranes with PBS-T five times over 25 min. The membranes
were then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T. Excess secondary antibody was removed by washing the membranes with PBS-T five times over 25 min. Immunoreactive proteins were detected by enhanced chemiluminescence. Blots shown are representative of a minimum of three independent experiments.

2.3.5 Quantification of apoptosis

After induction of apoptosis, CGNs were fixed in 4% paraformaldehyde for 30 min, and nuclei were stained with Hoechst dye (8 µg/ml final concentration) for 30 min. CGNs containing condensed and/or fragmented nuclei were scored as apoptotic. Typically, 800 cells were quantified from each 35-mm well by randomly counting five 40× fields. Final counts represent data obtained from at least three independent experiments performed in duplicate.

2.3.6 Immunocytochemistry

CGNs were plated at a density of 2.0 × 10⁶ cells/ml in 35-mm wells. After ToxB treatment, CGNs were fixed in 4% paraformaldehyde, washed once in PBS, and then permeabilized and blocked in PBS containing 0.2% Triton X-100 and 5% BSA. Primary antibodies were diluted in 2% BSA and 0.2% Triton X-100 in PBS, and cells were incubated in primary antibody overnight at 4 °C. Cells were subsequently washed five times in PBS and then incubated for 1 h with DAPI and either Cy3- or FITC-conjugated secondary antibody diluted in 2% BSA and 0.2% Triton X-100. The cells were washed five additional times with PBS before the addition of an antiquench solution composed of 0.1% p-phenylenediamine in 75% glycerol in PBS. Fluorescent images were captured using a 40× water oil immersion objective on a Zeiss Axioplan 2 microscope with a
Cooke Sensicam deep cooled charge-coupled device camera and a Slidebook software analysis program for digital deconvolution (Intelligent Imaging Innovations Inc., Denver, CO).

2.3.7 Preparation of nuclear and cytosolic extracts from CGNs

Nuclear and cytosolic extracts were prepared as described by Li et al. (2001). Briefly, CGNs were detached from culture dishes by a cell scraper and centrifuged at 250 × g for 5 min. The cell pellets were washed and homogenized with 15 strokes of a tightly fitting Dounce homogenizer to release nuclei. Next, the homogenate was centrifuged at 14,000 × g for 15 s to pellet the nuclei. The supernatants (cytosolic fractions) were removed, the pellets were resuspended in a HEPES/glycerol buffer, and nuclear proteins were extracted at 4 °C for 45 min. Insoluble nuclei were precipitated by centrifugation at 14,000 × g for 15 min, and the supernatants were dialyzed against a Tris/glycerol buffer for 3 h at 4 °C.

2.3.8 Adenovirus preparation and infection

Wild-type STAT5 and dominant negative STAT5 adenoviral constructs were prepared as described previously (Ahonen et al., 2003). CGNs were infected in vitro on day 6 with adenovirus carrying GFP, wild-type STAT5, or dominant negative STAT5 at a multiplicity of infection of 100. At 48 h of infection, cells were treated with 40 ng/ml ToxB. At 72 h of infection, cell lysates were prepared for immunoblot analysis, or cells were fixed for immunocytochemistry as described above.

2.3.9 Chromatin immunoprecipitation (ChIP)

ChIP assays were performed according to the manufacturer’s protocol using the ChIP assay kit from Active Motif (Carlsbad, CA). CGNs were treated with 40 ng/ml
ToxB for 0 and 8 h. Next, DNA-associated proteins were cross-linked with formaldehyde. Cross-linked chromatin was extracted, sheared enzymatically, and incubated with a ChIP grade STAT5 antibody overnight at 4 °C and protein G-agarose beads. After washing, immune complexes were eluted from the beads, heated to reverse the cross-links, and treated with proteinase K and RNase A to remove proteins and any contaminating RNA. DNA was analyzed by polymerase chain reaction (PCR) using the following primers provided by SABiosciences that generate a 114-bp product that corresponds to the promoter region of the rat Bcl-xL gene: forward primer, 5′-GAAGCTGACACCAGTGAGTGTCCGAACGGTAAATGCCTACGAAGCTGACACCAGTGAGTG-3′; and reverse primer, 5′-GTAGGCATTTACCGTTCGGA-3′. These primers were selected because they amplify a sequence of DNA that is near two predicted STAT5 binding sites on the promoter region of Bcl-xL variant 1 and variant 2. As a negative control, ChIP reactions were performed as described in the absence of STAT5 antibody followed by PCR. PCR was performed using the following conditions: one cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

2.3.10 Data analysis

Results represent the mean ± S.E. for the number (n) of independent experiments performed. Statistical differences between the means of unpaired sets of data were evaluated by one-way analysis of variance with a post hoc Tukey's test. A p value of <0.05 was considered statistically significant. Images and immunoblots are representative of at least three independent experiments. For the cycle threshold values reported in Fig. 2.10A, results represent the mean for four independent experiments, and statistical differences were evaluated by a Student's t test.
2.4 Results

2.4.1 STAT1 is up-regulated in JAK-dependent manner in CGNs treated with Rho family GTPase inhibitor ToxB

To evaluate the involvement of Rac in CGN survival, cultures were incubated with *C. difficile* toxin B. This cytotoxin monoglucosylates a key threonine residue in the switch 1 region of Rho GTPases, thus preventing any interactions with downstream effectors (Just et al., 1995; Jank and Aktories, 2008). We have shown previously that inhibition of the Rho family member Rac with ToxB elicits the derepression of a proapoptotic JAK/STAT pathway in CGNs (Loucks et al., 2006). However, the specific STAT family protein involved in this pathway has not yet been elucidated. As STAT1 is the most extensively described family member in studies of apoptosis (Sahni et al., 2001; Stephanou and Latchman, 2003; Sironi and Ouchi, 2004), we examined its expression following Rac inhibition. CGNs incubated with ToxB for 24 h exhibited a marked increase in the expression of STAT1 (Fig. 2.1A). Moreover, co-incubation with a small molecule pan-JI (structure shown in Fig. 2.1B) was sufficient to prevent the induction of STAT1 by ToxB, confirming that the increase in STAT1 is dependent on JAK activation (Fig. 2.1A).

In addition to provoking apoptosis in a variety of diverse cell types (Thomas et al., 2004; Kaganoi et al., 2007; Soond et al., 2007), activated STAT1 has been shown to play a proapoptotic role in neuronal death induced during ischemic brain injury (Takagi et al., 2002). To determine whether derepression of a JAK/STAT1 pathway exerts a similar proapoptotic effect in Rac-inhibited neurons, we examined the neuroprotective effects of pan-JI in ToxB-treated CGNs. Examination of nuclei by Hoechst staining
revealed increased apoptotic cell death in ToxB-treated CGNs as evidenced by nuclear fragmentation and/or condensation. The effect of ToxB on the morphology of CGN nuclei was significantly attenuated by co-treatment with the pan-JI (Fig. 2.1D, lower panels). As an additional means of identifying apoptotic cells, we examined caspase-3 activation as cleavage to its active proteolytic fragments is a hallmark of apoptosis and signifies commitment to cell death (Porter and Jänicke, 1999). CGNs treated with ToxB alone exhibited increased activation of caspase-3, and this effect was blocked by pan-JI (Fig. 2.1D, upper panels). Quantification of apoptosis in CGNs co-incubated with ToxB and pan-JI revealed that the pan-JI conferred significant neuroprotection and effectively reduced apoptosis to control levels (Fig. 2.1C). Taken together, these data suggest that inhibition of Rac with ToxB results in the activation of a proapoptotic JAK/STAT1 signaling pathway in CGNs.

To elucidate the specific JAK family member(s) that induces STAT activation downstream of Rac inhibition in CGNs, we evaluated the protective effects of more targeted JAK inhibitors against ToxB. We found that two specific JAK3 inhibitors (JAK3 inhibitor and tofacitinib) did not confer significant neuroprotection in ToxB-treated CGNs (Fig. 2.1E). However, we found that the JAK1/2 inhibitor (ruxolitinib) modestly protected CGNs from ToxB-mediated apoptosis, although this effect did not quite reach statistical significance. Based on the marked protective effects of the pan-JI (which inhibits JAK1–3 and Tyk2 with similar potency) and our results showing that more targeted inhibition of specific JAK family members (JAK1–3) is not overtly protective, these data suggest a possible contribution of Tyk2 in mediating the apoptosis downstream of ToxB-mediated Rac inhibition in primary CGNs.
Figure 2.1 STAT1 is up-regulated in JAK-dependent manner during CGN apoptosis induced by Rho family GTPase inhibitor ToxB. A. CGNs were incubated for 24 h in complete medium containing 25 mm KCl and serum (control (Con) medium) ± ToxB (40 ng/ml) or ToxB + pan-JI (1 µm). Cells were then lysed, and proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membrane was probed with an antibody against STAT1. STAT1 expression was up-regulated when treated with ToxB, and this effect was blocked by pan-JI. B. Molecular structure of pan-JI. C. Quantification of apoptosis in CGNs incubated for 24 h as described in A. **, p < 0.01 compared with control (Con); ††, p < 0.01 compared with ToxB. D. CGNs were incubated for 24 h as described in A. For 4 independent experiments following incubation, cells were incubated with a polyclonal antibody against active caspase-3 (shown in red) and a monoclonal antibody against β-tubulin (shown in green), and nuclei were stained with DAPI (shown in blue). Lower panels show decolorized DAPI staining of nuclei for clarity. CGNs incubated with ToxB exhibited many condensed and/or fragmented nuclei and displayed increased immunoreactivity for active caspase-3. CGNs co-incubated with pan-JI were significantly protected from apoptosis and displayed nuclear morphology similar to that in controls. Scale bar, 10 µm. E. CGNs were incubated for 24 h in control medium ± ToxB (40 ng/ml) or ToxB + JAK3 inhibitor (JAK3 Inh; 100 µm), ruxolitinib (Rux; 10 µm), or tofacitinib (Tof; 10 µm). Following incubation, cells were fixed with 4% paraformaldehyde, and the nuclei were stained with Hoechst dye. Apoptotic cells were scored as those with condensed and/or fragmented nuclei. Values are mean ± S.E. for 6 independent experiments. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.2 STAT1 is not activated by tyrosine phosphorylation nor does it translocate into nucleus of ToxB-treated CGNs

Next, we examined the phosphorylation status and localization of STAT1 in CGNs exposed to ToxB. To translocate into the nucleus and influence gene expression, members of the STAT family must be activated via tyrosine phosphorylation (Rawlings et al., 2004). Whereas total STAT1 expression increased in CGNs treated for 24 h with ToxB, time course experiments performed for up to 24 h did not show significant activation of STAT1 as assessed by tyrosine phosphorylation (Fig. 2.2A). Furthermore, using immunostaining to examine STAT1 localization, we found that STAT1 remained cytosolic and perinuclear in CGNs following ToxB treatment (Fig. 2.2B). These observations are striking in that they suggest that the observed up-regulation of STAT1 expression may not be directly involved in apoptosis following ToxB-mediated inhibition of Rac.
Figure 2.2 STAT1 does not display increased tyrosine phosphorylation nor does it translocate into nucleus of ToxB-treated CGNs. A. A 24h time course of ToxB treatment (40 ng/ml) was performed in CGNs. Cell lysates were resolved by SDS-PAGE, and proteins were transferred to PVDF membranes. The blots were probed with antibodies against pSTAT1 (Tyr-701) and STAT1. Little to no increase in the tyrosine phosphorylation of STAT1 was observed in CGNs subjected to ToxB for up to 24 h. B. CGNs were incubated with control (Con) medium ± ToxB (40 ng/ml) for 8 h. Cells were fixed, and their nuclei were stained with DAPI. A primary antibody against pSTAT1 (Tyr-701) and a Cy3-conjugated secondary antibody were used to visualize pSTAT1. Incubation of CGNs with ToxB did not result in translocation of pSTAT1 into the nucleus. Scale bar, 10 µm. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.3 CGNs from STAT1 knock-out mice are susceptible to ToxB-induced apoptosis

To definitively establish whether the up-regulation of total STAT1 expression in the absence of enhanced tyrosine phosphorylation or nuclear translocation is involved in ToxB-mediated apoptosis, we measured the effects of ToxB on primary cultures of CGNs isolated from STAT1 KO mice versus their wild-type (WT) littermates. CGNs from WT mice demonstrated a marked increase in STAT1 expression following ToxB treatment, whereas CGNs from STAT1 KO mice did not demonstrate any expression of STAT1 in either the absence or presence of ToxB (Fig. 2.3A), thus confirming effective knock-out of STAT1 expression in these mice. Unexpectedly, CGNs from both WT and STAT1 KO mice equally succumbed to apoptosis in response to ToxB treatment (Fig. 2.3B), whereas inclusion of the pan-JI was equally neuroprotective for both cell types (Fig. 2.3C). Collectively, these data indicate that although total STAT1 is up-regulated a different member of the STAT family mediates JAK-dependent apoptosis in Rac-inhibited CGNs.
Figure 2.3 CGNs from STAT1 knock-out and wild-type mice are equally susceptible to ToxB-induced apoptosis. A. CGNs from WT and STAT1 KO mice were treated for 0, 24, or 48 h with ToxB (40 ng/ml). Cell lysates were resolved by SDS-PAGE, and proteins were transferred to PVDF membranes. The blot was probed with a primary antibody against STAT1. The Western blot shows induced expression of STAT1 in WT CGNs after treatment with ToxB. B. WT and STAT1 KO CGNs were incubated for 48 h in control (Con) medium ± ToxB (40 ng/ml) or with ToxB + pan-JI (1 µm). Cells were fixed, and nuclei were stained with Hoechst. Both WT and KO cells treated with ToxB displayed condensed and/or fragmented nuclear morphology. WT and KO CGNs co-incubated with pan-JI exhibited nuclear morphologies similar to those of control cells. Scale bar, 10 µm. C. Quantification of apoptosis in CGNs incubated for 24 or 48 h with control medium ± ToxB (40 ng/ml) or ToxB + pan-JI (1 µm). STAT1 KO cells succumbed to apoptosis as readily as WT CGNs after treatment with ToxB. When co-incubated with pan-JI, STAT1 KO and WT CGNs showed similar protection from ToxB. **, \( p < 0.01 \) compared with control; ††, \( p < 0.01 \) compared with ToxB. \textit{Inh}, inhibitor. Values are mean ± S.E. for 3 independent experiments. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.4 STAT3 is tyrosine phosphorylated in response to ToxB, and this effect is blocked by Pan-JI

As our data demonstrate that STAT1 up-regulation does not significantly contribute to apoptosis in Rac-inhibited CGNs, we next evaluated the involvement of additional members of the STAT family. Recent evidence suggests that STAT3 mediates β-amyloid-induced apoptosis in mouse cortical neurons (Wan et al., 2010). To determine whether ToxB-targeted inhibition of Rac acts to ultimately activate a similar proapoptotic JAK/STAT3 pathway, we analyzed the activation of STAT3 in response to ToxB treatment. Time course experiments demonstrated that STAT3 was tyrosine phosphorylated as early as 6 h after ToxB treatment (Fig. 2.4A), and inclusion of pan-JI blocked the phosphorylation of STAT3 at both 6 and 8 h in ToxB-treated CGNs (Fig. 2.4B).
Figure 2.4 STAT3 is tyrosine phosphorylated in response to ToxB in CGNs, and this effect is blocked by pan-JI. A. CGNs were incubated for various periods of time up to 8 h with ToxB in control medium. Cells were lysed, and proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The blot was probed with antibodies against pSTAT3 (Tyr-705) or STAT3. The expression of pSTAT3 increased in response to ToxB. B. CGNs were incubated for 6 or 8 h in control (Con) medium ± ToxB (40 ng/ml) or ToxB + pan-JI (1 µm). Cell lysates were resolved by SDS-PAGE, and proteins were transferred to PVDF membranes. The blot was probed with antibodies against pSTAT3 (Tyr-705) or STAT3. The pan-JI inhibitor blocked ToxB-induced STAT3 phosphorylation. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.5 The reputed STAT3 inhibitor JSI-124 protects CGNs from ToxB-induced apoptosis

To further define the potential role of STAT3 in the proapoptotic pathway activated downstream of Rac inhibition, we examined whether or not the reputed STAT3 inhibitor JSI-124 (Blaskovich et al., 2003) exerted a protective effect in ToxB-treated CGNs (Fig. 2.5A). Following a 24-h incubation period with ToxB alone, many CGNs displayed condensed and/or fragmented nuclei consistent with apoptosis. In contrast, CGNs co-incubated with JSI-124 were essentially completely protected from apoptosis, and their nuclei were morphologically similar to those in control cells (Fig. 2.5B). Inclusion of JSI-124 reduced CGN apoptosis in the presence of ToxB to ~10% (Fig. 2.5C). These data support a model in which a proapoptotic JAK/STAT3 pathway mediates apoptosis in Rac-inhibited CGNs.
Figure 2.5 The reputed STAT3 inhibitor, JSI-124, protects CGNs from ToxB-induced apoptosis. A. Molecular structure of JSI-124. B. CGNs were incubated for 24 h in control (Con) medium ± ToxB (40 ng/ml) or ToxB + JSI-124 (JSI; 5 µm). Cells were fixed, and their nuclei were stained with Hoechst dye. CGNs treated with ToxB exhibited significantly more condensed and/or fragmented nuclei than control CGNs. CGNs co-incubated with JSI-124 were significantly protected from apoptosis, and their nuclei were morphologically similar to those of control cells. Scale bar, 10 µm. C. Quantification of apoptosis in CGNs treated with ToxB or ±JSI-124. Values are mean ± S.E. JSI-124 significantly protected CGNs from ToxB-induced apoptosis. **, p < 0.01 compared with control; ††, p < 0.01 compared with ToxB. Values are mean ± S.E. for 5 independent experiments. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.6 Phosphorylated STAT3 does not translocate into nucleus of CGNs following treatment with ToxB, and additional STAT3 inhibitors do not protect CGNs from apoptosis induced by ToxB

To further investigate the role of STAT3 in apoptosis, we examined whether or not pSTAT3 translocates into the nucleus following ToxB treatment. Nuclear fractionation experiments demonstrated that although STAT3 was tyrosine phosphorylated pSTAT3 remained cytosolic following ToxB targeted inhibition of Rac (Fig. 2.6A). Furthermore, two additional STAT3 inhibitors, STA-21 and a STAT3 inhibitory peptide, did not protect CGNs from ToxB-induced apoptosis (Fig. 2.6, B and C). These results indicate that STAT3 does not directly regulate apoptosis downstream of Rac inhibition. This finding was particularly surprising as the selective STAT3 inhibitor JSI-124 exerted a neuroprotective effect in ToxB-treated CGNs. However, further evaluation of the effects of JSI-124 in CGNs revealed that this compound in fact had no significant inhibitory effect on ToxB-induced STAT3 phosphorylation (Fig. 2.6D). These data suggest that the mechanism by which JSI-124 protects CGNs from apoptosis is unrelated to attenuation of activated STAT3 and may involve a different member of the STAT family.
Figure 2.6 Phosphorylated STAT3 does not translocate into nucleus of CGNs following treatment with ToxB, and additional STAT3 inhibitors do not protect CGNs from apoptosis. A. CGNs were incubated in control (Con) medium ± ToxB (40 ng/ml) for 8 h. Following incubation, the cells were fractionated into nuclear and cytosolic (cyto) fractions as described under “Experimental Procedures.” A monoclonal antibody against pSTAT3 (Tyr-705) was used for Western blotting. pSTAT3 did not translocate into the nucleus following treatment with ToxB. The purity of the nuclear fractions was verified by immunoblotting for LAP-2. B. CGNs were incubated for 24 h in control medium ± ToxB (40 ng/ml) or ToxB + STA-21 (20 µm). Cells were fixed, and nuclei were stained with Hoechst dye to quantify apoptosis. STA-21 did not protect CGNs from ToxB-induced apoptosis. Results shown are mean ± range (n = 2). C. CGNs were incubated for 24 h in control medium ± ToxB (40 ng/ml) or ToxB + STAT3 inhibitory peptide (Inh Pep; 100 µm). Cells were fixed, and nuclei were stained with Hoechst dye to quantify apoptosis. STAT3 inhibitory peptide did not protect CGNs from ToxB-induced apoptosis. Results shown are mean ± range (n = 2). D. CGNs were incubated for 24 h in control medium ± ToxB (40 ng/ml), JSI-124 (JSI; 5 µm), or ToxB + JSI-124. The cells were lysed, and proteins were separated by SDS-PAGE and transferred to PVDF membranes. Blots were probed for pSTAT3 (Tyr-705). ToxB increased the expression of pSTAT3. This effect was not blocked by co-incubation with JSI-124. NS, nonspecific band shown to indicate equal protein loading. Values are mean ± range for 2 independent experiments. I acknowledge Alex Loucks for assistance with generating data for this figure.
**2.4.7 Tyrosine phosphorylation of STAT5 in response to ToxB in CGNs is blocked by JSI-124 and putative STAT5 inhibitor roscovitine**

Recent evidence demonstrates a necessary role for STAT5 in apoptosis induced by oncostatin M in osteosarcoma cells (Chipoy et al., 2007). In addition, both STAT5 isoforms have been implicated in the down-regulation of the prosurvival Bcl-xL protein in thymocytes (Nelson et al., 2004). Therefore, we next examined STAT5 and found that STAT5 was tyrosine phosphorylated in ToxB-treated CGNs. Interestingly, although blotting for total STAT5 clearly showed the presence of both STAT5A (94 kDa) and STAT5B (92 kDa) in CGNs (Fig. 2.7A, *lower blot*), Western blots for phospho-STAT5 only indicated a single tyrosine phosphorylated isoform in response to ToxB (Fig. 2.7A, *upper blot*). Therefore, we next sought to determine whether STAT5A or STAT5B was phosphorylated in response to ToxB. To establish which STAT5 isoform was phosphorylated, we compared lysates that were immunoprecipitated for either STAT5A or STAT5B with whole cell lysates and Western blotted for total STAT5. Using this approach, we were able to determine where the STAT5A and STAT5B isoforms were detected by immunoblot (Fig. 2.7B, *left panel*). Despite efficient immunoprecipitation of each of the STAT5 isoforms from CGNs using isoform-specific antibodies, we were not able to observe tyrosine phosphorylation of either isoform in response to ToxB. However, in whole cell lysates obtained following 8 h of ToxB treatment, we observed a pronounced increase in the tyrosine phosphorylation of the higher molecular weight STAT5A isoform but not STAT5B using a phosphospecific antibody to STAT5 (Fig. 2.7B, *right panel*). The inability to observe STAT5A tyrosine phosphorylation following immunoprecipitation may reflect the physical association of STAT5A with one or more
protein tyrosine phosphatases, any one of which may be insensitive to the phosphatase inhibitors present in our lysis buffer, as described previously (Chughtai et al., 2002; Rigacci et al., 2003). Our data are consistent with several previous reports demonstrating that STAT5A and STAT5B can be individually activated in a stimulus- and cell type-specific manner (Rosen et al., 1996; al-Shami et al., 1997; Storz et al., 1999; Grimley et al., 1999; Faderl et al., 2003).

In addition, co-treatment of CGNs with either JSI-124 or the putative STAT5 inhibitor roscovitine (Mohapatra et al., 2003) was sufficient to block the tyrosine phosphorylation of STAT5A in response to ToxB (Fig. 2.7, A and B). To further substantiate the involvement of STAT5 in ToxB-treated CGNs, we examined STAT5 phosphorylation and localization by immunocytochemistry. In control CGNs, the expression of pSTAT5 detected by immunofluorescence was negligible. In contrast, following incubation of CGNs with ToxB, there was a profound increase in the immunoreactivity for pSTAT5, and this transcription factor was principally localized to CGN nuclei (Fig. 2.7C). Collectively, these data indicate that STAT5 is tyrosine phosphorylated in response to ToxB and localizes to the nucleus under these conditions to modulate gene transcription. Moreover, the tyrosine phosphorylation of STAT5 appears to be specific for the STAT5A isoform and is sensitive to inhibition by either JSI-124 or roscovitine.
Figure 2.7 STAT5A shows enhanced tyrosine phosphorylation in response to ToxB in CGNs that is blocked by JSI-124 and STAT5 inhibitor roscovitine. A. CGNs were incubated in control (Con) medium ± ToxB (40 ng/ml) or ToxB + JSI-124 (JSI; 5 µm) for 24 h. Cells were lysed, and proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed for pSTAT5 (Tyr-694) and total STAT5. STAT5 was phosphorylated in response to ToxB, and this effect was blocked by JSI-124. B. CGNs were incubated in control medium ± ToxB (40 ng/ml) or ToxB + roscovitine (Rosc; 30 µm) for 24 h. Following incubation, cells were lysed and immunoprecipitated (IP; right panel) for either total STAT5A or STAT5B. Immunoprecipitated proteins and a lane containing the whole cell lysate (WCL) were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed for total STAT5. In the left panel, proteins were lysed following a 24-h incubation period and resolved by SDS-PAGE. Next, proteins were transferred to PVDF membranes. Blots were probed for pSTAT5 (Tyr-694) and total STAT5. STAT5 was phosphorylated in response to ToxB, and this effect was blocked by roscovitine. NS, nonspecific band shown to indicate equal protein loading; 5A, STAT5A; 5B, STAT5B. C. CGNs were incubated in control medium ± ToxB (40 ng/ml) for 24 h. Cells were fixed and incubated with a polyclonal antibody against pSTAT5 (Tyr-694; shown in green). Nuclei were stained with DAPI (shown in blue). pSTAT5 translocated into the nucleus in response to ToxB. Scale bar, 10 µm. IB, immunoblotted.
2.4.8 Roscovitine significantly protects CGNs from ToxB-induced apoptosis

Consistent with STAT5 playing a key role in ToxB-induced apoptosis, CGNs co-treated with ToxB and roscovitine displayed healthy, intact nuclei (Fig. 2.8A). Quantification of these results shows that roscovitine significantly protected CGNs from ToxB-induced apoptosis (Fig. 2.8B). In addition, ToxB treatment for 24 h resulted in the cleavage of procaspase-3 to its active fragments, an effect largely attenuated by roscovitine (Fig. 2.8C). Generally, roscovitine is used to suppress the cell cycle through inhibition of cyclin-dependent kinases (CDKs) (Edamatsu et al., 2000). To ensure that the protection conferred by roscovitine was not through CDK suppression but was a result of STAT5 inhibition, CGNs were also treated with ToxB ± purvalanol A, a closely related CDK inhibitor. Although remarkably similar in structure, purvalanol A did not protect CGNs from ToxB-induced apoptosis and in fact significantly enhanced cell death (Fig. 2.8D). These results suggest that the neuroprotective effects of roscovitine are unrelated to CDK suppression and are likely due to STAT5 inhibition.
Figure 2.8 Roscovitine significantly protects CGNs from ToxB-induced apoptosis. A. CGNs were incubated for 24 h in control (Con) medium ± ToxB (40 ng/ml) or ToxB + Roscovitine (Rosc; 30 µm). Following incubation, cells were fixed, and the nuclei were stained with Hoechst dye. Scale bar, 10 µm. B. Quantification of apoptosis in CGNs treated with ToxB alone or co-treated with roscovitine. Values are mean ± S.E. (n = 6). Roscovitine significantly protected CGNs from ToxB-induced apoptosis. **, p < 0.01 compared with control; ††, p < 0.01 compared with ToxB. C. CGNs were treated as described in A and lysed. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membrane was immunoblotted (IB) for caspase-3. Roscovitine blocked the ToxB-induced processing of caspase-3 to its active fragments. fl, full length. D. CGNs were incubated for 24 h in control medium ± ToxB (40 ng/ml) or ToxB + purvalanol A (Purv; 30 µm). Following incubation, cells were fixed with 4% paraformaldehyde, and the nuclei were stained with Hoechst dye. Purvalanol A did not protect CGNs from ToxB-induced apoptosis. Values are mean ± S.E. for 3 independent experiments. I acknowledge Alex Loucks for assistance with generating data for this figure.
2.4.9 Adenoviral dominant negative STAT5 protects CGNs from ToxB-induced apoptosis

As a more specific approach to definitively establish the proapoptotic role of STAT5 following disruption of Rac activity, we examined the neuroprotective effects of a dominant negative mutant of STAT5 containing a C-terminal truncation at residue 713 that removes the entire transcriptional activation domain of STAT5 (Ad-DN STAT5; Ref. Wang et al., 1996). First, adenoviruses expressing either WT STAT5 or DN STAT5 were grown and overexpressed in HEK293AD cells, and these cells were compared with cells that were infected with the Ad-GFP control. Infection with Ad-WT STAT5 increased expression of full-length STAT5, and Ad-DN STAT5 appeared as a lower molecular weight form of STAT5 (Fig. 2.9A). Next, we infected CGNs with the Ad-GFP control, WT STAT5, and DN STAT5 and observed similar high level expression of the constructs (Fig. 2.9B). Importantly, none of the adenoviral constructs induced any significant increase in basal apoptosis of CGNs on their own (Fig. 2.9C). As we have consistently shown, ToxB treatment resulted in condensed and fragmented nuclei indicative of apoptosis. Not unexpectedly, preincubation of CGNs with WT STAT5 did not confer neuroprotection from ToxB treatment (Fig. 2.9E). However, CGNs preincubated with Ad-DN STAT5 and subsequently treated with ToxB displayed nuclear morphology strikingly more similar to that of control CGNs (Fig. 2.9E). Quantification of apoptosis revealed that inhibiting the transcriptional activity of endogenous STAT5 through overexpression of Ad-DN STAT5 significantly protected CGNs from ToxB-induced apoptosis (Fig. 2.9D). The protective effect of Ad-DN STAT5 was not as complete as that with the chemical JAK/STAT inhibitors used previously. This was likely a reflection of the infection efficiency of CGNs, which we observed to be 50–60% in cells infected
with Ad-GFP. These data highlight a novel proapoptotic function for STAT5 downstream of Rac inhibition in primary cerebellar neurons.
Figure 2.9 Adenoviral dominant negative STAT5 protects CGNs from ToxB-induced apoptosis. A. HEK293AD cells were infected for 48 h with an adenovirus carrying GFP (Ad-GFP), wild-type STAT5 (Ad-WT STAT5), or dominant negative STAT5 (Ad-DN STAT5). Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted (IB) for STAT5 expression. There was a marked increase in STAT5 expression in HEK cells infected with either Ad-WT or Ad-DN STAT5. *Endo*, endogenous; *trunc*, truncated. B. CGNs were infected for 48 h with the same adenoviruses as described in A, and cell lysates were probed for STAT5 expression. C. CGNs were incubated for 48 h with adenoviruses as described above. The cells were fixed, and the nuclei were stained with Hoechst. Apoptotic cells were scored as those with condensed and/or fragmented nuclei. There was no significant difference in basal apoptosis in CGNs infected with Ad-GFP, Ad-WT STAT5, or Ad-DN STAT5. D. CGNs were infected for 48 h with either Ad-WT STAT5 or Ad-DN STAT5. After the initial 24 h of incubation, each experimental condition was treated ±ToxB (40 ng/ml) for 24 h. At the end of the incubation, cells were fixed, and nuclei were stained with DAPI. Apoptotic cells were scored as those with condensed and/or fragmented nuclei. The fold change in apoptosis for ToxB-treated cells versus control (Con) cells was quantified. CGNs incubated with dominant negative STAT5 were protected from ToxB-induced apoptosis. Data shown represent the mean ± S.E. for *n* = 3 experiments. **, *p* < 0.01 versus WT STAT5. E. CGNs were incubated as describe in D. Cells were then fixed, and nuclei were stained with Hoechst dye. Images shown are representative of three independent experiments. Scale bar, 10 µm. Values are mean ± S.E. for 6 independent experiments.
2.4.10 STAT5 transcriptionally represses Bcl-xL during ToxB-induced apoptosis

Recent studies suggest that under certain conditions STAT5 transcriptionally represses prosurvival members of the Bcl-2 family (Chipoy et al., 2007; Nelson et al., 2004; Moucadel and Constantinescu, 2005). Disturbances in the ratio of these proteins to their corresponding proapoptotic family members can induce apoptosis. The prosurvival members of the Bcl-2 family have an important and well documented role in promoting cell survival through binding and inactivating the proapoptotic members of their same family (Korsmeyer et al., 1993). To evaluate whether STAT5 similarly represses Bcl-2 family proteins in Rac-inhibited CGNs, we isolated and purified DNA with a ChIP grade antibody to STAT5 in control CGNs or those treated with ToxB for 8 h. There was no significant difference between STAT5 bound to the promoter region of Bcl-2 in control versus ToxB-treated CGNs (data not shown). In the case of Bcl-xL, the input levels of Bcl-xL promoter DNA amplified from the non-immunoprecipitated control and ToxB-treated CGNs were not significantly different. However, the cycle threshold values obtained for amplification of the Bcl-xL promoter from STAT5 ChIP samples were significantly lower for ToxB treatment compared with the control, indicating a significant increase in STAT5 binding to the Bcl-xL promoter in Rac-inhibited CGNs (Fig. 2.10A). Indeed, we report that there was an apparent although not statistically significant 9.5 ± 4.1 (n = 4)-fold increase in STAT5 recruitment to the Bcl-xL promoter in response to ToxB treatment after 8 h (Fig. 2.10B). Consistent with these data, Bcl-xL expression was down-regulated at 16 h and more significantly at 24 h after ToxB treatment (Fig. 2.10C). In agreement with a relatively slow mechanism of death due to the transcriptional repression of Bcl-xL, we report that ToxB did not induce caspase-3 activation or
significant apoptotic morphology until 16 h of incubation after STAT5 is bound to the promoter region of Bcl-xL in CGNs (Fig. 2.10, B and C).
Figure 2.10 STAT5 transcriptionally represses Bcl-xL during ToxB-induced apoptosis. A. CGNs were incubated in control (Con) medium ± ToxB (40 ng/ml) for 8 h. As a control for ChIP experiments (“Input”), ChIP-quantitative PCR was performed as described under “Experimental Procedures” in the absence of the STAT5 antibody to determine the total concentration of input Bcl-xL promoter DNA. The graph shows the mean ± S.E. of the results of four independent experiments. The concentration of input DNA did not differ significantly between the control and ToxB samples. ChIP: STAT5, CGNs were incubated in control medium ± ToxB (40 ng/ml) for 8 h. The relative binding of STAT5 to the Bcl-xL promoter was assessed via ChIP-quantitative PCR analysis as described under “Experimental Procedures.” The graph shows the mean ± S.E. of the results for four independent experiments. STAT5 binding to the Bcl-xL promoter was significantly increased in response to ToxB treatment for 8 h. *, p < 0.05 versus control evaluated by a two-tailed Student’s t test. B. The -fold enrichment of STAT5 recruitment to the Bcl-xL promoter was quantified from the cycle threshold (Ct) value. STAT5 binding to the promoter region of Bcl-xL increased 9.5 ± 4.1-fold in response to 8-h ToxB treatment, although it was not statistically significant. C. CGNs were incubated for 0, 16, and 24 h with ToxB (40 ng/ml). Cells were then lysed, and proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membrane was probed with an antibody against Bcl-xL. Subsequently, the blot was stripped and reprobed for active caspase-3 and actin (loading control). Bcl-xL expression decreased in response to ToxB, and active caspase-3 expression increased. The values above the Bcl-xL blot represent optical densities (O.D.) of the Bcl-xL expression. The normalized optical density for the time “0” control was set to 1.0. Values are mean ± S.E. for 3 independent experiments.
2.5 Discussion

Several studies have demonstrated a critical role for Rho GTPases (i.e. Rac, Rho, and Cdc42) in promoting neuronal survival (Kobayashi et al., 2004; Kanekura et al., 2005; Cheung et al., 2007). Consistent with these findings, we have shown previously that Rac activity is critical for the survival of primary CGNs (Linseman et al., 2001), and Rac signaling functions to repress a proapoptotic JAK/STAT pathway in these neurons (Loucks et al., 2006). Similarly, studies from other groups support a role for the activation of a prodeath JAK/STAT pathway in neurons (Colodner and Feany, 2010; Mäkelä et al., 2010). For instance, Takagi et al. (2002) reported that STAT1 was phosphorylated on activating tyrosine and serine residues and translocated into neuronal nuclei following ischemic brain injury in mice. Moreover, STAT1 KO mice displayed a decrease in the volume of ischemic brain injury and reduced TUNEL staining when compared with WT mice subjected to middle cerebral artery occlusion. Thus, STAT1 can play a proapoptotic role in neurons exposed to ischemic stress. In a similar manner, cortical neurons cultured from STAT1 KO mice showed marked resistance to interferon γ-stimulated neurotoxicity of the human immunodeficiency virus type 1 proteins gp120 and Tat (Giunta et al., 2006). A more recent study demonstrated that exposure of SH-SY5Y neuroblastoma cells to interferon β induced STAT1 tyrosine phosphorylation and caspase-dependent apoptosis (Dedoni et al., 2010). Both STAT1 activation and caspase activation were prevented by a JAK inhibitor. Similar to STAT1, STAT3 also has the potential to have a proapoptotic effect in neurons under certain conditions. Wan et al. (2010) recently showed that a proapoptotic Tyk2/STAT3 pathway mediates β-amyloid peptide-induced neuronal death. Specifically, siRNA knockdown of STAT3 protected
PC12 cells from β-amyloid, and a STAT3 inhibitory peptide protected cultured cortical neurons. Finally, cortical neurons isolated from Tyk2-null mice were significantly less sensitive than WT neurons to β-amyloid-induced apoptosis. Collectively, these studies demonstrate that both STAT1 and STAT3 have the potential of acting as proapoptotic mediators in neurons under specific pathological conditions. In contrast to these previous reports, we found that although total STAT1 levels are up-regulated and STAT3 is activated via tyrosine phosphorylation in response to ToxB neither transcription factor is responsible for the neuronal apoptosis induced in CGNs by inhibition of Rac function.

Instead, we report that ToxB induces the enhanced tyrosine phosphorylation of STAT5, which subsequently translocates into the nucleus of CGNs. Interestingly, we found that the phosphorylation of STAT5 was prevented by the reputed STAT3 inhibitor JSI-124. This finding is perhaps not surprising given that JSI-124 has been reported to exert effects outside of STAT3 inhibition (Graness and Goppelt-Struebe, 2006). In fact, this compound was recently shown to inhibit the neuroprotective effects of growth hormone against glutamate-induced toxicity in hippocampal neurons, an effect that is dependent on STAT5 activity (Byts et al., 2008). In addition to the neuroprotective effects of JSI-124, we report that roscovitine similarly inhibits STAT5 phosphorylation induced by ToxB and protects CGNs against ToxB targeted Rac inhibition. The effects of roscovitine were dissociated from its effects as a CDK inhibitor because the similarly structured compound purvalanol A did not protect CGNs from ToxB. Our data with roscovitine are consistent with a previous study showing its ability to suppress STAT5 activation in a leukemia cell line (Mohapatra et al., 2003). Finally, we show that adenoviral infection with a dominant negative mutant of STAT5, but not WT STAT5,
significantly protects CGNs from Rac inhibition with ToxB. To our knowledge, these findings are novel in that they are the first to identify a proapoptotic function for STAT5 in neurons.

Our data indicate that of the two STAT5 isoforms only STAT5A is activated via phosphorylation in CGNs exposed to ToxB-mediated Rac blockage. Although the two isoforms are highly homologous, this is consistent with several previous reports that have revealed isoform-specific STAT5 activation. Similar to our results in CGNs, JAK/STAT signaling has been shown to induce caspase-dependent apoptosis in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) in OCIM2 acute myeloid leukemia cells (Faderl et al., 2003). However, although GM-CSF has been shown to preferentially activate STAT5A in human monocytes (Rosen et al., 1996), this factor was shown to induce specific STAT5B signaling in human neutrophils (al-Shami et al., 1997). In a similar manner, insulin was demonstrated to activate STAT5B, but not STAT5A, in Kym-1 rhabdomyosarcoma cells (Storz et al., 1999). Furthermore, targeted gene disruptions of either STAT5A or STAT5B in mice have shown functional differences in vivo. For example, deletion of STAT5A disrupts prolactin-derived mammary gland maturation, whereas disruption of STAT5B diminishes growth hormone effects on hepatic function and body mass in male mice (Grimley et al., 1999). Thus, our data are consistent with several previous reports highlighting isoform-specific activation and functions of STAT5.

STAT5 is activated by many diverse cytokines and growth factors, and it is well established that this transcription factor has an important role throughout the body. Although few studies have examined the role of STAT5 in the central nervous system,
the majority of reports are paradoxical to our present study and suggest that STAT5 chiefly exerts a pro-survival effect in neurons. For example, STAT5 is required in conjunction with Akt to mediate the neurotrophic and neuroprotective effects of both growth hormone in hippocampal neurons (Byts et al., 2008) and erythropoietin in differentiated neuroblastoma cells (Um and Lodish, 2006). Furthermore, STAT5 was shown to elicit a pro-survival effect via induction of Bcl-2 and Bcl-xL in neural progenitor cells subjected to apoptotic stimulation (Choi et al., 2011). Thus, the relatively few studies conducted on STAT5 in neuronal models indicate that it mainly functions to transmit pro-survival signals. However, critical studies in non-neuronal cells suggest that the downstream effects of STAT5 are much more complex. STAT5 sensitizes osteosarcoma cells to apoptosis following treatment with oncostatin M (Chipoy et al., 2007). Moreover, in a mouse model of familial amyotrophic lateral sclerosis, a JAK3 inhibitor was shown to extend the lifespan of mutant mice when compared with those that did not receive the inhibitor. Although the effects of this inhibitor on the activation of specific STAT family members was not evaluated in this particular study, JAK3 has been shown to activate STAT5 in various studies (Bingisser et al., 1998; Cavalcanti et al., 2010; Martín et al., 2010). In evaluating various JAK isoform-selective inhibitors, we did not observe any significant inhibition of ToxB-induced apoptosis in CGNs except with a pan-JI. Thus, our data suggest a possible contribution of Tyk2 or multiple JAK isoforms in the apoptosis of neurons subjected to Rac inhibition. In conjunction with our present findings, these previous studies suggest that STAT5 functions in a cell type- and stimulus-specific manner, and the specific role of JAK/STAT5 in the central nervous
system and in particular with respect to neuronal survival is likely determined by many factors.

Although we have identified Rac as a repressor of a proapoptotic JAK/STAT5 pathway, the mechanism of STAT5 activation following Rac inhibition remains incompletely understood. Suppressors of cytokine signaling (SOCS) exist as endogenous STAT inhibitors (Krebs and Hilton, 2001; Cooney, 2002), and previous evidence suggests that SOCS protein expression can be regulated by ERK (Terstegen et al., 2000; Borland et al., 2009). This is consistent with our previous finding that a prosurvival MEK/ERK pathway functions downstream of Rac GTPase to actively repress proapoptotic JAK/STAT signaling in healthy CGNs (Loucks et al., 2006). Whether SOCS proteins are indeed mediators of the Rac-dependent repression of proapoptotic JAK/STAT signaling in neurons remains to be investigated.

In agreement with our work identifying a proapoptotic JAK/STAT5 pathway in CGNs, recent examination of the genetic targets of STAT5 demonstrates transcriptional regulation of prosurvival members of the Bcl-2 family. For example, STAT5 has been shown to negatively regulate the levels of Bcl-2 in osteosarcoma cells sensitized to undergo apoptosis (Chipoy et al., 2007). Similarly, both STAT5 isoforms were shown to bind to the promoter region of Bcl-x, a gene that is transcribed into prosurvival Bcl-xL and proapoptotic Bcl-extra short (Bcl-xS) (Boise et al., 1993; Nelson et al., 2004; Moucadel and Constantinescu, 2005). Therefore, it was of interest to determine whether Bcl-2 and/or Bcl-xL were transcriptional targets of STAT5 in CGNs subjected to Rac inhibition with ToxB. Our ChIP data show that STAT5 is recruited to the Bcl-xL promoter in ToxB-treated CGNs. These results suggest that STAT5 may transcriptionally
repress Bcl-xL to tip the delicate balance between prosurvival and proapoptotic Bcl-2 proteins toward apoptosis. Consistent with this interpretation, we observed caspase-3 activation and classical apoptotic morphology subsequent to down-regulation of Bcl-xL protein levels. Although we show that STAT5 binding to the promoter region of Bcl-xL increases in response to Rac inhibition, an alternative mechanism by which STAT5 may mediate transcriptional repression of Bcl-2 family proteins in the nucleus is through competitive binding to the transcriptional co-activator p300/cAMP-response element-binding protein (CREB)-binding protein (CBP) (Zhu et al., 1999; Zhang et al., 2008). At the present time, we cannot exclude this as a possible mechanism of STAT5-dependent transcriptional repression of Bcl-xL during Rac inhibition.

Another potential mechanism by which STAT5 has been proposed to repress Bcl-xL transcription involves a cooperative interaction with the glucocorticoid receptor (GR). A recent report found that glucocorticoid treatment in lymphoid cells inhibited the transcription of Bcl-xL and decreased the ratio of Bcl-xL to the proapoptotic Bcl-xS in a STAT5B-dependent manner to induce apoptosis (Rocha-Viegas et al., 2006). Conversely, another report found that the GR can interact with STAT5 to up-regulate Bcl-xL as part of a prosurvival pathway activated by the GR agonist methylprednisolone in oligodendrocytes (Xu et al., 2009). Given the evidence suggesting that the GR can act in a cooperative manner with STAT5 to regulate the transcription of Bcl-xL, we sought to determine whether or not the GR was involved in STAT5A-mediated apoptotic signaling in CGNs subjected to ToxB treatment. In CGNs exposed to ToxB, the GR antagonist mifepristone provided no significant protection, indicating that the STAT5-dependent
effects on Bcl-xL transcription are likely GR-independent in this cell system (data not shown).

In summary, we show that ToxB-induced inactivation of Rac GTPase results in the derepression of a novel proapoptotic JAK/STAT5 pathway. Although STAT1 was induced and STAT3 was phosphorylated following ToxB treatment, neither protein was responsible for inducing apoptosis of CGNs downstream of Rac inhibition. Rather, our data support a model in which ToxB-induced Rac inhibition in CGNs activates a novel proapoptotic JAK/STAT5 pathway that, following STAT5A phosphorylation and translocation to the nucleus, results in the transcriptional repression of prosurvival Bcl-xL and subsequent activation of caspase-3 and apoptosis. To our knowledge, our findings are novel in that they are the first to demonstrate a proapoptotic function for a JAK/STAT5 pathway in neurons.
CHAPTER THREE: NEURONAL APOPTOSIS INDUCED BY SELECTIVE INHIBITION OF RAC GTPASE VERSUS GLOBAL SUPPRESSION OF RHO FAMILY GTPASES IS MEDIATED BY ALTERATIONS IN DISTINCT MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING CASCADES

3.1 Abstract

Rho family GTPases play integral roles in neuronal differentiation and survival. We have previously shown that *Clostridium difficile* toxin B (ToxB), an inhibitor of RhoA, Rac1, and Cdc42, induces apoptosis of cerebellar granule neurons (CGNs). In the current study, we compared the effects of ToxB to a selective inhibitor of the Rac-specific guanine nucleotide exchange factors (GEFs), Tiam1 and Trio (NSC23766). In a manner similar to ToxB, selective inhibition of Rac induces CGN apoptosis that is associated with enhanced caspase-3 activation and reduced phosphorylation of the Rac effector, p21-activated kinase (PAK). In contrast to ToxB, caspase inhibitors do not protect CGNs from targeted inhibition of Rac. Also dissimilar to ToxB, selective inhibition of Rac neither inhibits MEK1/2/ERK1/2 nor activates JNK/c-Jun. Targeted inhibition of Rac instead suppresses distinct MEK5/ERK5, p90Rsk and Akt-dependent signaling cascades known to regulate the localization and expression of the Bcl-2 homology-3 domain (BH3)-only protein Bad. Adenoviral expression of a constitutively active mutant of MEK5 is sufficient to attenuate neuronal cell death induced by selective inhibition of Rac with NSC23766, but not apoptosis induced by global inhibition of Rho GTPases with ToxB. Collectively, these data demonstrate that global suppression of Rho family GTPases with ToxB causes a loss of MEK1/2/ERK1/2 signaling and activation of JNK/c-
Jun, resulting in diminished degradation and enhanced transcription of the BH3-only protein Bim. In contrast, selective inhibition of Rac induces CGN apoptosis by repressing unique MEK5/ERK5, p90Rsk, and Akt-dependent pro-survival pathways, ultimately leading to enhanced expression, dephosphorylation, and mitochondrial localization of pro-apoptotic Bad.

3.2 Introduction

Rho family GTPases belong to the Ras superfamily of monomeric G-proteins and include the well described proteins Rac, Rho, and Cdc42. While Rho GTPases are best recognized for their prominent roles in regulating actin cytoskeletal dynamics, more recent studies have demonstrated an important role for Rho GTPases in regulating neuronal survival (Linseman and Loucks, 2008). Indeed, apoptosis induced by inhibitors of 3-HMG-CoA reductase (statins) was correlated with loss of plasma membrane-associated Rho GTPase family members in rat cortical neurons (Tanaka et al., 2000), and downregulation of Rho family GTPases is closely associated with an increase in neuronal apoptosis (Desagher et al., 2005). More recently, Rac has been highlighted as a specific member of the Rho GTPase family critical for maintaining neuronal survival. For example, prior studies demonstrated that either dominant negative Rac expression or siRNA-mediated knockdown of the Rac GEF, Alsin (ALS2), induces apoptosis of cultured motor neurons (Topp et al., 2004; Jacquier et al., 2006). The critical involvement of Rac in neurodegeneration is further evidenced by studies demonstrating that loss-of-function mutations in ALS2 are causative in juvenile onset ALS (Yamanaka et al., 2003). Collectively, these data highlight Rac as a crucial modulator of neuronal
survival and suggest that Rac dysregulation may underlie some forms of neurodegenerative disease.

In previous studies, we have examined the neurotoxic effects of the large cytotoxin Clostridium difficile toxin B (ToxB) in CGNs. ToxB monoglucosylates a key threonine residue in the switch 1 region of Rho GTPases, preventing Rho, Rac, and Cdc42 from interacting with their downstream effectors (Just et al., 1995). Global inhibition of Rho GTPases with ToxB induces CGN apoptosis through dysregulation of critical pro-survival and pro-apoptotic signaling cascades (Loucks et al., 2006; Stankiewicz et al., 2012). Specifically, ToxB induces downregulation of Rac1 GTPase, as well as elements of a Rac-dependent mitogen-activated protein (MAP) kinase pathway, including the p21-activated kinase (PAK), MAP kinase kinase (MEK)1/2, and extracellular signal-regulated kinase (ERK)1/2 signaling cascade. Inhibition of this pathway in CGNs induces apoptosis, in part, through reduced degradation of the pro-apoptotic BH3-only protein Bim. In addition to repression of a pro-survival MEK1/2/ERK1/2 signaling cascade, we have previously reported that broad Rho GTPase inhibition with ToxB induces CGN death through activation of a c-Jun N-terminal kinase (JNK)/c-Jun pathway that stimulates transcription of Bim (Le et al., 2005). Thus, ToxB globally suppresses Rho GTPase function and induces CGN apoptosis through dysregulation of specific MAP kinase signaling cascades, leading to enhanced expression and diminished degradation of the pro-apoptotic BH3-only protein Bim.

The focus of the current study was to compare the effects of ToxB on neuronal survival to those of a more targeted inhibitor of Rac GTPase. While ToxB has been shown to inhibit Rac, Rho, and Cdc42, NSC23766 suppresses a more discrete pool of
Rho family GTPases through inhibition of the Rac-specific GEFs, Tiam1 and Trio, which are two of the most prominent regulators of Rac in the brain (Schmidt and Hall, 2002). The application of this targeted Rac inhibitor allows for a refined understanding of the Rho-family GTPase-regulated signaling pathways essential to neuronal survival. Contrary to our results with ToxB, we demonstrate that targeted inhibition of Rac with NSC23766 does not turn off pro-survival MEK1/2/ERK1/2 signaling in CGNs, nor does it activate the JNK/c-Jun cascade. Instead, NSC23766 induces apoptosis via repression of the distinct MAP kinase pathway, MEK5/ERK5. Further establishing these findings, adenoviral expression of constitutively active MEK5 significantly protects CGNs from NSC23766-mediated Rac inhibition but is unable to protect CGNs from ToxB-mediated global Rho GTPase inhibition. We report that loss of MEK5/ERK5 signaling in NSC23766-treated CGNs results in deactivation of the downstream effectors p90Rsk and Akt, leading to induction, dephosphorylation, and mitochondrial localization of the BH3-only, pro-apoptotic protein Bad. These findings are novel in that they are the first to distinguish the precise MAP kinase signaling pathways that regulate neuronal apoptosis in response to selective inhibition of Rac versus global suppression of Rho family GTPases.

3.3 Materials and Methods

3.3.1 Reagents

NSC23766, JNK inhibitor II (SP600125), caspase inhibitors BOC, LEHD, and QVD (Non-O-Methylated) were obtained from Calbiochem (San Diego, CA). Clostridium difficile Toxin B was prepared using a method published previously (Just et al., 1995). The monoclonal antibody for active Rac1 was procured from NewEast Biosciences.
Hoechst dye 33258 and 4,5-di-amidino-2-phenylindole (DAPI) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to active caspase-3 was from Promega (Madison, WI). Cy3- and FITC-conjugated secondary antibodies for immunofluorescence were from Jackson Immunoresearch Laboratories (West Grove, PA). The CellQuanti-MTT viability assay kit was obtained from Bioassay Systems (Hayward, CA). The monoclonal β-tubulin antibody conjugated to Alexa Fluor® 555, polyclonal antibodies for pPAK1 (Ser144)/pPAK2 (Ser 141), PAK1/2/3, pERK1/2 (Thr202/Tyr204), pMEK1/2 (Ser217/ 221), pERK5 (Thr218/Tyr220), pAkt (Ser473), ERK5, and the monoclonal antibodies against Actin, c-Jun, and pBad (Ser136), pP90Rsk (Ser380) were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies for pMEK5 (S311/T315) and MAP2 monoclonal were obtained from Abcam (Cambridge, MA). The monoclonal antibody used to detect β-tubulin was purchased from Sigma (St. Louis, MO), The rabbit monoclonal antibody for Bad (N-term) was purchased from Millipore (Billerica, MA). Horseradish peroxidase-linked secondary anti-bodies and reagents for enhanced chemiluminescence detection were from Amersham Bio-sciences (Piscataway, NJ, USA).

3.3.2 CGN culture

CGNs were isolated from 7-day old Sprague-Dawley rat pups of both sexes (15-19 g) as described previously (Linseman et al., 2001). Briefly, neurons were plated on 35-mm diameter plastic dishes coated with poly-L-lysine at a density of approximately 2.0 x 10^6 cells/mL in basal modified Eagle’s medium containing 10% fetal bovine serum, 25mM KCl, 2mM L-glutamine, and penicillin (100 units/mL)/streptomycin (100 µg/mL).
(Life Technologies, Grand Island, NY). At 24 h after plating the CGNs, cytosine arabinoside (10 µM) was added to the culture medium to limit the growth of non-neuronal cells. In general, experiments were performed after 6-7 days in culture.

### 3.3.3 Cell lysis and immunoblotting

Following treatments as described in the Results section, cell lysates were prepared for Western blotting as previously described (Loucks et al., 2006). The protein concentration of the samples was determined using a protein assay kit (BCA, Thermoscientific, PA, USA) and SDS-polyacrylamide gel electrophoresis was performed using equal amounts of protein followed by transfer to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The entire PVDF membranes, once processed, were blocked in PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA) for 1 h at room temperature (25°C). Subsequently, membranes were incubated for 1 h with primary antibodies diluted in blocking solution. Excess primary antibody was removed by washing the membranes with PBS-T five times over 25 min. The corresponding horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T were incubated with the membranes for 1 h following the washes. Excess secondary antibody was removed by washing with PBS-T five times over 25 min. Immunoreactive proteins were detected by enhanced chemiluminescence.

### 3.3.4 Immunofluorescence microscopy

After treatment, CGNs were washed once with PBS (1 mL/well) and then fixed in 4% paraformaldehyde (1 mL/well) for 1 h at room temperature (25°C). Cells were washed once with PBS (1 mL/well) and then permeabilized and blocked in 0.2% Triton X-100 and 5% BSA in PBS (pH 7.4). Primary antibodies were diluted in 2% BSA and
0.2% Triton X-100 in PBS. Cells were incubated in the primary antibody for 1 h at room temperature or overnight at 4°C depending on the antibody. After incubation, cells were washed five times in PBS (5 minutes per wash) and then incubated for 1 h at 25°C with Cy3- or FITC-conjugated secondary antibodies and DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS. Following the second incubation, neurons were washed an additional five times with PBS (5 minutes per wash) before the addition of anti-quench (0.1% p-phenylenediamine in 75% glycerol) in PBS. Fluorescent images were captured by using a 63x oil immersion objective on a Zeiss Axioplan 2 microscope equipped with a Cooke Sensicam deep-cooled charge-coupled device (CCD) camera and a Slidebook software-analysis program for digital deconvolution (Intelligent Imaging Innovations, Inc., Denver, CO).

3.3.5 Quantification of apoptosis

Following treatment, Hoechst dye (8µg/mL, final concentration) was added directly to each well and incubated at 25°C for 30 minutes to stain CGN nuclei. Cells were then washed with PBS (1mL/well) and fixed by incubation in 4% paraformaldehyde (in PBS) for 30 minutes at 25°C. Cells were subsequently washed twice with PBS (1mL/well). To quantify apoptosis CGNs displaying condensed chromatin or nuclear fragmentation were scored as apoptotic. Approximately 300 neurons were counted in a minimum of three 63x fields of a 35-mm dish, and final counts represent data collected from a minimum of three independent experiments performed in duplicate.

3.3.6 MTT viability assay

Following treatment, 187.5 µL of CellQuanti-MTT assay reagent (calculated at 15 µL per 80 µL of culture medium) was added directly to each well. CGNs were incubated
for 4 h at 37°C in 10% CO₂. Blanks, wells containing control media alone, were incubated with 187.5 µL of assay reagent along with samples. Subsequently, the media was aspirated and DMSO was added (2mL/well). Following solubilization (approximately 10 minutes at room temperature), 200 µL of each blank or sample was diluted and mixed in spectrophotometer cuvettes containing 800 µL of sterile water. Samples and blanks were made up in triplicate. Absorbance readings were taken at a wavelength of 570 nm. Data represent five independent experiments performed in triplicate.

3.3.7 Adenovirus preparation and infection

Adenoviral constitutively active MEK5 was purchased from Cell BioLabs (San Diego, CA) and Adenoviral GFP was a kind gift from Marja T. Nevalainen (Thomas Jefferson University, PA, USA) and was prepared as previously described (Ahonen et al., 2003). CGNs were infected on day 5 in vitro with 35 moi (multiplicity of infection) of adenovirus carrying GFP or constitutively active MEK5. At 48 h post-infection, some cells were treated with 175 µM NSC23766 for an additional 48 h. At 72 h post-infection, another group of cells were treated with 60 ng/mL ToxB for an additional 24 h. At 96 h post-infection, cells were fixed for fluorescent imaging and quantification of apoptosis, as described above.

3.3.8 Data analysis

In general, western blotting observations were based upon three independent experiments. Graphical data represent the mean ± SEM for the number of independent experiments performed (n). Statistical differences between the means of unpaired sets of
data were assessed by one-way ANOVA, and further analyzed using a post hoc Tukey’s test. A \( p \)-value of <0.05 was considered statistically significant.

3.4 Results

3.4.1 The specific Rac inhibitor NSC23766 induces inactivation of Rac-GTP resulting in CGN apoptosis

The targeted Rac inhibitor, NSC23766, selectively and reversibly hinders GDP/GTP exchange carried out by the Rac-specific guanine nucleotide exchange factors (GEFs), Tiam1 and Trio. Specifically, this cell-permeable pyrimidine compound targets a tryptophan within the GEF binding site, prohibiting Tiam1 and Trio from binding and activating Rac (Fig. 3.1) (Gao et al., 2004). Consistent with this mechanism of inhibition, CGNs incubated for 48 h with NSC23766 (200 \( \mu \)M) exhibited significantly decreased immuno-reactivity for active Rac (Rac-GTP) compared to control CGNs (Fig. 3.2A). The maintenance of an intact microtubule network, visualized by \( \beta \)-tubulin and MAP2 immunoreactivity, confirmed that the relative lack of Rac-GTP staining observed in NSC23766-treated cells was not due to the global absence or disruption of cellular components (Fig. 3.2B, upper panels). Moreover, examination of nuclei by Hoechst staining revealed significantly increased apoptotic cell death in NSC23766-treated CGNs compared to untreated controls as evidenced by nuclear fragmentation and/or condensation (Fig. 3.2B, lower panels and 3.2C). Collectively, these data indicate that the targeted inhibition of a select pool of Rac GTPase activated by Tiam1 and Trio is sufficient to induce CGN apoptosis.
Figure 3.1 NSC23766 acts by specifically inhibiting the activation of Rac by GEFs Tiam1 and Trio. A. Chemical structure of NSC23766. B. The cell-permeable pyrimidine compound selectively and reversibly hinders GDP/GTP exchange carried out by the Rac-specific guanine nucleotide exchange factors (GEFs), Tiam 1 and Trio. Specifically, NSC23766 targets a key tryptophan residue within the GEF binding site, prohibiting Tiam1 and Trio from binding and activating Rac.
Figure 3.2 The specific Rac inhibitor NSC23766 reduces activation of Rac1, resulting in CGN apoptosis. CGNs were incubated for 48 h in either control medium alone (Con) or containing NSC23766 (200 µM; NSC). A. Cells were then fixed and their nuclei stained with DAPI. Active Rac1 was visualized using a monoclonal antibody that specifically detects Rac1-GTP and a FITC-conjugated secondary antibody. Scale bar = 10 microns. B. Cells were fixed and their nuclei stained with DAPI. The microtubule network was visualized using a monoclonal antibody for β-tubulin conjugated with Cy3 Alexa Fluor 555 and a mouse monoclonal MAP2 antibody followed by a FITC-conjugated secondary antibody. While the microtubule network remains largely intact, CGNs display condensed or fragmented nuclei characteristic of apoptosis following treatment with NSC23766. Bottom panels: black and white images of DAPI-stained nuclei. Scale bar = 10 microns. C. Quantification of CGN apoptosis. Hoechst-stained CGNs displaying condensed and/or fragmented nuclei were scored as apoptotic. Data represent the mean ± SEM of four independent experiments. **Significantly different from control (p< 0.01). I acknowledge Anandi Ramaswami for assistance with generating data for this figure.
3.4.2 CGN death induced by NSC23766 is largely caspase-independent

To determine whether NSC23766-mediated CGN death occurs via a mechanism that is dependent upon activation of caspases, we examined activation of caspase-3 as its proteolytic cleavage signifies commitment to apoptotic cell death (Porter and Jänicke, 1999). In agreement with this mechanism of cell death, CGNs treated with NSC23766 displayed increased immunoreactivity for active (cleaved) caspase-3 (Fig. 3.3A). To definitively establish the involvement of caspase-3 activation in NSC23766-mediated CGN death, we next examined the potential protective effects of caspase inhibitors. CGN viability assessed by an MTT assay was notably reduced in NSC23766-treated cells compared to untreated controls; however, inclusion of either BOC or QVD, pan-caspase inhibitors, had no apparent protective effects against this Rac inhibitor (Fig. 3.3B). In marked contrast, BOC and LEHD, a pan-caspase and caspase-9-selective inhibitor, respectively, significantly protected CGNs treated with the global Rho GTPase inhibitor ToxB (Fig. 3.3C). These data suggest that CGN death induced by targeted inhibition of Rac GTPase occurs via a mechanism that involves a dispensable activation of caspase-3.
Figure 3.3 CGN death induced by NSC23766 is largely caspase-independent, unlike Toxin B-induced apoptosis. A. Cells were incubated for 48 h in either control medium alone (Con) or containing NSC23766 (200 µM; NSC). Cells were then fixed and their nuclei stained with DAPI. Active caspase-3 was visualized using a polyclonal antibody and a Cy3-conjugated secondary. NSC23766 treatment causes increased activation of caspase-3 in CGNs (See arrows) Scale bar = 10 microns. B. Cells were incubated for 48 h in either Con medium or medium containing NSC23766 (200 µM; NSC) ± either BOC (50 µM) or QVD (20 µM). Percentage cell viability was measured by the reduction of MTT. The Con was set as the standard at 100% cell viability. Data represent the mean ± SEM of five independently performed experiments. *Significantly different from control (p < 0.05); NS = No significant difference between NSC and NSC co-treated with caspase inhibitors (BOC or QVD). C. Cells were incubated for 24 h in either Con medium or medium containing ToxB (60 ng/mL) ± BOC (50 µM) or LEHD (100 µM; a caspase-9 inhibitor). Data represent the mean ± SEM of four independently performed experiments. *Significantly different from control (p < 0.05); †† Significantly different from ToxB (p < 0.01). I acknowledge Anandi Ramaswami for assistance with generating data for this figure.
3.4.3 NSC23766 reduces the phosphorylation of PAK, but does not suppress the downstream MEK1/2 and ERK1/2 signaling cascade in CGNs

We have previously reported that broad Rho GTPase inhibition with ToxB deactivates a pro-survival MAP kinase signaling cascade involving PAK1/2/3, MEK1/2, and ERK1/2. Thus, we examined if this signaling cascade was similarly suppressed in NSC23766-treated CGNs. While decreased levels of pPAK1/2/3 were detected in CGNs incubated with NSC23766 for 48 h, specific Rac inhibition did not result in decreased phosphorylation of MEK1/2 or ERK1/2 (Fig. 3.4). These data suggest that targeted inhibition of a select pool of Rac activated by Tiam1 and Trio in CGNs induces cell death through a mechanism that is distinct from global inhibition of Rho GTPases with ToxB.
Figure 3.4 NSC23766 reduces the phosphorylation of PAK, but does not suppress the downstream MEK1/2 and ERK1/2 signaling cascade in CGNs. CGNs were incubated for 48 h in either control medium alone (Con) or containing NSC23766 (200 µM; NSC). Cells were then lysed, proteins were resolved using SDS-PAGE, and PVDF membranes were immunoblotted with antibodies to pPAK1/2, total PAK1/2/3, pMEK1/2, pERK1/2, and Actin. The blots shown are representative of three independent experiments. I acknowledge Anandi Ramaswami for assistance with generating data for this figure.
3.4.4 NSC23766, but not ToxB, decreases MEK5/ERK5 phosphorylation in CGNs

The above results suggested the possibility that the more targeted Rac inhibitor may work through suppressing MEK5 and ERK5, components of an alternative MAP kinase signaling cascade, to repress pro-survival signaling downstream of Rac and PAK. Indeed, immunoblot analysis indicated that NSC23766 treatment caused decreased phosphorylation of both MEK5 and ERK5 in CGNs. Conversely, ToxB treatment had little to no effect on either pMEK5 or pERK5 expression in CGNs (Fig. 3.5). These data indicate that the targeted inhibition of Rac GTPase may elicit cell death via deactivation of an alternative pro-survival MAP kinase pathway downstream of PAK, MEK5/ERK5, when compared to ToxB-treated CGNs.
Figure 3.5 NSC23766, but not ToxB, decreases MEK5/ERK5 phosphorylation in CGNs. CGNs were incubated in either control medium alone (Con), medium containing NSC23766 (200 µM; NSC) for 48 h, or medium containing ToxB (60 ng/mL) for 24 h. Cells were then lysed, proteins were resolved using SDS-PAGE, and PVDF membranes were immunoblotted with antibodies to pMEK5, pERK5, and total ERK5. The blots shown are representative of three independent experiments. I acknowledge Anandi Ramaswami for assistance with generating data for this figure.
3.4.5 NSC23766 treatment in CGNs induces dephosphorylation of p90Rsk and Akt, leading to the upregulation and mitochondrial localization of the pro-apoptotic BH3-only protein Bad

A recent study demonstrated that neuronal cell death induced by deletion of ERK5 is correlated with a marked decrease in phosphorylation of p90Rsk and Akt (Finegan et al., 2009). To determine if the loss of ERK5 phosphorylation induced by selective inhibition of Rac has a similar effect in CGNs, we examined the phosphorylation of p90Rsk and Akt in NSC23766-treated CGNs. Consistent with this previous study, we report distinct decreases in phosphorylated p90Rsk and Akt in NSC23766-treated CGNs when compared to controls (Fig. 3.6A and 3.6B). One of the shared pro-survival functions of p90Rsk and Akt is to suppress the expression of Bad via repression of Bad transcription by the cAMP response element-binding protein (CREB) (Bonni et al., 2009; Finegan et al., 2009). Bad is a member of the Bcl-2 family of proteins that promotes apoptosis through its ability to heterodimerize with, and thus inhibit, pro-survival members of this family, such as Bcl-xL (Kelekar et al., 1997; Kitada et al., 1998). We report that treatment with the targeted Rac inhibitor, NSC23766, resulted in upregulation of pro-apoptotic Bad, an effect that was not observed with ToxB (Fig. 3.6C). Interestingly, both of the inhibitors also triggered the cleavage of Bad (Fig. 3.6C), producing a 15kDa truncated form of the protein which is reportedly a more potent inducer of apoptosis (Condorelli et al., 2001).

We next examined Bad phosphorylation as it has been demonstrated that the addition of a phosphate group at either Ser112 or Ser136 inactivates the pro-apoptotic function of Bad by sequestering the protein away from the mitochondria to 14-3-3
scaffolding proteins in the cytosol (Zha et al., 1996). While we did not detect a change in pBad (Ser112; data not shown), expression of pBad (Ser136) was markedly reduced in NSC23766-treated, but not ToxB-treated, CGNs (Fig. 3.6D). Prior reports have characterized a critical pro-survival function for Akt–dependent phosphorylation of Bad at Ser136 (Datta et al., 1997; del Peso et al., 1997). In agreement with these studies, NSC23766-treated CGNs also displayed a marked reduction in pAkt (Ser473; Fig. 3.6B). In contrast, we have previously reported that ToxB treatment does not alter the phosphorylation of Akt in CGNs (Linseman et al., 2001). Because the Akt-mediated phosphorylation of Bad targets it to 14-3-3 scaffolding proteins in the cytosol, we next analyzed if the dephosphorylation of Bad induced by inhibition of Rac was associated with translocation of this BH3-only protein to mitochondria. Immunofluorescent staining for Bad in control CGNs revealed a diffuse localization in the cytosol of the cell bodies with little detectable staining in cell processes (Fig. 3.6E, left panels). In ToxB-treated CGNs, a similar distribution of Bad was observed in cells not actively undergoing apoptosis, while clearly apoptotic cells displayed undetectable levels of Bad expression (Fig. 3.6E, right panels). In marked contrast to these results, CGNs incubated with the selective Rac inhibitor, NSC23766, showed a striking redistribution of Bad to neuronal processes and an overall punctuate distribution of the protein (Fig. 3.6E, middle panels). To establish if the punctuate appearance of Bad induced by the inhibition of Rac was due to its translocation to mitochondria, CGNs were subfractionated into mitochondrial and cytosolic fractions which were then immunoblotted for Bad. In control CGNs Bad was exclusively localized to the cytosolic fraction, while in NSC2376-treated CGNs Bad was exclusively found in the mitochondrial fraction (Fig. 3.6F). Collectively, these data
indicate that targeted inhibition of Rac with NSC23766 induces CGN apoptosis by turning off p90Rsk and Akt, likely downstream of MEK5/ERK5, leading to enhanced expression, dephosphorylation, and mitochondrial localization of pro-apoptotic Bad. In contrast, neither Bad dephosphorylation nor Bad translocation to mitochondria appears to be induced by ToxB.
Figure 3.6 NSC23766 treatment in CGNs deactivates p90Rsk and Akt leading to increased expression, dephosphorylation, and mitochondrial localization of the pro-apoptotic BH3-only protein Bad. A. CGNs were incubated in either control medium alone (Con) or medium containing NSC23766 (200 µM; NSC) for 48 h. Cells were then lysed, proteins were resolved using SDS-PAGE, and PVDF membranes were immunoblotted with antibodies to phospho-p90Rsk and subsequently stripped and reprobed for β-tubulin as a loading control. B. CGNs were incubated in either control medium alone (Con) or medium containing NSC23766 (200 µM; NSC) for various periods up to 48 h. Cell lysates were prepared for immunoblotting as described in (A) and PVDF membranes were blotted for pAkt (Ser473). Subsequently, membranes were stripped and reprobed for Actin as a loading control. C. CGNs were treated and prepared for immunoblotting as described in (A) with the addition of a ToxB-treated condition for 24 h. Cell were lysed and PVDF membranes were blotted for total Bad and subsequently stripped and reprobed for Actin as a loading control. D. CGNs were incubated as described in (C). Cell lysates were prepared and PVDF membranes were blotted for pBad (Ser136) and subsequently stripped and reprobed for Actin as a loading control. E. CGNs were incubated as described in (C). Cells were then fixed and their nuclei stained with DAPI. Bad was visualized using a polyclonal antibody and a Cy3-conjugated secondary. Images are representative of three independent experiments. Scale bar = 10 microns. F. CGNs were incubated as described in (C) and cells were subfractionated into mitochondrial and cytosolic fractions. Subcellular fractions were immunoblotted for Bad and OPA1 (mitochondrial marker). Blots shown are representative of three independent experiments.
3.4.6 Unlike ToxB, NSC23766 treatment does not activate a pro-apoptotic JNK/c-Jun/Bim pathway in CGNs

We have previously shown that ToxB-mediated repression of MEK1/2/ERK1/2 signaling induces intrinsic apoptosis of CGNs via decreased phosphorylation, and thus diminished degradation, of the pro-apoptotic BH3-only protein Bim (Loucks et al., 2006). In addition, we revealed that ToxB-treated CGNs displayed increased activation of a JNK/c-Jun signaling cascade that led to enhanced transcription of Bim (Le et al., 2005). Therefore, we next examined whether targeted inhibition of Rac in CGNs had a similar effect on c-Jun and/or Bim. NSC23766-treated CGNs did not display increased c-Jun expression at any time point up to 48 h. As expected, ToxB treatment elevated c-Jun expression in CGNs within 8 h of treatment (Fig. 3.7A). To investigate the potential involvement of JNK activation in promoting CGN death downstream of Rac inhibition, we examined the neuroprotective effects of the JNK inhibitor SP600125 (JNK Inh). While inclusion of the JNK Inh did not significantly protect CGNs from selective Rac inhibition with NSC23766, the inhibitor significantly mitigated CGN apoptosis in response to ToxB treatment (Fig. 3.7B, and 3.7C). Consistent with activation of a pro-death JNK/c-Jun signaling pathway acting upstream of the BH3-only protein Bim, the expression of Bim_{short} was elevated in ToxB-treated CGNs. In contrast, we did not detect an increase in Bim expression in NSC23766-treated CGNs at any time point up to 48 h (Fig. 3.7D). These results indicate that broad inhibition of Rho GTPases with ToxB elicits CGN apoptosis primarily through the upregulation of Bim in a JNK/c-Jun-dependent manner, while neither JNK/c-Jun signaling nor Bim appear to play a significant role in NSC23766-mediated CGN death.
Figure 3.7 Unlike ToxB, NSC23766 treatment does not activate a pro-apoptotic JNK/c-Jun/Bim pathway in CGNs. A. CGNs were incubated in either control medium alone (Con), medium containing ToxB (60 ng/mL), or medium containing NSC23766 (200 µM; NSC) for various periods up to 8 h and 48 h, respectively. Cells were then lysed, proteins were resolved using SDS-PAGE, and PVDF membranes were immunoblotted with an antibody against c-Jun and subsequently stripped and reprobed for Actin as a loading control. B. CGNs were incubated in either control medium alone (Con), medium containing ToxB (40 ng/mL) for 24 h, or medium containing NSC23766 (200 µM; NSC) for 48 h. In addition, some cells were exposed to NSC or ToxB in the presence of the JNK inhibitor (JNK inhibitor; 20 µM). Cells were then fixed and their nuclei stained with Hoescht dye. C. Quantification of CGN apoptosis from three independent experiments conducted as described in (B). *** Significantly different from Con (p<0.001); ††† Significantly different from ToxB (p<0.001); NS = no significant difference from NSC. D. CGNs were incubated in either control medium alone (Con), medium containing ToxB (60 ng/mL) for 8 h, or medium containing NSC23766 (200 µM; NSC) for various periods up to 48 h. Cells were then lysed, proteins were resolved using SDS-PAGE, and PVDF membranes were immunoblotted with an antibody against Bim and subsequently stripped and reprobed for β-tubulin as a loading control. Blots shown are representative of three independent experiments.
To definitively establish the precise MAP kinase signaling pathway that is deactivated in CGNs exposed to NSC23766-targeted Rac inhibition versus ToxB-mediated global Rho GTPase inhibition, we examined the protective effects of an adenoviral constitutively active mutant of MEK5 (Ad-CA MEK5), in which the dual phosphorylation site S311/S315 has been changed to D311/D315. Infection with Ad-CA MEK5, but not Ad-GFP, increased the expression of MEK5 in CGNs (Fig. 3.8A). It is important to note that neither adenoviral construct increased the basal level of apoptosis on their own (data not shown). As we have previously shown, global inhibition of Rho GTPases with ToxB resulted in significant CGN apoptosis (Fig. 3.8B). Consistent with ToxB inducing death through a mechanism independent of MEK5/ERK5 signaling, pre-incubation of CGNs with either Ad-GFP or Ad-CA MEK5 did not confer significant protection against ToxB treatment (Fig. 3.8B and 3.8C). Conversely, while CGNs exposed to selective inactivation of Rac GTPase with NSC23766 showed condensed and fragmented nuclei indicative of apoptotic cell death, CGNs pre-incubated with Ad-CA MEK5, but not Ad-GFP, displayed nuclear morphology strikingly more similar to controls (Fig. 3.8D). Quantification of these results demonstrated that adenoviral infection with Ad-CA MEK5 significantly protected CGNs from NSC23766 treatment (Fig. 3.8E). It is worth noting that the protection conferred in CGNs pre-incubated with Ad-CA MEK5 and subsequently treated with NSC23766 was not complete, presumably because the infection efficiency was approximately 40-50% as estimated by the percentage of CGNs expressing Ad-GFP. Nonetheless, these results further corroborate
that selective inactivation of Rac GTPase versus global inhibition of Rho GTPases induces CGN death through deactivation of unique pro-survival MAP kinase signaling pathways.
Figure 3.8 Adenoviral infection with constitutively active MEK5 protects CGNs from NSC23766 treatment, but not ToxB. A. CGNs were incubated in control medium (Con) or infected for 48 h with an adenovirus carrying GFP (Ad-GFP) or constitutively active MEK5 (Ad-CA MEK5). Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted for MEK5 expression. Blots were subsequently stripped and reprobed for actin expression. B. CGNs were infected for 48 h with either Ad-GFP or Ad-CA MEK5. After the 48 h incubation period, CGNs were treated with ToxB (40ng/mL) for an additional 24 h as indicated. At the end of the incubation, cells were fixed and nuclei were stained with DAPI. Scale bar = 10 microns. C. Quantification of apoptosis from cells treated as described in (B). D. CGNs were infected for 48 h with either Ad-GFP or Ad-CA MEK5. After the 48 h incubation period, CGNs were treated with NSC23766 (NSC; 175 µM) for an additional 48 h as indicated. At the end of the incubation, cells were fixed and nuclei were stained with DAPI. Scale bar = 10 microns. E. Quantification of apoptosis from cells treated as described in (D). Data represent the mean ± S.E. of three independently performed experiments. *Significantly different from control ($p < 0.05$); **Significantly different from control ($p < 0.001$); † Significantly different from NSC ($p < 0.05$). NS denotes not significant.
3.5 Discussion

While a number of studies have highlighted a critical pro-survival role for Rho family GTPases in various neuronal cell types (Linseman et al., 2001; Kobayashi et al., 2004; Kanekura et al., 2005; Le et al., 2005; Loucks et al., 2006; Stankiewicz et al., 2012), the mechanisms by which these G-proteins induce their neuroprotective effects remain largely unknown. In previous studies, we have utilized *Clostrium difficile* ToxB as a selective inhibitor of Rac, Rho, and Cdc42 to reveal that PAK/MEK/ERK and JNK/c-Jun signaling cascades, components of the Rac-activated MAP kinase pathway, critically regulate CGN survival (Le et al., 2005; Loucks et al., 2006). To advance our understanding of Rho family GTPases in neuronal survival, we compared the effects of ToxB to NSC23766, an inhibitor which specifically prevents Rac activation by the GEFs Tiam1 and Trio (Gao et al., 2004). Initial results confirmed that NSC23766 promotes CGN death through the loss of GTP-bound (active) Rac. However, while both ToxB and NSC23766 induce CGN death in the presence of serum and depolarizing potassium, our current study indicates that CGNs succumb to cell death via unique signaling pathways in response to specific Rac inhibition versus global Rho GTPase inhibition.

Similar to ToxB, NSC23766 induces activation of caspase-3 in CGNs; however, while ToxB-induced CGN death was sensitive to caspase inhibitors, the effects of NSC23766 on neuronal death were not mitigated to a significant degree by co-incubation with pan-caspase inhibitors. This is a key point of divergence between the effects of ToxB and NSC23766 on neuronal survival and indicates that NSC23766 is capable of promoting CGN death through a caspase-independent pathway. Given the antagonistic relationship between Rac and Rho signaling, it is possible that specific inhibition of only
Rac via NSC23766 unmask a Rho-dependent pathway that does not rely on caspase activation for the execution of CGN death. Indeed, Rac directly suppresses Rho activity through binding and activating the Rho GAP, p190RhoGAP, in HeLa cells (Wildenberg et al., 2006). Moreover, the downstream effector of Rac, PAK, has been shown to regulate the activation of Rho through GEFs in a variety of cell types (Hakoshima et al., 2003). Consistent with this idea, Rho has been demonstrated to sensitize corticohippocampal neurons to cell death (Barberan et al., 2011), and inhibition of the Rho effector, ROCK, exerts a neuroprotective effect in various in vitro and in vivo models of neuronal cell death such as, cerebral ischemia and excitotoxicity (Rikitake et al., 2005; Jeon et al., 2013). In addition to ROCK, Rho GTPase has many other downstream effectors that could contribute to neuronal death via a mechanism that is independent of caspase activation.

The second contrast between the activities of ToxB and NSC23766 was apparent in their divergent effects upon MAP kinase signaling. While ToxB suppressed MEK1/2/ERK1/2 signaling, the selective Rac inhibitor had no effect of this MAP kinase pathway and instead suppressed a unique MEK5/ERK5 cascade. Indeed, adenoviral expression of constitutively active MEK5 was only sufficient to mitigate the toxic effects of the selective Rac inhibitor, NSC23766, in CGNs and displayed no protective effect against global Rho GTPase inhibition with ToxB. It is possible that this stark contrast may be attributed to differential regulation of PAK. Upstream of the MEK/ERK signaling cascades, both Rho family GTPase inhibitors reduced the phosphorylation of PAK in CGNs. However, one limitation of these findings is that although we have utilized an antibody that detects phosphorylation of PAK1/2/3, there are six known isoforms of
PAK. Thus, regulation of unique MAP kinase pathways in response to Rac inhibition may be a result of differential inactivation of specific PAK isoforms. Furthermore, an additional member of the Rho GTPase family that is inhibited by ToxB, Cdc42, also regulates PAK activity in a manner similar to Rac (Kreis et al., 2007). Inactivation of distinct PAK isoforms in response to targeted inhibition of Rac versus global inhibition of Rho GTPases may represent a key point at which the signaling cascades regulated by these inhibitors deviate.

Although deactivation of specific PAK isoforms is a potential point at which the signaling cascades regulated by distinct Rho family GTPases may diverge, this could also be accomplished downstream of PAK signaling through mitogen-activated protein kinase kinase kinases (MEKKs). MEKKs are upstream regulators of MAP kinase pathways, such as MEK/ERK, c-Jun NH$_2$-terminal kinases (JNK), and p38 (Fanger et al., 1997; Sun et al., 2001). As the most well studied MAP kinase signaling pathway, MEK1/2 activation has largely been attributed to MEKKs that belong to the Raf family of proteins (i.e., A-Raf, Raf-1, B-Raf). This may be a critical point of divergence in MEK/ERK signaling as recent studies have demonstrated that MEKK2 and MEKK3 specifically activate the downstream effector MEK5 via heterodimerization of a PB1 domain that is specific to MEKK2, MEKK3, and MEK5 (Sun et al., 2001; Nakamura and Johnson, 2003). Furthermore, while both MEKK2 and MEKK3 bind to MEK5 and activate the MEK5/ERK5 pathway, it was shown that MEKK2 binds MEK5 with higher affinity, stimulating ERK5 activity to a greater extent (Sun et al., 2001). Collectively, these previous studies suggest that the distinct MAP kinase pathways which are deactivated by
either ToxB or NSC23766 in CGNs may diverge downstream of PAK at the level of specific MEKK activation.

Overall, these data suggest that CGN death triggered by the targeted inhibition of a select pool of Rac activated by the GEFs Tiam1 and Trio, involves the suppression of a MEK5/ERK5 signaling cascade to induce apoptosis. This is in agreement with previous studies highlighting a critical pro-survival function for ERK5 in dopaminergic neurons, as well as, sympathetic neurons (Cavanaugh et al., 2006; Finegan et al., 2009). Moreover, past studies have indicated that ERK5 is activated by MEK5 but not by MEK1 or MEK2, speaking to the highly specific nature of the MEK5/ERK5 interaction (English et al., 1995; Zhou et al., 1995). Finally, others have demonstrated that Rac1 can signal to MEK5/ERK5 (Cheng et al., 2012). This Rac effect presumably occurs through PAK-mediated activation of MEKK2/3 and their subsequent activation of MEK5 (Porter et al., 1999; Nakamura and Johnson, 2003). Thus, selective inhibition of Rac is capable of suppressing a putative PAK/MEKK2/3/MEK5/ERK5 pro-survival pathway.

Previous experimentation has shown that ERK5 is capable of triggering a phosphorylation cascade that results in the phosphorylation of the MAPK-activated kinase, p90Rsk, and the subsequent activation of the transcription factor cAMP response element binding protein (CREB), which regulates transcription of pro-survival and pro-apoptotic genes in neurons, including repression of the pro-apoptotic Bad gene (Watson et al., 2001; Park and Cho, 2006; Ranganathan et al., 2006; Ha and Redmond, 2008). Accordingly, recent findings in neurons indicate that, in the absence of ERK5, phosphorylation of p90Rsk is impaired and cell death is elicited in a Bad-dependent manner (Finegan et al., 2009). Consistent with the observed reduction in phosphorylated
p90Rsk in response to NSC23766, we report that NSC23766 treatment also elicited upregulation of Bad in CGNs. Given that ToxB did not enhance Bad expression, it appears that deactivation of p90Rsk is unique to NSC23766-treated CGNs. Interestingly, both NSC23766 and ToxB induced the cleavage of Bad, generating elevated levels of a 15kDa cleavage product. This is consistent with a previous report which revealed that caspase cleavage of Bad at its N-terminus produces a 15 kDa truncated form of Bad that is a more effective inducer of apoptosis than the full length protein (Condorelli et al., 2001). However, given that pan-caspase inhibitors do not protect NSC23766-treated CGNs to a significant extent, our data suggest that caspase cleavage of Bad is not the prominent mechanism of death in CGNs exposed to NSC23766. Together, these data suggest an important role for the MEK5/ERK5 pathway in neuronal survival. Specifically, targeted inhibition of a discrete pool of Rac activated by Tiam1 and Trio appears to trigger CGN death through repression of a novel pro-survival pathway involving PAK, possibly MEKK2/3, and the MEK5/ERK5 signaling cascade including its downstream targets, p90Rsk and Bad (Fig. 3.9).
Figure 3.9 Conclusion: comparing CGN death induced by NSC23766 and ToxB. The concluding schematic illustrates the Rac-dependent signaling cascades examined in this study. A. Pathway regulated by the Rho GTPases Rac, Rho, and Cdc42. B. Pathway regulated by the specific pool of Rac activated by the GEFs Tiam1 and Trio. Dashed lines represent hypothesized effects. Our findings suggest that targeted inhibition of the specific pool of Rac activated by the GEFs, Tiam1 and Trio, promotes CGN death through an alternative pathway to that employed by the broad-spectrum Rho family inhibitor, ToxB. These pathways diverge downstream of PAK, with NSC23766 repressing the MEK5/ERK5 signaling cascade while ToxB inhibits MEK1/2 and ERK1/2. ToxB also induces the activation of JNK/c-Jun, a pathway that is not activated by NSC23766. ToxB ultimately results in decreased degradation and enhanced transcription of Bim while NSC23766 promotes the dephosphorylation, mitochondrial localization, and induction of Bad.
In addition to regulation of Bad at the transcriptional level via CREB, p90Rsk also catalyzes the phosphorylation of Bad at serine 112, suppressing Bad-mediated apoptosis in neurons (Bonni et al., 1999, Bertolotto et al., 2000). However, we did not detect any decrease in the phosphorylation of Bad at Ser112 (data not shown). Instead, we observed that NSC23766-treated CGNs exhibited a marked reduction in levels of pBad (Ser136). Interestingly, several studies have highlighted that phosphorylation of Bad at Ser136 may be the more critical 14-3-3 binding site which enhances the association of Bad with cytosolic 14-3-3 scaffolding proteins, effectively sequestering Bad away from mitochondria (Zha et al., 1996, Masters et al., 2001). Several reports have indicated that phosphorylation of Bad at this particular site is largely attributed to Akt activation (Datta et al., 1997; del Peso et al., 1997). This is consistent with our data demonstrating reduced phosphorylation (activation) of Akt (Ser473) in NSC23766-treated CGNs. As we have previously reported that ToxB does not regulate Akt signaling in CGNs (Linseman et al., 2001), the finding that NSC23766-treated CGNs display a marked reduction in activated Akt highlights an additional signaling pathway that is differentially regulated in response to specific Rac inhibition versus global Rho GTPase inhibition. However, the particular mechanism by which Akt is deactivated in NSC23766-treated CGNs is currently unknown. For instance, although it is well documented that Akt can be phosphorylated and activated by Rac through phophatidylinositol-3 kinase (del Peso et al., 1997; Kanekura et al., 2005), ERK5 has also been shown to modestly activate Akt to promote survival of non-neuronal cells (Lennartsson et al., 2010). Thus, the precise mechanism by which Akt is dephosphorylated following Rac inhibition warrants further investigation. Despite these considerations, our data
indicate that inhibition of a specific pool of Rac activated by the GEFs Tiam1 and Trio induces apoptosis through a mechanism consistent with Akt deactivation and subsequent dephosphorylation and translocation of Bad to the mitochondria.

Finally, in addition to the distinct differences in MEK/ERK, p90Rsk, and Akt signaling, ToxB and NSC23766 also appear to dissimilarly regulate JNK/c-Jun signaling in CGNs. While we previously reported that ToxB induced mitochondrial-dependent CGN apoptosis through a mechanism consistent with JNK/c-Jun-dependent transcriptional upregulation of the BH3-only protein Bim (Le et al., 2005), we currently report that c-Jun was not induced in CGNs exposed to the specific Rac inhibitor NSC23766. Moreover, inclusion of the JNK inhibitor, SP600125, did not exert a protective effect in NSC23766-treated CGNs. Furthermore, although we report an increase in expression of the pro-apoptotic protein Bim in response to ToxB in CGNs, Bim was not elevated above control levels in NSC23766-treated CGNs. Thus, while ToxB appears to induce CGN death through a mechanism dependent upon JNK/c-Jun-mediated induction of Bim, Bim does not appear to execute cell death in CGNs exposed to the specific Rac inhibitor NSC23766. Many studies have indicated that Rac and Cdc42 can regulate JNK activation; thus this distinction in signaling may be due to the intrinsic differences between inactivation of specifically Rac versus global inhibition of Rho GTPases.

In conclusion, the specificity with which NSC23766 suppresses Rac activity allowed us to further investigate signaling pathways regulated by this Rho family GTPase which are involved in neuronal survival. We have revealed that CGNs exposed to ToxB or NSC23766 succumb to neuronal death through inactivation of distinct MAP kinase
signaling pathways. Comparison of the mechanisms behind specific Rac inhibition with those associated with broader Rho family GTPase inhibition has been central to elucidating the signaling pathways that regulate survival in CGNs. It is interesting to note that inhibition of the select pool of Rac activated by Tiam1 and Trio is sufficient to induce dysregulation of multiple signaling pathways including MEK5/ERK5, p90Rsk, and Akt. Furthermore, the critical function of Rac in maintaining neuronal survival is highlighted by the fact that targeted inhibition of Rac is sufficient to induce CGN death in the presence of both serum and depolarizing potassium. Accordingly, this study serves to advance our understanding of the key pro-survival roles of Rho family GTPases in neurons. These findings may ultimately contribute to the discovery of novel signaling molecules which could be targeted therapeutically for debilitating neurodegenerative diseases, such as amyotrophic lateral sclerosis, for which loss of Rac function is thought to play a significant role in neuronal cell death.
CHAPTER FOUR: C-TERMINAL BINDING PROTEINS ARE ESSENTIAL PRO-SURVIVAL FACTORS THAT UNDERGO CASPASE-DEPENDENT DOWNREGULATION DURING NEURONAL APOPTOSIS

4.1 Abstract

C-terminal binding proteins (CtBPs) are transcriptional co-repressors that are subject to proteasome-dependent downregulation during apoptosis. Alternative mechanisms that regulate CtBP expression are currently under investigation and the role of CtBPs in neuronal survival is largely unexplored. Here, we show that CtBPs are downregulated in cerebellar granule neurons (CGNs) induced to undergo apoptosis by a variety of stressors. Moreover, antisense-mediated downregulation of CtBP1 is sufficient to cause CGN apoptosis. Similarly, the CtBP inhibitor, 4-methylthio-2-oxobutyric acid, induces expression of the CtBP target Noxa and causes actinomycin-sensitive CGN apoptosis. Unexpectedly, we found that the mechanism of CtBP downregulation in CGNs undergoing apoptosis varies in a stimulus-specific manner involving either the proteasome or caspases. In the case of CGNs deprived of depolarizing potassium (5K apoptotic condition), caspases appear to play a dominant role in CtBP downregulation. However, incubation in 5K does not enhance the kinetics of CtBP1 degradation and recombinant CtBP1 is not cleaved in vitro by caspase-3. In addition, 5K has no significant effect on CtBP transcript expression. Finally, mouse embryonic stem cells display caspase-dependent downregulation of CtBP1 following exposure to staurosporine, an effect that is not observed in DGCR8 knockout cells which are deficient
in miRNA processing. These data identify caspase-dependent downregulation of CtBPs as an alternative mechanism to the proteasome for regulation of these transcriptional co-repressors in neurons undergoing apoptosis. Moreover, caspases appear to regulate CtBP expression indirectly, at a post-transcriptional level, and via a mechanism that is dependent upon miRNA processing. We conclude that CtBPs are essential pro-survival proteins in neurons and their downregulation contributes significantly to neuronal apoptosis via the de-repression of pro-apoptotic genes.

4.2 Introduction

C-terminal binding proteins (CtBPs) were originally identified as binding partners for the adenovirus E1A transforming proteins (Schaeper et al., 1995). CtBP1 and CtBP2 are highly homologous transcriptional co-repressors that function as partners for repressor proteins like ZEB and basic krüppel-like factor (BKLF) (Postigo and Dean, 1999 and Turner and Crossley, 1998). CtBPs recruit a number of key chromatin modifying enzymes (e.g., histone deacetylases) to gene promoters primarily via PXDLS-dependent interactions with the CtBP hydrophobic cleft (Kuppuswamy et al., 2008 and Quinlan et al., 2006). In a similar manner, CtBPs repress p300-dependent transcriptional activation by directly binding to a PXDLS motif in the bromodomain of this co-activator (Kim et al., 2005). CtBPs are directed to the nuclear compartment either via a nuclear localization signal (which is unique to CtBP2) or by binding to PXDLS motif-containing partners like BKLF (Verger et al., 2006). CtBPs are functional dehydrogenases which bind to NADH with greater than 100-fold higher affinity than NAD+ (Fjeld et al., 2003, Kumar et al., 2002 and Zhang et al., 2002). Binding of NAD(H) appears to stabilize the protein and promotes dimerization of CtBPs which is
required for transcriptional repression (Fjeld et al., 2003, Kuppuswamy et al., 2008 and Mani-Telang et al., 2007). Thus, CtBPs act as redox-sensitive transcriptional co-repressors of a specific subset of target genes.

CtBPs are key transcriptional co-repressors of epithelial and pro-apoptotic gene expression programs (Bergman and Blaydes, 2006 and Grootecaes et al., 2003). By repressing epithelial cell adhesion (via repression of E-cadherin) and concomitantly suppressing apoptosis and anoikis, CtBPs promote cancer cell migration, invasion, and survival (Grootecaes and Frisch, 2000 and Straza et al., 2010). In the context of apoptosis, CtBPs act as co-repressors at several pro-apoptotic Bcl-2 family member gene promoters, such as Bax and the Bcl-2 homology-3 domain (BH3)-only proteins, Noxa, Bik, Bim, and Bmf (Bergman and Blaydes, 2006, Grootecaes et al., 2003 and Kovi et al., 2010). Murine embryonic fibroblasts (MEFs) isolated and immortalized from Ctbp1/Ctbp2 double knockout embryos show constitutive upregulation of Bax and Noxa, and demonstrate enhanced sensitivity to diverse apoptotic stimuli (Grootecaes et al., 2003). Both the increased expression of Bax and Noxa, as well as the enhanced susceptibility to apoptosis, were reversed by Ctbp1 or Ctbp2 rescue expression.

To date, relatively few studies have examined the roles of CtBPs in CNS development or neuronal survival. Based largely on the results of genetic deletion experiments, it appears that Ctbp1 and Ctbp2 display both duplicative and independent roles in mouse development including maturation of the CNS (Hildebrand and Soriano, 2002). Ctbp2 homozygous null mice display delayed development of the forebrain and midbrain, and typically die by E10.5. In contrast, Ctbp1 homozygous null mice are viable and fertile. In a genetic interaction experiment, increasing the dosage of Ctbp1 decreased
the severity of the Ctbp2 null phenotype. For instance, Ctbp1+/− Ctbp2−/− embryos did not complete neural tube closure and arrested at the turning stage while Ctbp1+/+ Ctbp2−/− embryos completed both processes. In the context of cell survival, CtBPs are targeted for proteasomal degradation in response to pro-apoptotic stimuli that induce p53-independent apoptosis in non-neuronal cells (Paliwal et al., 2006, Wang et al., 2006, Zhang et al., 2003 and Zhang et al., 2005). In contrast, the role of CtBPs in neuronal apoptosis has not previously been explored. Here, we identify a novel caspase-dependent pathway for CtBP downregulation during neuronal apoptosis and further show that loss of CtBP function is sufficient to induce neuronal cell death.

4.3 Materials and Methods

4.3.1 Reagents

Clostridium difficile Toxin B (ToxB) and Clostridium sordellii lethal toxin (LTox) were kindly provided by Dr. Klaus Aktories (Albert-Ludwigs-Universität Freiburg, Germany). The high-throughput immunoblotting screen was performed by BD Pharmingen (Palo Alto, CA, USA) and monoclonal antibodies used for subsequent western blotting of CtBP1 and CtBP2 were obtained from BD Biosciences (San Diego, CA, USA). Polyclonal antibody against actin was obtained from Cell Signaling (Beverly, MA, USA). The polyclonal antibody used to detect Noxa was from Abcam (Cambridge, MA, USA). Horseradish peroxidase-linked secondary antibodies and reagents for enhanced chemiluminescence detection were from Amersham Biosciences (Piscataway, NJ, USA). The polyclonal antibody used to detect active caspase-3 was from Promega (Madison, WI, USA). 4,6-Diamidino-2-phenylindole (DAPI), Hoescht dye
33258, monoclonal antibody against β-tubulin, 1-methyl-4-phenylpyridinium (MPP +), 6-hydroxydopamine (6-OHDA), 4-methylthio-2-oxobutyric (MTOB), staurosporine, actinomycin D, and recombinant PARP were from Sigma (St. Louis, MO, USA). Cy3- and FITC-conjugated secondary antibodies for immunofluorescence were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). HA14-1 and BOC were obtained from Alexis (San Diego, CA, USA). MG-132, sodium nitroprusside (SNP), and recombinant caspase-3 were from Calbiochem (Darmstadt, Germany). The polyclonal antibody to PARP was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Morpholino-antisense oligonucleotides and the EndoPorter delivery reagent were obtained from Gene Tools (Philomath, PA, USA). Wild type and DGCR8 knockout mouse embryonic stem cells, as well as, recombinant CtBP1 were obtained from Novus Biologicals (Littleton, CO, USA).

4.3.2 Cerebellar granule neuron (CGN) culture

Rat CGNs were isolated from 7-day-old Sprague-Dawley rat pups of both sexes (15–19 g) as previously described (Linseman et al., 2001). CGNs were plated on 35-mm diameter plastic dishes coated with poly-L-lysine at a density of 2.0 × 10^6 cells/ml in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY, USA). Cytosine arabinoside (10 µM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. With use of this protocol, the cultures were approximately 95% pure for granule neurons. In general, experiments were performed after 6–7 days in culture.
4.3.3 BD Pharmingen PowerBlot™ analysis

CGNs were incubated in either control medium or medium containing 40 ng/ml *Clostridium difficile* Toxin B (ToxB) for 24 h and subsequently lysed according to the manufacturer's protocol. Lysates from three independent experiments were pooled and subjected to high-throughput immunoblotting against a panel of 1009 purified monoclonal antibodies (BD PowerBlot™). Raw data was obtained from the manufacturer in the form of image files of the actual blots and densitometric measurements of the immunoreactive proteins. The blots shown for CtBP1, CtBP2, and G protein-coupled receptor kinase-interacting protein-z-short are representative of $2 \times 2$ comparisons of duplicate control and ToxB lysates.

4.3.4 Cell lysis and immunoblotting

Following treatment, whole cell lysates of CGNs were prepared essentially as previously described (Loucks et al., 2006). Briefly, protein concentrations were determined by a commercially available protein assay kit (BCA; Thermo Fisher Scientific, Waltham, MA, USA), and SDS-polyacrylamide gel electrophoresis was performed using equal amounts of protein followed by transfer to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked in PBS-T (1X phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Tween 20) containing 1% BSA and 0.01% sodium azide for 1 h at room temperature (22 °C). Membranes were incubated for 1 h in primary antibody diluted in blocking solution. Membranes were subsequently washed 5 times over 30 min in PBS-T to remove excess primary antibody. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T. Following secondary incubation, membranes were washed 5 times over
30 min in PBS-T to remove excess secondary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence. Blots shown are representative of a minimum of three independent experiments.

4.3.5 Immunofluorescence microscopy

CGNs were plated at a density of 4.5 × 10⁵ cells per ml on glass coverslips coated with polyethylene-imine. After treatment, cells were fixed in 4% paraformaldehyde, washed once in PBS, and permeabilized and blocked in 0.2% Triton X-100 and 5% BSA in PBS (pH 7.4). Cells were incubated for 16 h at 4 °C in primary antibodies diluted in 2% BSA and 0.2% Triton X-100 in PBS. They were subsequently washed 5 times in PBS over 30 min and then incubated for 1 h at room temperature with Cy3- or FITC-conjugated secondary antibodies and DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS. The cells were washed five additional times over 30 min with PBS before mounting coverslips onto slides with anti-quench composed of 0.1% p-phenylenediamine in 75% glycerol in PBS. Fluorescent images were captured using a 63x oil immersion objective on a Zeiss Axioplan 2 epifluorescence microscope that was equipped with a Cooke Sensicam deep-cooled charge-coupled device (CCD) camera and a Slidebook software analysis program for digital deconvolution (Intelligent Imaging Innovations Inc., Denver, CO, USA).

4.3.6 Morpholino-antisense oligonucleotide treatment

CGNs were plated at a density of 4.5 × 10⁵ cells per ml on glass coverslips coated with polyethyleneimine. On day 6 in vitro, cells were treated with EndoPorter reagent (6 µM) alone or in combination with morpholino-antisense or inverse oligonucleotides (3 µM final concentration). After 72 h of treatment, cells were fixed with 4%
paraformaldehyde and nuclei were stained with Hoechst dye. CGNs containing condensed and/or fragmented nuclei were scored as apoptotic.

4.3.7 N27 dopaminergic cell culture

N27 cells were maintained in culture in RPMI medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

4.3.8 6-Hydroxydopamine treatment

For treatment with 6-OHDA, the compound was diluted in a vehicle solution consisting of ddH₂O containing 0.15% ascorbic acid and 10 mM DETAPAC. This solution was purged with nitrogen gas for 30 min while on ice. After removal of oxygen, 6-OHDA was added to the solution and used to treat N27 cells at a concentration of 25 µM or 50 µM for 24 h.

4.3.9 Pulse-chase assay

CGNs were incubated for 4 h in cysteine-methionine-free medium containing ³⁵S-methionine. Following this labeling “pulse”, cells were washed and medium was replaced with control (25K) or apoptotic (5K) medium for the “chase” period. At the times indicated, cells were lysed, CtBP1 was immunoprecipitated, and immune complexes were resolved by SDS-PAGE followed by transfer to polyvinylidene difluoride membranes. Membranes were exposed directly to film and bands representing ³⁵S-CtBP1 were quantified by densitometry.

4.3.10 In vitro caspase-3 cleavage assay

In separate microcentrifuge tubes, 5 µg of recombinant PARP or 1.5 µg of recombinant CtBP1 were incubated alone or in combination with 100 units of recombinant caspase-3. All samples were queued to 100 µl with caspase buffer (100 mM
NaCl, 5 mM DTT, 50 mM EDTA, 20 mM PIPES, 1% Chaps, and 10% sucrose in ddH$_2$O). Samples were incubated for 4 h at 37 °C in a thermomixer. Following incubation, proteins were resolved by SDS-PAGE, proteins transferred onto membranes, and immunoblotted as previously described.

4.3.11 RT-PCR

CGNs were incubated in control (25K) or apoptotic (5K) medium for 24 h. RNA was isolated using a miRCURY RNA isolation kit purchased from Exiqon (Woburn, MA, USA). cDNA was synthesized from the isolated RNA samples using an Omniscript reverse transcriptase kit that was purchased from Qiagen (Valencia, CA, USA). DNA was analyzed by polymerase chain reaction (PCR) using an Accuprime Pfx Supermix kit from Life Technologies (Grand Island, NY, USA). Primers were purchased from Integrative DNA Technologies (Coralville, IA, USA) corresponding to CtBP1, CtBP2, and beta-actin. The forward primer to CtBP1 was 5′-TTGGGCAT CATTGGACTAGGTGCTG-3′ and the reverse primer was 5′-TCAGGTGGTCCTTTGTT GACACAGT-3′. The forward primer to CtBP2 was 5′-TGTGATGCACAGTCCACTCAG GAA-3′ and the reverse primer was 5′-CCATTGAACACGGACATTGCACCA-3′. The forward primer to beta-actin was 5′-CCATTG AACACGGCATTGCACCA-3′ and the reverse primer was 5′-ACTCCTGCTTGCTGA TCCACATCT-3′. PCR was performed using the following conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min.
4.3.12 Mouse embryonic stem cell culture

Wild type and DGCR8 knockout mouse embryonic stem cells were purchase from Novus Biologicals (Littleton, CO, USA). Cell culture plates were coated with a solution containing 1% gelatin that was purchased from Millipore (Billerica, MA, USA). Cells were maintained in basal modified Eagle's medium containing 10% fetal bovine serum, 1X non essential amino acids, 1% beta-mercaptoethanol, and 1000 units/ml leukemia inhibitory factor (Millipore). In general, the culture medium of both wild type and DGCR8 knockout cells was replaced daily.

4.3.13 Data analysis

Results represent the mean ± S.E. for the number \((n)\) of independent experiments performed. Statistical differences between the means of unpaired sets of data were evaluated by one-way analysis of variance with a post hoc Tukey's test. A \(p\) value of < 0.05 was considered statistically significant. Images and immunoblots shown are representative of at least three independent experiments.

4.4 Results

4.4.1 CtBP1 and CtBP2 are downregulated in CGNs exposed to Clostridium difficile

Toxin B, an inhibitor of Rho family GTPases

In our initial studies using a high throughput immunoblotting screen (BD PowerBlot™), we identified CtBP1 and CtBP2 as two proteins that were significantly downregulated in CGNs following a 24 h incubation with the Rho family GTPase inhibitor, Clostridium difficile toxin B (Fig. 4.1A). Toxin B is a monoglucosyltransferase that directly glucosylates and inhibits the small GTPases Rho, Rac, and Cdc42 (Just et al., 1995). We have previously shown that toxin B induces intrinsic apoptosis in CGNs that is
largely dependent on its capacity to inhibit pro-survival signaling by Rac GTPase (Le et al., 2005, Linseman et al., 2001, Loucks et al., 2006 and Stankiewicz et al., 2012). CtBP1 appeared on polyacrylamide gels as a single band of approximately 48 kDa and CtBP2 appeared as a doublet of approximately 48/50 kDa. In the initial PowerBlot™ analysis, the expression of each of these proteins was significantly decreased by at least two-fold in CGNs incubated for 24 h with toxin B (Fig. 4.1B). In a separate experiment using a distinct preparation of primary CGN cultures, the toxin B-induced decrease in CtBP1 and CtBP2 expression was confirmed (Fig. 4.1C).
Figure 4.1 CtBP1 and CtBP2 are downregulated in CGNs exposed to *Clostridium difficile* Toxin B, an inhibitor of Rho family GTPases. A. CGNs were incubated for 24 h in control medium containing 25 mM KCl and 10% FBS (Con) in the absence or presence of Toxin B (Tox B; 40 ng/ml). Cells were then lysed and protein extracts were subjected to PowerBlot™ analysis. Incubation with Tox B resulted in the downregulation of both CtBP1 (indicated by the black arrows) and CtBP2 (a doublet indicated by the asterisks). G protein-coupled receptor kinase-interacting protein-z-short (GITz-short; white arrows) is shown to demonstrate equal protein loading of the PowerBlot™ gels. B. Densitometric quantification of CtBP1 and CtBP2 protein expression in Con and Tox B-treated CGNs. Results shown are the mean ± range from duplicate PowerBlot™ gels and are expressed as the fold change in CtBP expression between Tox B-treated cells and Con. C. To confirm the results of the PowerBlot™, CGNs obtained from a different primary culture than those utilized in the PowerBlot™ analysis were incubated for 24 h in either Con medium or medium containing Tox B. After incubation, CtBP1 and CtBP2 expression was analyzed by western blotting. Blots were then stripped and re-probed for actin as a loading control. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.2 Multiple pro-death stimuli induce downregulation of CtBPs in CGNs

Next, we examined the effects of a number of diverse pro-death stimuli on the expression of CtBPs in CGNs. Primary CGN cultures require serum-derived growth factors and depolarizing extracellular potassium for their survival. Removal of depolarizing potassium (i.e., 5K conditions) induces apoptosis of CGNs through an intrinsic pathway (D'Mello et al., 1993 and Linseman et al., 2002). Upon removal of serum and depolarizing potassium (5K conditions), the expression of both CtBP1 and CtBP2 was significantly decreased (Fig. 4.2A). Similar reductions in CtBP expression were observed when CGNs were incubated with the BH3 mimetic, HA14-1, the nitric oxide donor, sodium nitroprusside (SNP), or the complex I inhibitor, 1-methyl-4-phenylpyridinium (MPP +) (Fig. 4.2B). The expression of CtBP1 was also analyzed by immunofluorescent staining under control, 5K, and MPP + treatment conditions. In control CGNs, CtBP1 was localized almost exclusively to the nucleus (Fig. 4.2C, upper panels). However, whether CGNs were exposed to either 5K or MPP +, two completely distinct stressors, CtBP1 immunoreactivity essentially disappeared in cells that demonstrated condensed and/or fragmented chromatin indicative of apoptotic morphology (Fig. 4.2C, middle and lower panels). These results demonstrate the downregulation of CtBP1 and CtBP2 in neurons undergoing apoptosis and further show that CtBPs are downregulated in response to a number of mechanistically distinct pro-death stimuli.
Figure 4.2 Multiple pro-death stimuli induce downregulation of CtBPs in CGNs. A. CGNs were incubated for 24 h in either control medium containing 25 mM KCl and 10% FBS (Con) or apoptotic medium containing only 5 mM KCl and lacking FBS (5K). Cells were then lysed, proteins resolved by SDS-PAGE, and immunoblotted for CtBP1 and CtBP2. Blots were subsequently stripped and re-probed for actin to show equal loading. B. CGNs were incubated for 24 h in either Con medium alone or containing the Bcl-2 inhibitor HA14-1 (HA14; 15 µM), the nitric oxide donor sodium nitroprusside (SNP; 100 µM), or the complex I inhibitor 1-methyl-4-phenylpyridinium (MPP+; 150 µM). After incubation, protein extracts were western blotted for CtBP1 and CtBP2. Blots were also stripped and re-probed for actin as a loading control. C. CGNs were incubated for 24 h in either Con medium, 5K medium, or medium containing MPP+, as described above in (A) and (B). Following incubation, cells were fixed with 4% paraformaldehyde and their nuclei stained with DAPI. The microtubule network was stained with a polyclonal antibody to β-tubulin and a Cy3-conjugated secondary antibody. CtBP1 was detected using a monoclonal antibody to CtBP1 and a secondary antibody conjugated to FITC. White arrows indicate cells with condensed and/or fragmented nuclei indicative of apoptotic cell death. Note that regardless of the stimulus, CGNs with apoptotic nuclear morphology displayed essentially no detectable CtBP1. Scale bar = 10 microns. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.3 Antisense oligonucleotides to CtBP1 induce CGN apoptosis

To determine if forced downregulation of CtBP1 is sufficient to trigger CGN apoptosis, we transfected cells with morpholino-antisense oligonucleotides to rat CtBP1 using an EndoPorter delivery reagent. As negative controls, CGNs were transfected with inverse morpholino oligonucleotides or were exposed to the EndoPorter reagent alone. Transfections and subsequent 24 h incubations were performed in control medium containing 25 mM KCl and 10% FBS. CGNs transfected with morpholino-antisense oligonucleotides to rat CtBP1 underwent significant apoptosis characterized by nuclear condensation and fragmentation (Fig. 4.3A and 4.3B). In contrast, CGNs exposed to the EndoPorter reagent alone or transfected with inverse morpholino oligonucleotides displayed a similar level of apoptosis to untreated control CGNs. Although the morpholino-antisense oligonucleotides were fluorescently labeled with fluorescein, we were unable to accurately assess the extent of CtBP1 downregulation within the transfected cell population using immunofluorescent staining for CtBP1. This was primarily because the cells that fluoresced positive for the morpholino-antisense oligonucleotides were most often apoptotic and their nuclei were essentially devoid of CtBP1 immunoreactivity. However, given that CtBP1 nuclear staining disappears in apoptotic cells (see Fig. 4.2C), we cannot discern whether the CtBP1 staining is lost due to the antisense treatment or due to the fact that the cell is undergoing apoptosis. Given this limitation, our data suggest that antisense-mediated down-regulation of CtBP1 is sufficient to induce significant CGN apoptosis.
Figure 4.3 Morpholino-antisense oligonucleotides to CtBP1 induce CGN apoptosis. A. CGNs were incubated for 24 h in Con medium alone or containing the vehicle Endoporter (EP; 6 µl/ml), EP plus morpholino-antisense oligonucleotides to CtBP1 (Anti; 10 µM), or EP plus inverse control morpholino-oligonucleotides (Inv; 10 µM). Apoptosis was quantified from 3 separate experiments each performed in triplicate. Antisense treatment induced significant CGN apoptosis (*p < 0.05) when compared to Inv, EP, or Con treatments. B. Representative images of CGNs following incubation with morpholino-antisense oligonucleotides or appropriate controls, as described in (A). Cells were fixed and nuclei stained with Hoechst. CGNs containing nuclei that were visibly condensed and/or fragmented were scored as apoptotic. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.4 The CtBP inhibitor, 4-methylthio-2-oxobutyric acid (MTOB), induces actinomycin D-sensitive apoptosis of CGNs

As an alternative means of knocking out CtBP function in CGNs, we next incubated cells with the putative CtBP inhibitor, MTOB. This compound is a CtBP dehydrogenase substrate which acts as a CtBP inhibitor and is toxic to cancer cells at high (1–10 mM) concentrations (Straza et al., 2010). In agreement with this previous study, incubation of CGNs with MTOB for 24 h revealed that significant apoptosis was induced at a concentration of 5 mM (Fig. 4.4A). Moreover, the CGN apoptosis induced by this concentration of MTOB was delayed and required 24 h of incubation (Fig. 4.4B). In accordance with MTOB inducing apoptosis of CGNs, its toxic effects were completely suppressed by blocking the transcription of new genes with actinomycin D (Fig. 4.4C and D). Moreover, consistent with MTOB inhibiting the co-repressor function of CtBP, incubation with this compound induced a late induction of the CtBP target, the BH3-only protein Noxa, at 24 h post-treatment (Fig. 4.4E). Collectively, these results demonstrate that inhibition of CtBP co-repressor function is capable of inducing actinomycin D-sensitive CGN apoptosis.
Figure 4.4 The CtBP inhibitor, MTOB, induces CGN apoptosis. A. MTOB dose response: CGNs were incubated for 24 h in Con medium containing increasing concentrations of MTOB. Apoptosis was quantified as the percentage of cells with condensed and/or fragmented nuclei. B. MTOB time course: CGNs were incubated for increasing durations with 5 mM MTOB and apoptosis was quantified. C. Representative fluorescence images of CGNs incubated for 24 h in Con medium alone or containing 5 mM MTOB ± actinomycin D (AD). D. Quantification of CGN apoptosis for the experiment described in (C). E. CGNs were incubated for increasing durations with 5 mM MTOB. Cell lysates were prepared and western blotted for Noxa and actin. For (A), (B), and (D); **p < 0.01 compared to Con, ††p < 0.01 compared to MTOB. The results shown represent the means ± SEM of three independent experiments each performed in duplicate.
4.4.5 In addition to the recognized proteasome degradation pathway, CtBPs are also downregulated through a novel caspase-dependent mechanism in CGNs

Several previous reports have demonstrated that CtBPs are downregulated via proteasomal degradation during p53-independent apoptosis in non-neuronal cells (Paliwal et al., 2006, Wang et al., 2006, Zhang et al., 2003 and Zhang et al., 2005). However, the mechanism by which CtBPs are downregulated in neurons undergoing apoptosis has not previously been explored. To address this question, we analyzed the effects of inhibitors of the proteasome or caspases on the downregulation of CtBPs induced by various pro-death stimuli in CGNs. Downregulation of CtBP1 and CtBP2 induced under 5K apoptotic conditions in CGNs was completely prevented by the pan-caspase inhibitor, BOC, but was only partially attenuated by the proteasome inhibitor MG132 (Fig. 4.5A). In response to apoptosis induced by toxin B or the related Clostridium sordellii lethal toxin which also inhibits Rac GTPase (Just et al., 1996), the observed downregulation of CtBP1 and CtBP2 was significantly blocked by caspase inhibition with BOC or zVAD but was unaffected by proteasome inhibition with MG132 (Fig. 4.5B and 4.5C). In contrast to 5K and the Clostridial toxins which are each known to activate caspases in CGNs, we have previously shown that CGN death induced by MPP + occurs through a caspase-independent mechanism (Harbison et al., 2011). In agreement with this, the downregulation of CtBP1 and CtBP2 induced by MPP + in CGNs was unaltered by BOC but was significantly inhibited by MG132 (Fig. 4.5D). These data indicate that distinct pro-death stimuli act through a proteasome-dependent pathway and/or an alternative caspase-dependent pathway to downregulate CtBPs in neurons. To our knowledge, this is
the first demonstration of CtBP expression being regulated via a caspase-dependent mechanism.
Figure 4.5 Caspases and the proteasome contribute to CtBP downregulation in a stimulus-specific manner and CtBPs are downregulated in an exclusively proteasome-dependent manner following exposure to the complex I inhibitor MPP+. A. CGNs were incubated for 24 h in Con medium or 5K medium ± the pan-caspase inhibitor BOC (50 µM) or the proteasome inhibitor MG132 (10 µM). B. CGNs were incubated for 24 h in Con medium alone or containing ToxB (40 ng/ml) ± either BOC or MG132. C. CGNs were incubated for 24 h in Con medium alone or containing Clostridium sordelli lethal toxin (400 µg/ml) ± the pan-caspase inhibitor zVAD (50 µM) or MG132. Following incubation, cells were lysed, proteins resolved by SDS-PAGE and transferred onto PVDF membranes. Blots were probed for CtBP1 and CtBP2 followed by stripping and re-probing for actin to indicate equal loading. D. CGNs were incubated for 24 h in Con medium alone or containing MPP + (150 µM) ± BOC or MG132. Following incubation, cells were lysed, proteins resolved by SDS-PAGE, and immunoblotted using CtBP1 and CtBP2 antibodies. Blots were stripped and re-probed for actin to show equal loading. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.6 6-hydroxydopamine (6-OHDA) induces caspase-dependent downregulation of CtBPs in the dopaminergic N27 cell line

In order to establish that the novel caspase-dependent mechanism of CtBP downregulation observed in CGNs undergoing apoptosis was not unique to this cell system, we next investigated the mode of CtBP downregulation in an in vitro model relevant to Parkinson's disease. The N27 cell line is a large T-antigen-immortalized, mesencephalon-derived cell line with characteristics of dopaminergic neurons (Zhou et al., 2000). Exposure of N27 cells to the neurotoxin, 6-OHDA, induces caspase-dependent apoptosis (Latchoumycandane et al., 2011). Incubation of N27 cells with 6-OHDA resulted in the downregulation of CtBP1 and CtBP2 in a dose-dependent manner (Fig. 4.6A). In addition, the downregulation of CtBPs induced by 6-OHDA was largely prevented by the pan-caspase inhibitor, BOC, but was actually accentuated by the proteasome inhibitor MG132 (Fig. 4.6B). Thus, caspase-dependent downregulation of CtBPs occurs in diverse neuronal cell systems and in response to multiple pro-apoptotic stressors.
Figure 4.6 Incubation of N27 dopaminergic cells with 6-OHDA induces downregulation of CtBP1 and CtBP2 through a caspase-dependent mechanism. A. N27 cells were incubated for 24 h in Con medium alone or containing either 25 µM or 50 µM concentrations of 6-OHDA. Because of cell detachment induced by 6-OHDA, medium containing floating cells was obtained in a microcentrifuge tube and pelleted. The supernatant was then discarded and cells remaining in the 6-well dish were lysed, scraped, and added to the previously pelleted cells which had detached. Combined contents in the microcentrifuge tube were then lysed, and proteins were resolved by SDS-PAGE. Once transferred onto PVDF membranes, samples were immunoblotted with antibodies to CtBP1 and CtBP2. Membranes were stripped and re-probed for actin to show equal loading. B. N27 cells were incubated for 24 h in Con medium alone or containing 6-OHDA (25 µM) ± BOC (50 µM) or MG132 (10 µM). Cells lysates were immunoblotted as described in (A). I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.7 CtBP downregulation induced by 5K apoptotic conditions in CGNs does not occur through an enhanced rate of protein degradation

The downregulation of CtBP1 observed under 5K apoptotic conditions occurred in a relatively protracted manner over the course of many hours (Fig. 4.7A). One principal mechanism by which proteins are downregulated is by an enhanced rate of degradation. Given that the downregulation of CtBPs under 5K apoptotic conditions was sensitive to caspase inhibition, we utilized pulse-chase methodology to determine if CtBPs were being actively degraded during CGN apoptosis. Quantification of a discrete pool of $^{35}$S-labeled CtBP1 (pulse) following incubation in either 25K or 5K serum-free medium (chase) resulted in nearly identical $t_{1/2}$ values for CtBP1 of approximately 10 h (Fig. 4.7B and 4.7C). These data suggest that CtBP1 does not undergo an enhanced rate of degradation during 5K-induced apoptosis in CGNs. Instead, the downregulation of CtBPs under these conditions is likely due to decreased synthesis of new protein.
Figure 4.7 Pulse-chase analysis of the kinetics of CtBP1 downregulation in CGNs incubated in apoptotic (5K) medium. A. CGNs were incubated in 5K medium for various times over the course of 56 h. CtBP1 protein levels were assessed by western blotting and membranes were stripped and re-probed for actin to show equal loading. B. CGNs were initially incubated for 4 h in cysteine-methionine-free medium containing $^{35}$S-methionine. Following this “pulse” incubation, cells were washed and medium was replaced with serum-free Con medium or 5K medium. At various time points during this “chase” incubation, cells were lysed and CtBP1 was immunoprecipitated using a monoclonal antibody. Immunoprecipitated samples were resolved on a 10% polyacrylamide gel, transferred onto a PVDF membrane, and exposed directly to film. C. Quantification of $^{35}$S-CtBP1 densitometry from (B) was graphed to compare the kinetics of loss of CtBP1 during the 24 h “chase” incubation period. Values are plotted as a percentage of the time 0 band density. CtBP1 displayed a half-life of approximately 10 h in CGNs incubated in either Con or 5K medium. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.8 CtBP1 is not directly cleaved by caspase-3 in vitro

Upon examination of the CtBP1 amino acid sequence, a consensus DXXD caspase-3 cleavage motif was noted in human CtBP1 at residues $^{103}$DNID$^{106}$. This consensus caspase-3 cleavage site is highly conserved and is present in CtBP1 and CtBP2 proteins of human, mouse, and rat species (Fig. 4.8A). An identical DNID motif present in the adenomatous polyposis coli protein was previously shown to be directly cleaved by caspase-3 (Webb et al., 1999). To determine if this $^{103}$DNID$^{106}$ site serves as a viable caspase-3 cleavage site in CtBP1 we performed an in vitro proteolysis experiment with recombinant caspase-3 and recombinant CtBP1. As a positive control for caspase-3 activity, we utilized poly(ADP-ribose) polymerase (PARP) which is cleaved by caspase-3 to generate an 85 kDa fragment (Tewari et al., 1995). As expected, recombinant caspase-3 cleaved PARP to produce the 85 kDa fragment but it failed to cleave CtBP1 to any appreciable extent (Fig. 4.8B). Thus, despite the presence of a consensus caspase-3 cleavage motif, CtBP1 is not a direct substrate of caspase-3. This result is in agreement with the above data and further suggests that the downregulation of CtBPs observed in CGNs undergoing apoptosis is not due to direct caspase-mediated degradation.
Despite the presence of a highly conserved, consensus caspase-3 cleavage site, CtBP1 is not directly cleaved by recombinant caspase-3 in vitro. A. CtBP1 and CtBP2 sequences across numerous species indicate a highly conserved caspase-3 cleavage site (DXXD; shaded residues). B. Recombinant CtBP1 (1.5 µg) or recombinant PARP (5 µg) were incubated in the absence or presence of recombinant caspase-3 (rCasp3; 100 U) for 4 h at 37 °C. Recombinant proteins were then run on 7.5% polyacrylamide gels and western blotted with monoclonal antibodies to CtBP1 and PARP. The full length CtBP1 and PARP proteins are indicated by “fl”. The expected 85 kDa PARP cleavage fragment produced by caspase-3-dependent proteolysis is denoted as “clv”. I acknowledge Emily Schroeder for assistance with generating data for this figure.
4.4.9 CtBP transcript levels are not significantly decreased during 5K-induced apoptosis in CGNs

If the downregulation of CtBPs under 5K apoptotic conditions is due to decreased synthesis of new protein, then one possible point of regulation is at the level of CtBP gene transcription. To determine if CtBP1 and CtBP2 mRNA levels are reduced when CGNs are incubated in 5K apoptotic medium, we performed RT-PCR analysis. Surprisingly, even after 24 h incubation in 5K medium, no significant decrease in either CtBP1 or CtBP2 transcript expression was observed (Fig. 4.9A and 4.9B). Similar results were also obtained using quantitative real-time PCR analysis (data not shown). These results indicate that the downregulation of CtBPs observed during CGN apoptosis is not due to decreased gene transcription.
Figure 4.9 Incubation of CGNs in apoptotic (5K) medium has no significant effect on CtBP1 or CtBP2 transcript expression. A. CGNs were incubated in either Con or 5K medium for 24 h. Total RNA was isolated and subjected to RT-PCR using primers specific for CtBP1, CtBP2, and actin mRNA transcripts. Representative bands are shown. B. Quantification of three independent experiments performed in duplicate as described in (A). CtBP1 and CtBP2 transcript expression levels were divided by the corresponding actin signals for normalization.
4.4.10 CtBP1 undergoes caspase-dependent downregulation in staurosporine-treated, wild type (WT) mouse embryonic stem cells (mESCs) but not DGCR8 knockout (KO) mESCs

The above findings indicate that CtBP function is essential for CGN survival and furthermore, during CGN apoptosis CtBPs undergo an indirect, caspase-dependent downregulation that occurs via a post-transcriptional mechanism. Micro RNAs (miRNAs) are small noncoding RNAs that act as negative post-transcriptional regulators by binding to the 3′ UTRs of target mRNAs (Lai, 2002). Mature miRNAs are produced by a series of tightly regulated processing events. First, the primary miRNA transcript (pri-miRNA) is cleaved by the Microprocessor, a protein complex consisting of the ribonuclease Drosha and its essential cofactor, DiGeorge Critical Region 8 (DGCR8) (Gregory and Schiekhattar, 2005). Microprocessor cleavage of pri-miRNAs generates intermediate precursor miRNAs (pre-miRNAs) which are in turn, processed by Dicer to produce the mature miRNAs (Triboulet and Gregory, 2010). As a first step to determine if the caspase inhibitor-sensitive downregulation of CtBPs induced during apoptosis might be mediated via a miRNA-dependent pathway, we compared the expression of CtBP1 in WT mESCs to DGCR8 KO mESCs which are deficient in miRNA biogenesis (Wang et al., 2007). Incubation of WT mESCs with the classical apoptosis inducer, staurosporine, caused a marked downregulation of CtBP1 that was prevented by the pan-caspase inhibitor QVD (Fig. 4.10A, left blot). In contrast, incubation of DGCR8 KO mESCs with staurosporine failed to have any significant effect on the expression of CtBP1 (Fig. 4.10A, right blot). It is important to note that staurosporine treatment caused significant cell death in both cell lines that was partially blunted by co-treatment with
QVD (Fig. 4.10B). These data indicate that the caspase-dependent downregulation of CtBP1 induced under apoptotic conditions requires intact miRNA processing and biogenesis machinery.
Figure 4.10 DGCR8 knockout mouse embryonic stem cells (mESCs) do not display caspase-dependent downregulation of CtBP1 in response to staurosporine treatment. A. Wild-type (WT) and DGCR8 knockout (KO) mESCs were incubated for 24 h in Con medium alone or containing staurosporine (STS; 100 nM) ± the pan-caspase inhibitor QVD (20 µM). Following incubation, cell lysates were resolved by SDS-PAGE and western blotted for CtBP1. Membranes were subsequently stripped and reprobed for actin as a loading control. B. WT or KO mESCs were treated as described in (A). Representative bright field images are shown following a 24 h treatment period. Scale bar = 10 microns.
4.5 Discussion

CtBPs act as transcriptional co-repressors of a number of pro-apoptotic genes including the Bcl-2 family members Bax, Noxa, Bik, Bim, and Bmf (Bergman and Blaydes, 2006, Grootecaes et al., 2003 and Kovi et al., 2010). Therefore, it is not surprising that CtBPs might be downregulated in cells undergoing apoptosis. However, to our knowledge the present study is the first to document this effect during neuronal apoptosis. Previous studies of CtBP function in the nervous system have been mostly limited to the role of these proteins in development. For instance, the Drosophila CtBP (dCtBP) significantly impacts development of the fly peripheral nervous system by negatively regulating formation of mechanosensory bristles, perhaps by influencing extra sensory organ precursor cell fate (Biryukova and Heitzler, 2008 and Stern et al., 2009).

CtBPs are expressed throughout the developing avian CNS, often in overlapping regions but sometimes in unique localizations such as CtBP1 expression in dorsal root ganglia and CtBP2 expression in emigrating neural crest cells (Van Hateren et al., 2006). The functional significance of CtBP expression in the developing chick CNS is demonstrated by the key role that these proteins play in regulating the transition of neural precursor cells in the ventricular zone of the dorsal spinal cord from a proliferative to a differentiated state (Xie et al., 2011). In a similar manner, CtBP1 and CtBP2 display both duplicative and independent roles in mouse CNS development including maturation of the forebrain and midbrain (Hildebrand and Soriano, 2002). Additional studies have demonstrated interactions of CtBPs with a number of neuronal proteins including neuronal nitric oxide synthase, actin-related protein alpha, and calsenilin, although the physiological significance of these interactions is not well defined (Oma et al., 2003, 2006).
Finally, CtBPs have been loosely associated with some types of CNS injury. For example, CtBP expression has been shown to decline rapidly following spinal cord injury in the mouse (Cai et al., 2012). In another study, the glycolytic inhibitor, 2-deoxy-D-glucose, suppressed seizure activity in a rat kindling model of temporal lobe epilepsy via an NRSF/CtBP-dependent repression of the BDNF gene promoter (Garriga-Canut et al., 2006). These studies demonstrate that CtBPs are key factors in CNS and peripheral nervous system development; however, the role of CtBPs in determining neuronal survival and death has not been explicitly investigated.

In the present study, we have identified CtBPs as essential pro-survival proteins in CGNs. The expression of CtBP1 and CtBP2 was significantly downregulated in CGNs exposed to a number of pro-apoptotic stressors. Moreover, forced downregulation of CtBP1 using morpholino-antisense oligonucleotides was sufficient to induce CGN apoptosis. In a similar manner, incubation of CGNs with the CtBP inhibitor, MTOB, induced the upregulation of pro-apoptotic Noxa and triggered actinomycin D-sensitive CGN apoptosis. These results are consistent with CtBPs functioning in CGNs as pro-survival factors which are subject to downregulation during neuronal apoptosis. One may consider the downregulation of CtBPs as a major factor in determining whether neurons succumb to apoptosis since loss of CtBP function ultimately leads to the de-repression of pro-apoptotic genes.

In non-neuronal cells, CtBPs have previously been shown to be targeted for proteasomal degradation in response to pro-apoptotic stimuli that induce p53-independent apoptosis (Paliwal et al., 2006, Wang et al., 2006, Zhang et al., 2003 and Zhang et al., 2006).
2005). In contrast, CGNs exposed to Rac GTPase-inhibitory Clostridial toxins displayed downregulation of CtBP1 and CtBP2 that was insensitive to proteasome inhibition but sensitive to caspase inhibition. This novel caspase-dependent downregulation of CtBPs also predominated under 5K apoptotic conditions in CGNs and in N27 dopaminergic cells exposed to 6-OHDA. In contrast, the downregulation of CtBPs induced in CGNs by the complex I inhibitor, MPP +, was completely insensitive to caspase inhibition but was significantly attenuated by proteasome inhibition. These results demonstrate that the downregulation of CtBPs during neuronal apoptosis occurs via a stimulus-specific mechanism with both proteasome-dependent and caspase-dependent modes of downregulation.

Previous studies on the regulation of CtBP expression have focused largely on the proteasome-dependent degradation of these transcriptional co-repressors. During p53-independent apoptosis of non-neuronal cells, CtBPs are phosphorylated on Ser422 or Ser 428 (CtBP1 or CtBP2, respectively) by either homeodomain interacting protein kinase-2 or c-Jun NH2-terminal kinase, targeting these proteins for subsequent ubiquitinylation and proteasomal degradation (Wang et al., 2006, Zhang et al., 2003 and Zhang et al., 2005). Under some conditions, targeting of CtBP to the proteasome may require interaction with additional proteins such as the tumor suppressor ARF (Paliwal et al., 2006). Recently, additional pathways have been suggested to regulate CtBP expression via the proteasome. For instance, Akt1 has recently been shown to cooperate with the SUMO E3 ligase Pc2 to induce phosphorylation and ubiquitinylation of CtBP resulting in its enhanced degradation (Merrill et al., 2010). Another pathway for CtBP degradation involves its interaction with the X-linked inhibitor of apoptosis protein which directly
ubiquitinylates CtBP and targets it for proteasomal degradation (Lee et al., 2012). On the other hand, B-cell lymphoma-3 (Bcl-3) is a proto-oncogene that has recently been shown to interact with and stabilize CtBP by preventing its ubiquitinylation and subsequent proteasomal degradation (Choi et al., 2010). Interestingly, CtBP1 protein levels were downregulated in HEK293T cells incubated with the apoptosis inducer etoposide, but this effect was prevented by overexpression of Bcl-3. Thus, proteasome-dependent degradation appears to be a dominant pathway for turnover of CtBPs in non-neuronal cells undergoing apoptosis.

In marked contrast to these previous studies, we have identified a novel caspase-dependent pathway for CtBP downregulation in neurons undergoing apoptosis. Despite the presence of a conserved caspase-3 consensus cleavage site in the CtBP1 and CtBP2 proteins, CtBPs do not appear to be direct caspase-3 substrates in vitro. Although it cannot be ruled out that other caspase family members may directly cleave CtBPs in neurons undergoing apoptosis, the rate of degradation of CtBP1 in CGNs is not significantly altered in 5K (apoptotic) medium suggesting that the downregulation of CtBPs observed under these conditions is not due to enhanced proteolysis. Intriguingly, CtBP1 and CtBP2 mRNA transcripts are not significantly decreased in CGNs subjected to 5K apoptotic conditions indicating that the downregulation of CtBPs likely occurs via a post-transcriptional mechanism. Consistent with this idea, the caspase-dependent downregulation of CtBP1 observed in mESCs exposed to staurosporine does not occur in DGCR8 KO cells that are deficient in miRNA biogenesis. Collectively, these data suggest that the caspase-dependent downregulation of CtBPs observed in neurons undergoing apoptosis may occur via a miRNA-dependent mechanism.
Several recent studies add support to the hypothesis that CtBPs are subject to significant post-transcriptional regulation by miRNAs. For instance, the expression of miR-137 was found to inversely correlate with CtBP1 expression in melanoma cell lines. Moreover, miR-137 suppressed CtBP1 3’ UTR luciferase-reporter activity and overexpression of miR-137 decreased CtBP1 levels and caused a corresponding increase in expression of the CtBP1 target Bax (Deng et al., 2011). Interestingly, the miR-137 gene is found on chromosome 1p22 which is a known susceptibility region for melanoma and furthermore, miR-137 was found to be under expressed in a subset of patient-derived melanomas (Bemis et al., 2008, Chan et al., 2011 and Walker et al., 2004). In a similar manner, the miR-141-200c cluster was recently shown to downregulate the expression of CtBP2 and its transcriptional repressor partner, ZEB, in PANC-1 human pancreatic carcinoma cells (Sass et al., 2011). ZEB is a key inducer of the epithelial-to-mesenchymal transition which is thought to promote malignant tumor progression, particularly in pancreatic, colorectal, and breast cancer (Burk et al., 2008). These studies indicate that specific miRNAs may act as tumor suppressors in part by targeting CtBPs for translational repression. It is noteworthy that miR-137 has recently been shown to act as a key regulator of embryonic neural stem cell fate (Sun et al., 2011). However, whether miR-137 regulates CtBP expression in the CNS is presently unknown. In the future, it will be important to determine if downregulation of CtBPs is associated with particular neurodegenerative diseases, particularly those for which caspases are implicated in the underlying pathogenesis.

Finally, elucidating the mechanism by which caspases indirectly influence CtBP expression during neuronal apoptosis will require additional study. One possibility is that
caspases degrade a protein that under healthy conditions acts as a suppressor of specific miRNAs. Once this suppressor is degraded, miRNAs are induced and target CtBPs for translational repression. This in turn, leads to the de-repression of a subset of CtBP target pro-apoptotic genes that contribute to the execution of neuronal apoptosis. The existence of “RNA silencing suppressor” (RSS) proteins is evidenced by the HIV-1 Tat and Rex proteins which suppress specific siRNAs or miRNAs by competing for their binding with Dicer or other proteins of the RNA-induced silencing complex (Houzet and Jeang, 2011 and Rawlings et al., 2011). Thus, it is reasonable to hypothesize that neurons may possess intrinsic RSS proteins that are degraded by caspases during neuronal apoptosis. Identification of these putative neuronal RSS proteins may be necessary to resolve the mechanism underlying caspase-dependent CtBP downregulation during neuronal apoptosis.
CHAPTER FIVE: DYSREGULATION OF RHO OR RAC ELICITS DEATH OF MOTOR NEURONS AND ACTIVATION OF THESE GTPASES IS ALTERED IN G93A MUTANT SOD1 MICE

5.1 Abstract

Numerous studies have demonstrated a critical function for Rho GTPase family members (i.e., Rac, Rho, Cdc42) in neuronal survival. Although a pro-survival function for Rac has been reported in several neuronal cell types, the antagonistic relationship between Rac and Rho/ROCK signaling in neuronal survival remains poorly understood. In the current study, we examined the effects of Rac GTPase inhibition on motor neuronal survival. We demonstrate that treatment with NSC23766, a selective inhibitor of the Rac-specific GEFs, Tiam1 and Trio, induces death of embryonic stem cell (ESC)-derived motor neurons which is characterized by caspase-3 activation, dephosphorylation of ERK5 and Akt, and nuclear translocation of the BH3-only protein Bad. We also examined the effects of a constitutive activator of Rho, CN03, on motor neuronal survival in vitro. In a manner similar to selective inhibition of Rac GTPase, treatment of ESC-derived motor neurons with CN03 results in a marked loss of neurites and significant cell death. Furthermore, inclusion of either ROCK inhibitor, Fasudil or Y-27632, significantly protects ESC-derived motor neurons against constitutive activation of Rho. These data suggest that the balance between Rac and Rho signaling is critical for motor neuron survival. Moreover, in the G93A mutant Cu,Zn-superoxide dismutase (SOD1) mouse model of amyotrophic lateral sclerosis (ALS), active Rac1-GTP immunoreactivity
is markedly decreased in choline acetyltransferase (ChAT)-positive motor neurons of the lumbar spinal cord when compared to age-matched wild type (WT) littermates. In addition, although total RhoB localizes principally to nuclei of ChAT-positive motor neurons from WT mice, RhoB appears to redistribute to motor neuronal processes in end-stage mice harboring the G93A SOD1 mutation. Collectively, our data demonstrate that Rac and Rho are critical regulators of neuronal survival and as a result, disruptions in the balance of their activities may contribute to the etiology of motor neurodegenerative diseases such as ALS.

5.2 Introduction

ALS is a devastating and progressive neurodegenerative disease characterized by severe muscle weakness and atrophy due to loss of motor neurons in the spinal cord, brain stem, and motor cortex. Following diagnosis, ALS patients typically survive 2-5 years and succumb to death due to respiratory failure. Approximately 90% of ALS cases are classified as sporadic while the remaining 10% appear to be caused by a genetic component. In particular, mutations in SOD1 account for approximately 20% of all familial cases of ALS. Currently, mice harboring G93A mutant SOD1 (mSOD1) are the most extensively described rodent model of the disease (Pandya et al., 2013). Although the mechanism of motor neuron loss underlying the etiology of ALS is presently unclear, recent findings suggest that dysregulated Rho family GTPase activity may be a contributing factor.

Rho family GTPases are monomeric G-proteins that belong to the Ras superfamily of GTPases. Within the Rho GTPase family, RhoA, Rac1, and Cdc42 are the best described members. Numerous studies in neurons have demonstrated that Rho
GTPases are critical regulators of growth cone dynamics, dendritic spine morphogenesis, and neuronal survival (Linseman and Loucks, 2008). Specifically, Rac GTPase elicits neurite outgrowth and neuronal survival while Rho GTPase provokes neurite retraction and neuronal apoptosis (Luo, 2000). Many studies suggest that this intricate balance between Rho family GTPase activities is important for maintaining neuronal survival. For example, we have previously shown in primary cerebellar granule neurons (CGNs) that inhibition of Rac utilizing large Clostridial cytotoxins or overexpression of a dominant negative Rac1 mutant induces mitochondrial-dependent apoptosis (Linseman et al., 2001; Le et al., 2005; Loucks et al., 2006; Stankiewicz et al., 2012). Conversely, RhoA GTPase activation in CGNs elicits apoptosis following glutamate-induced excitotoxicity (Semenova et al., 2007). These studies underscore the importance of maintaining tight regulation of Rho family GTPase activity in order to promote neuronal survival.

More recent data have demonstrated that a disrupted balance between Rho family GTPases, particularly diminished Rac activity and/or enhanced Rho activity, may contribute to the pathogenesis of neurodegenerative diseases such as ALS. Indeed, dominant negative Rac expression alone is sufficient to induce death of cultured motor neurons (Jacquier et al., 2006). Moreover, expression of G93A mSOD1 induces redox-sensitive apoptosis of SH-SY5Y neuroblastoma cells that is dependent on inactivation of Rac GTPase (Pesaresi et al., 2011). In addition to mutations in SOD1, the involvement of Rac GTPase in maintaining motor neuron survival is underscored by the fact that loss-of-function mutations in a Rac guanine nucleotide exchange factor (GEF), alsin (ALS2), are causative in juvenile-onset ALS (Yang et al., 2001; Yamanaka et al., 2003; Hadano et al., 2007). Although alsin functions as a GEF for both Rac and Rab5 (Topp et al., 2004),
overexpression of constitutively active Rac, but not Rab5, attenuates the cellular apoptosis and reduced axonal growth observed following siRNA knockdown of alsin in primary motor neurons (Jacquier et al., 2006). Furthermore, alsin has been specifically demonstrated to antagonize mutant SOD1-induced NSC34 motor neuronal death via activation of a Rac-dependent pro-survival signaling cascade (Kanekura et al., 2005). Finally, a recent study identified that ARHGEF16, an upstream regulator of Rac GTPase (Blanke and Jäckle, 2008), is hyper-methylated and downregulated in patients with sporadic ALS (Figueroa-Romero et al., 2012). Collectively, these findings indicate that a loss of Rac GTPase activity may be a common factor underlying the death of motor neurons in both familial and sporadic forms of ALS.

In accordance with previous studies demonstrating a pro-apoptotic function for Rho GTPase in neurons, expression of the Rho GTPase effector Rho kinase (ROCK) is elevated 221% in the spinal cord of G93A mSOD1 mice when compared to WT mice, suggesting a potential increase due to enhanced Rho GTPase activity (Hu et al., 2003). These data indicate that in a manner similar to loss of Rac GTPase activity, gain of Rho GTPase/ROCK signaling may contribute to the selective motor neuron death underlying the etiology of ALS. This hypothesis is consistent with prior studies demonstrating aberrant activation of the Rho/ROCK pathway in other animal models of neurodegenerative disease (Mueller et al., 2005; Rikitake et al., 2005; Satoh et al., 2007; Zhang et al., 2010; Rodriguez-Perez et al., 2013). Nonetheless, detailed studies examining the activation status of Rac and Rho GTPases during ALS disease progression have not previously been investigated.
In the present study, we aimed to determine the effects of altered Rho family GTPase activity on motor neuron survival in vitro and whether dysregulated activation of Rho family GTPases may contribute to ALS disease progression in vivo in the G93A mSOD1 mouse. We report that targeted inhibition of Rac GTPase via NSC23766 treatment or constitutive activation of Rho via CN03 treatment induced neurite retraction and apoptosis of ESC-derived motor neurons in vitro. Moreover, in vivo, active Rac1-GTP is significantly decreased in motor neurons from pre-symptomatic and end-stage G93A mSOD1 mice when compared to age-matched WT mice. Finally, we demonstrate that WT motor neurons express total RhoB in a diffuse distribution throughout the nucleus and soma while motor neurons of end-stage G93A mSOD1 mice demonstrate a significant redistribution of RhoB immunoreactivity to their axonal processes. Collectively, these findings demonstrate that Rac and Rho are critical mediators of motor neuronal survival. Consequently, diminished Rac GTPase activity or elevated Rho GTPase activity may contribute to the selective motor neuron degeneration observed in ALS.

5.3 Materials and Methods

5.3.1 Reagents

NSC23766, Fasudil, and the antibody used to detect ChAT were from EMD Millipore (Billerica, MA). Alexafluor 647 used to detect ChAT staining was from Life Technologies (Carlsbad, CA). CN03 and Y-27632 were purchased from Cytoskeleton (Littleton, CO). The polyclonal antibody used to detect active caspase-3 by immunocytochemistry was from Promega (Madison, WI). The polyclonal antibodies used
for immunostaining phosphorylated Akt (pAkt; S473), phosphorylated ERK5 (pERK5; T218/Y220), and Bad were from Cell Signaling Technology (Beverly, MA). The monoclonal antibodies used to detect active Rac1-GTP and active RhoA-GTP were purchased from NewEast Biosciences (King of Prussia, PA). The polyclonal antibodies used to detect total RhoB and the vesicular acetylcholine transporter (VACHT) were purchased from Santa Cruz Biotechnology (Dallas, Tx). 4,6-Diamidino-2-phenylindole (DAPI) and retinoic acid were purchased from Sigma (St. Louis, MO). The antibody utilized to detect CD11b was purchased from Abcam (Cambridge, MA). Anti-mouse Cy3- or anti-rat FITC-conjugated secondary antibodies utilized for immunofluorescence were from Jackson ImmunoResearch Laboratories (West Grove, PA). Sonic hedgehog (SHH), recombinant human brain-derived neurotrophic factor (BDNF), recombinant rat ciliary neurotrophic factor (CNTF), recombinant rat glial cell line-derived neurotrophic factor (GDNF), and recombinant human neurotrophic-3 (NT3) were all purchased from R&D Systems (Minneapolis, MN).

5.3.2 Hb9::GFP embryonic stem cell (ESC) culture

The Hb9 mouse embryonic stem cell (ESC) line was derived from a transgenic mouse line expressing green fluorescent protein (Hb9::GFP) cDNA under the motor neuron-specific Hb9 promoter and was kindly provided by Wichterle and colleagues. Hb9::GFP ESCs were maintained in an undifferentiated state in culture and propagated as previously described (Wichterle et al., 2002). Briefly, Hb9::GFP ESCs were grown on a monolayer of mouse embryonic fibroblasts in ESC-grade DMEM medium containing 4500 mg/L glucose and 2250 mg/L Na-bicarbonate supplemented with 15% hyclone fetal bovine serum (FBS), 2 mM L-glutamine, 1x Penicillin/Streptomycin, 1x non-essential
amino acids, 1x nucleosides, 0.1 mM 2-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor (EMD Millipore).

5.3.3 Hb9::GFP ESC differentiation

ESC-derived motor neurons were differentiated as previously described (Wichterle et al., 2002). Briefly, ESC cell colonies were partially dissociated and grown in aggregate culture in ADFNK medium [DMEM/F12 differentiation medium containing 4500 mg/L glucose and supplemented 2 mM L-glutamine, 1x Penicillin/Streptomycin, 0.1 mM 2-mercaptoethanol, 1x Insulin-Transferrin-Selenium supplement (Life Technologies), and 10% Knockout Serum Replacement (Life Technologies)] to promote the formation of embryoid bodies. After 2 days in culture, ADFNK medium was replaced and supplemented with retinoic acid (2µM; Sigma) to induce the formation of neuronal precursor cells. After 24 h, recombinant SHH and GDNF were added for an additional 4 days to promote the differentiation of neural precursor cells into spinal motor neurons. Embryoid bodies were dissociated with papain (Worthington Bio, Lakewood, NJ) into single cell suspensions and plated onto dishes coated with matrigel (BD Biosciences, San Jose, CA). Differentiated ESC-derived motor neurons were cultured in DMEM/F12 medium containing 4500 mg/L glucose and 2250 mg/L Na-bicarbonate supplemented with 5% horse serum, 1x Insulin-Transferrin-Selenium Supplement, 1x B27 Supplement, and neurotrophic factors (BDNF, CNTF, GDNF, NT3; each at 10 ng/mL). Consistent with the previously established protocol, typically 30% of the cells were EGFP-expressing motor neurons that could be maintained in culture for at least 7 days for experiments (Wichterle et al., 2002; Fig. 5.1).
5.3.4 Immunofluorescence microscopy

For green fluorescent images, live fluorescent images were captured using a 20× air objective on an Olympus IX71 microscope with a Diagnostics Insight Spot 2 charge-coupled device (CCD) camera and a Diagnostics capture software analysis program for digital deconvolution (Spot Imaging Solutions, Sterling Heights, MI).

For triple fluorescent images, motor neurons cultures were fixed in 4% paraformaldehyde, washed once in PBS, and then permeabilized and blocked in PBS containing 0.2% Triton X-100 and 5% BSA. Cell were incubated for 1 h in primary antibodies diluted in 2% BSA and 0.2% Triton X-100 in PBS and subsequently washed five times in PBS. Next, cells were incubated with DAPI and a Cy3-conjugated secondary antibody diluted in 2% BSA and 0.2% Triton X-100. The cells were washed five additional times with PBS before the addition of an antiquench solution composed of 0.1% p-phenylenediamine in 75% glycerol in PBS. Fluorescent images were captured using a 63× water immersion objective on a Zeiss Axioplan 2 microscope with a Cooke Sensicam deep cooled CCD camera and a Slidebook software analysis program for digital deconvolution (Intelligent Imaging Innovations Inc., Denver, CO).

5.3.5 Quantification of cell death and neurite integrity

As NSC23766- and CN03-induced cell death resulted in detachment of EGFP+ ESC-derived motor neurons from the culture dish, as an indicator of cell death and neurite integrity, the number of EGFP+ cells in ten randomly selected 20× fields was counted as was the number of motor neurons with neurites greater than three times the length of their soma. Typically, ~100-200 cells were quantified from each 22.73mm well.
Final counts represent the data obtained from at least three independent experiments performed in duplicate.

5.3.6 Animals

Wild type FVB (WT) and mice expressing G93A mutant human SOD1 (mSOD1) on an FVB background were obtained from Jackson Laboratories (Bar Harbor, ME). When two observers independently noted for two consecutive days that a mouse displayed either hind limb trembling or failure to extend either hind limb fully when held by its tail, the mouse was considered to display signs of disease onset (~90 days). Mice unable to right themselves within 15 seconds after being placed on their side were considered end-stage (~120 days). Male and female mice corresponding to pre-onset (~60 days), onset (~90 days), and end-stage (~120 days) were sacrificed in an isofluorane chamber followed by decapitation. Lumbar spinal cord sections were identified by vertebrae, isolated, and immediately preserved in OCT for immunohistochemical analysis.

5.3.7 Immunohistochemistry

For immunohistochemical studies, 18 μM lumbar spinal cord tissue sections obtained from paired animal sets (each G93A mSOD1 mouse and its WT littermate) were fixed in 4% paraformaldehyde. Next, the sections were blocked in PBS containing 5% BSA and 0.2% Triton X-100. Primary antibodies were diluted in PBS containing 2% BSA and 0.2% Triton X-100 and sections were incubated in primary antibody overnight at 4°C. Next, sections were washed five times in PBS. Sections were then incubated with Hoescht dye and Alexafluor 647-, Cy3-, or FITC-conjugated secondary antibody diluted in PBS containing 2% BSA and 0.2% Triton X-100. Sections were washed five
additional times with PBS before the addition of an antiquench solution composed of 0.1% \( p \)-phenylenediamine in 75% glycerol in PBS. Fluorescent images were captured using a 40× air objective on a Zeiss Axioplan 2 microscope with a Cooke Sensicam deep cooled CCD camera and a Slidebook software analysis program for digital deconvolution (Intelligent Imaging Innovations Inc., Denver, CO).

5.3.8 Immunohistochemical Quantification

For paired sets of animals (each G93A mSOD1 mouse and its WT littermate), identical exposure times were used to capture fluorescent fields containing a total of approximately 100 ChAT-positive motor neurons per mouse. The DAPI and ChAT signals were transposed onto a corresponding bright field image of the same field. To select a specific motor neuron for quantitative analysis, only cells with large nuclei and significant ChAT immunofluorescence were chosen. The bright field image was then used to free hand draw an outline of the cell. The outline was then transposed onto the triple fluorescent image. The Rac1-GTP or RhoA-GTP fluorescent signal (red) was then measured within the outlined cell in relative units of average fluorescence intensity per pixel using Adobe Photoshop CS. An average background red fluorescence signal was also calculated for each individual field imaged by measuring the average fluorescence intensity per pixel in regions of the field which were completely devoid of cell nuclei. This background value was then subtracted from each of the individual cell fluorescence values obtained from that specific field. Using this method, the Rac1-GTP and RhoA-GTP fluorescence was quantified from approximately 100 ChAT-positive motor neurons per mouse. Typically, images with high signal to noise ratios were most suitable for quantitative analysis.
5.3.9 Data Analysis

Results represent the mean ± S.E.M. for the number \((n)\) of independent experiments performed. Statistical differences between the means of unpaired sets of data were evaluated by one-way analysis of variance with a post hoc Tukey's test or a student’s t test. A \(p\) value of <0.05 was considered statistically significant. Images are representative of at least three independent experiments.

5.4 Results

5.4.1 Generation and differentiation of ESC-derived motor neurons

To examine the involvement of Rho GTPases in regulating the survival of motor neurons, we utilized the HBG3 mouse ESC cell line developed by Wichterle and Peljto (Wichterle et al., 2002; Wichterle and Peljto, 2008). This ESC line was derived from a transgenic mouse line expressing enhanced green fluorescent protein (EGFP) cDNA under control of the mouse Hb9 promoter. The homeobox gene, Hb9, is expressed selectively by postmitotic motor neurons and is a classical marker of mature motor neuron identity (Arber et al., 1999). ESC colonies were partially dissociated and grown in aggregate culture in differentiation medium to promote formation of embryoid bodies. After 2 days in aggregate culture, retinoic acid was added to promote differentiation into neural precursor cells (Fig. 5.1A; left panel). Following a 24 h exposure to retinoic acid, recombinant sonic hedgehog (SHH) and glial cell line-derived neurotrophic factor (GDNF) were added for an additional 4 days to promote differentiation and survival of spinal motor neurons (Fig. 5.1A; right panel). Embryoid bodies containing differentiated EGFP+ ESC-derived motor neurons (Fig. 5.1B; top image) were dissociated into single cell suspensions (Fig. 5.1B; middle image) and plated onto dishes coated with matrigel
(Fig. 5.1B; lower image and 5.1C). Following this protocol, approximately 30% of cells terminally differentiated into EGFP expressing motor neurons (Fig. 5.1B; lower panel and 5.1C). These EGFP+ motor neurons stain positively for Hb9, choline acetyltransferase (ChAT), and β3-tubulin (data not shown).
Figure 5.1  Generation and differentiation of ESC-derived motor neurons. A. HBG3 mouse ESCs harboring an EGFP transgene driven by the motor neuron-specific Hb9 promoter (Hb9::EGFP) were initially cultured on a monolayer of mouse embryonic fibroblasts in medium containing serum and leukemia inhibitory factor to suppress differentiation. After detachment with trypsin, ESCs were cultured in suspension with differentiation medium containing retinoic acid to induce development of motor neuron progenitors (left image). After 24 h of exposure to retinoic acid, sonic hedgehog (SHH) and glial cell line-derived neurotrophic factor (GDNF) were added to further promote the differentiation of neuronal precursor cells into spinal motor neurons (right image). After 48 h incubation with SHH, embryoid bodies typically were larger and contained more EGFP+ motor neurons than those maintained in medium containing only retinoic acid (compare right panels to left panels). Scale bar = 100 micron. B. Following the 48 h incubation period with SHH and GDNF (top panel), embryoid bodies (EBs) were collected and gently digested with papain to produce a single cell suspension (middle panel). These cells were then plated onto matrigel-coated culture dishes (bottom panel). After 24 h on matrigel, EGFP+ motor neurons appear healthy and extend long processes as well as multiple shorter neurites (lower panel). Scale bar = 20 micron. C. 2x magnification of bottom, right image from 1B. Scale bar = 20 micron.
5.4.2 A selective inhibitor of the Rac-specific GEFs, Tiam1 and Trio (NSC23766; Rac Inh), causes a marked loss of neurites and death of ESC-derived motor neurons

To establish the involvement of Rac-GTPase in maintaining survival of ESC-derived motor neurons, motor neuronal cultures were treated with the Rac inhibitor NSC23766. This compound selectively and reversibly hinders GDP/GTP exchange carried out by the Rac-specific GEFs, Tiam1 and Trio, which are significant regulators of Rac in the central nervous system (Schmidt and Hall, 2002; Gao et al., 2004). In a manner consistent with previous observations in CGNs (Chapter 3 of this thesis), ESC-derived motor neuron cultures succumbed to cell death following exposure to targeted Rac GTPase inhibition. Incubation with the Rac inhibitor for only 6 h caused significant shrinkage of motor neuron soma and fragmentation of neuronal processes (Fig. 5.2A). Following 24 h of Rac inhibition, these effects were even more pronounced with more than 50% of the motor neurons detached from the culture dish and essentially none of the remaining motor neurons exhibited significant neuronal processes (Fig. 5.2B). Consistent with a critical pro-survival function for Rac GTPase, we also observed activation of the death executioner caspase-3 in approximately 30% of the EGFP+ motor neurons that remained attached to the culture dish after a 24 h exposure to the Rac inhibitor (Fig. 5.4A). These results indicate that NSC23766-mediated inhibition of Rac GTPase in ESC-derived motor neurons elicits cell death via a mechanism consistent with both apoptosis and anoikis, a form of programmed cell death characterized by the detachment of cells from the extracellular matrix.
Figure 5.2 A selective inhibitor of the Rac-specific GEFs, Tiam1 and Trio (NSC23766; Rac Inh), causes a marked loss of neurites and death of ESC-derived motor neurons. A. ESC-derived motor neuron cultures were left untreated (Control) or incubated with the Rac Inh (200 µM) for 6 h. Note that incubation with the Rac Inh caused the motor neuron cell bodies to round up and induced marked fragmentation of neuronal processes. Scale bar = 20 micron. B. ESC-derived motor neuron cultures were left untreated (Con) or incubated with the Rac Inh (200 µM) for 24 h. After 24 h, the total number of EGFP+ motor neurons was quantified as was the number of motor neurons with neurites greater than three times the length of their soma. Results shown are the mean ± SEM of 3 independent experiments performed on duplicate wells. *p<0.05, **p<0.01 vs. Con.
5.4.3 Inhibition of Rho Kinase (ROCK) only modestly protects ESC-derived motor neurons subjected to selective Rac-GTPase inhibition

Recent evidence demonstrates that phenylalanine treatment of cortical neurons results in a reduction of Rac expression and an induction of mitochondrial-dependent apoptotic cell death in a RhoA/ROCK-dependent mechanism (Zhang et al., 2007; Zhang et al., 2010). Therefore, we next examined whether inhibition of Rac GTPase induces neurite retraction and subsequent cell death through unopposed Rho/ROCK signaling. In ESC-derived motor neuron cultures treated with NSC23766, inclusion of either ROCK inhibitor, Fasudil or Y-27632, showed a very modest attenuation of cell loss and neurite fragmentation/retraction (Fig. 5.3A). However, quantification revealed that neither ROCK inhibitor displayed a statistically significant protective effect against selective Rac inhibition (Fig. 5.3B and 5.3C). These data suggest that the principal mechanism by which ESC-derived motor neurons subjected to Rac inhibition undergo apoptosis is not due to unopposed activation of pro-apoptotic Rho/ROCK signaling.
Figure 5.3 Inhibition of ROCK only modestly protects ESC-derived motor neurons subjected to selective Rac-GTPase inhibition. A. ESC-derived motor neuron cultures were incubated in either control medium (Con), Con medium + Rac Inh (200 µM), Rac Inh + Fasudil (10µM), or Rac Inh + Y-27632 (10µM) for 24 h. Images of EGFP+ cells were captured at 24 h. Scale bar = 20 micron. B. ESC-derived motor neuron cultures were treated as described in (A). After a 24 h incubation, the total number of EGFP+ motor neurons was quantified. C. ESC-derived motor neuron cultures were treated as described in (A). After a 24 h incubation, the number of EGFP+ cells containing neurites at least 3x the length of the soma was quantified. Results shown are the mean ± SEM of 3 independent experiments performed on duplicate wells. Values are expressed as a percentage of the Con condition. **p<0.01, ***p<0.001 vs. Con.
5.4.4 Selective inhibition of Rac GTPase in ESC-derived motor neurons induces dephosphorylation of ERK5 and Akt and nuclear translocation of the BH3-only protein Bad

The modest protection afforded by ROCK inhibitors indicates that other pathways known to regulate cellular survival downstream of Rac GTPase may be altered in NSC23766-treated ESC-derived motor neurons. In previous studies carried out in primary CGNs, we have demonstrated that NSC23766-mediated Rac inhibition deactivates a pro-survival signaling cascade involving MEK5/ERK5 and Akt, ultimately leading to an induction, dephosphorylation, and mitochondrial localization of the pro-apoptotic BH3-only protein Bad (Chapter 3 of this thesis). As anticipated based on these prior results, incubation with NSC23766 similarly induced dephosphorylation (inactivation) of the pro-survival proteins ERK5 (T218/Y220) and Akt (S473) in ESC-derived motor neurons (Fig. 5.4B and 5.4C). Remarkably, NSC23766 treatment evoked a marked redistribution of the BH3-only protein Bad from a diffuse cytosolic pattern to an exclusively nuclear localization (Fig. 5.4D). These findings indicate that inhibition of Rac induces motor neuron death via a mechanism that involves inactivation of critical pro-survival ERK5- and Akt-dependent signaling pathways, in addition to a redistribution of the BH3-only protein Bad from the cytosol to the nucleus.
Figure 5.4 Selective inhibition of Rac GTPase in ESC-derived motor neurons induces activation of caspase-3, dephosphorylation of ERK5 and Akt, and nuclear translocation of the BH3-only protein Bad. ESC-derived motor neuron cultures were left untreated (Control) or incubated with the Rac Inh (200µM) for 24 h. Cells were fixed and signaling proteins were visualized with a Cy3-conjugated secondary antibody as indicated (shown in red). Nuclei were stained with DAPI (shown in blue). Arrows indicate EGFP+ motor neurons. A. Active (cleaved) form of caspase-3. B. pERK5 (T218/Y220). C. pAkt (S473). D. BH3-only protein Bad. Scale bar = 20 micron.
5.4.5 Constitutive activation of Rho via CN03 treatment induces a marked loss of neurites and death of ESC-derived motor neurons

In a manner similar to loss of active Rac GTPase, previous studies have demonstrated that activation of Rho GTPase is sufficient to provoke apoptosis in several neuronal models (Bertrand et al., 2007; Semenova et al., 2007; Sanno et al., 2010; Barberan et al., 2011). Thus, we examined whether a specific activator of Rho GTPase, CN03, is toxic to ESC-derived motor neurons. CN03 is a fusion protein made up of the catalytic domain of a bacterial cytotoxic necrotizing factor and a proprietary cell penetration moiety. CN03 potently and selectively activates Rho GTPase by deamidating glutamine 63 within the switch 2 region of the protein, thus locking it in an active conformation (Flatau et al., 1997; Schmidt et al., 1997). Following a 24 h incubation period with CN03, ESC-derived motor neuron cultures displayed an approximately 35% reduction in EGFP+ motor neurons and a nearly 50% decrease in the number of EGFP+ motor neurons with neurites at least three times the length of their soma (Fig. 5.5A). Quantification revealed that constitutive activation of Rho GTPase induces significant cell death and neurite retraction (Fig. 5.5B). As anticipated, these data highlight that in a manner similar to inhibition of Rac function, constitutive activation of Rho triggers motor neuron death.
Figure 5.5 Constitutive activation of Rho via CN03 treatment induces a marked loss of neurites and death of ESC-derived motor neurons. A. ESC-derived motor neuron cultures were left untreated (Control) or incubated with the CN03 (1 µg/mL) for 24 h. Scale bar = 20 micron. B. ESC-derived motor neuron cultures were left untreated (Con) or incubated with CN03 (1 µg/mL) for 24 h After 24 h, the total number of EGFP+ motor neurons was quantified as was the number of motor neurons with neurites greater than three times the length of their soma. Results shown are the mean ± SEM of 3 independent experiments performed on duplicate wells. *p<0.05, **p<0.01 vs. Con.
5.4.6 Inhibition of ROCK significantly protects ESC-derived motor neurons subjected to constitutive Rho GTPase activation

Recent evidence suggests a necessary role for ROCK in apoptosis induced by Rho activation (Godin and Ferguson, 2010; Zhang et al., 2010). To definitively establish whether downstream activation of ROCK is required to induce cell death in ESC-derived motor neurons subjected to Rho activation, we evaluated whether co-incubation with either ROCK inhibitor, Fasudil or Y-27632, exerted a protective effect against CN03 treatment. In ESC-derived motor neurons treated with CN03, co-incubation with either Fasudil or Y-27632 protected against membrane blebbing and neurite retraction (Fig. 5.6A). Quantification of cell loss and neurite fragmentation/retraction in ESC-derived motor neurons co-incubated with CN03 and Fasudil or Y-27632 revealed that inhibition of ROCK conferred significant neuroprotection against Rho activation (Fig. 5.6B and 5.6C). These data support a model in which activation of Rho induces motor neurite retraction, fragmentation, and cell loss that is dependent on activity of the downstream effector ROCK.
Figure 5.6 Inhibition of ROCK significantly protects ESC-derived motor neurons subjected to constitutive Rho GTPase activation. A. ESC-derived motor neuron cultures were incubated in either control medium (Con), Con medium + CN03 (1µg/mL), CN03 + Fasudil (10µM), or CN03 + Y-27632 (10µM) for 24 h. At 24 h, images of EGFP+ cells were captured. Scale bar = 20 micron. B. ESC-derived motor neuron cultures were treated as described in (A). After the 24 h incubation, the total number of EGFP+ motor neurons was quantified. C. ESC-derived motor neuron cultures were treated as described in (A). After the 24 h incubation, the number of EGFP+ cells containing neurites at least 3x the length of the soma was quantified. Results shown are the mean ± SEM of 3 independent experiments performed on duplicate wells. Values are expressed as a percentage of the Con condition. ***p<0.001 vs. Control, #p<0.05, ###p<0.001 vs. CN03.
5.4.7 Rac1-GTP immunoreactivity is decreased in ChAT-labeled motor neurons from G93A mSOD1 mice

Our data in ESC-derived motor neurons suggest that Rac GTPase activity is essential to maintain motor neuronal survival. To begin to unravel the complex role of Rac GTPase in ALS disease progression, lumbar spinal cord sections from end-stage G93A mSOD1 mice (G93A) and their age-matched wild type (WT) littermates were stained for DAPI, active Rac1-GTP, and ChAT. Quantification of Rac1-GTP intensity revealed that there is markedly reduced Rac1-GTP immunoreactivity in ChAT-positive motor neurons in the spinal cord of end-stage G93A mSOD1 mice when compared to WT mice (Fig. 5.7A and 5.7B). Furthermore, a similar reduction in Rac1-GTP immunoreactivity was observed at 90 days (disease onset) but not 60 days (presymptomatic; Fig 5.7B). To our knowledge, these findings are novel in that they are the first to identify a substantial and progressive loss of active Rac1-GTP specifically in spinal motor neurons of G93A mSOD1 mice.
Figure 5.7 Rac1-GTP immunoreactivity is decreased in ChAT-labeled motor neurons from G93A mSOD1 mice. A. Spinal cords from end-stage (~120 days) mice harboring the G93A SOD1 mutation (G93A) or wild type (WT) littermates were cryosectioned into 18µM sections. Sections were stained for DAPI, active Rac1-GTP (red), and choline acetyltransferase (ChAT; to label motor neurons; green). Note that Rac1-GTP immunoreactivity is consistently high in WT motor neurons but is substantially decreased in ChAT-positive cells from G93A mutant mice. Arrows indicate prominent ChAT-positive motor neurons. Scale bar = 10 micron. B. Lumbar spinal cord tissue was stained as described in (A) and quantified for paired animal sets (each pair consisting of an end-stage G93A mSOD1 mouse and its age-matched WT littermate) at end-stage (~120 days), disease onset (~90 days), and pre-symptomatic (~60 days). The graph shown represents the average fluorescence intensity (per pixel) per ChAT-positive cell and is presented as the mean ± SEM for 4 paired sets of mice at each disease stage. Values are expressed as a percentage of WT. *p<0.05 compared to WT by a paired t-test. NS denotes not significant.
5.4.8 Rac1-GTP immunoreactivity is enhanced in CD11b-positive microglia of end-stage G93A mSOD1 mice when compared to WT littermates

Collectively, our data in mouse ESC-derived motor neurons indicate an essential pro-survival function for Rac GTPase. Yet, previous studies have also shown that Rac1 activation within microglia can activate NADPH oxidase to enhance the production of reactive oxygen species (ROS) and significantly contribute to the death of neighboring neurons (Harraz et al., 2008). To determine whether increased activation of Rac1 in microglia may contribute to the progression of disease in the G93A mSOD1 mouse model of ALS, lumbar spinal cord sections were stained for DAPI, Rac1-GTP, and CD11b. In agreement with aberrant Rac1 GTPase activity contributing to ALS disease progression, we observed increased Rac1-GTP immunoreactivity in CD11b-labeled microglia in the spinal cord of end-stage G93A mSOD1 mice (G93A) when compared to WT mice (Fig. 5.8). Collectively, our data support a model in which aberrant Rac1 activity may contribute to ALS disease progression in a cell type-specific manner; through loss of its pro-survival function in motor neurons and enhancement of its ROS-generating function (via NADPH oxidase or Nox activity) in glial cells.
Figure 5.8 Rac1-GTP immunoreactivity is enhanced in CD11b-positive microglia of end-stage G93A mSOD1 mice when compared to WT littermates. Spinal cords from end-stage (~120 days) mice harboring the G93A SOD1 mutation (G93A) or wild type (WT) littermates were cryosectioned into 18µM sections. Sections were stained for DAPI, active Rac1-GTP (red), and CD11b (to label microglia; green). Scale bar = 10 micron. Right image: 2.5X magnification of demarcated boxes from the upper image. Note that Rac1-GTP immunoreactivity is essentially absent in WT microglia but is very high in CD11b-positive cells from G93A mutant mice. Arrow indicates CD11b-labeled microglia. Scale bar = 10 micron.
5.4.9 RhoA-GTP immunoreactivity is not altered in ChAT-labeled motor neurons from G93A mSOD1 mice

Given the antagonistic relationship between Rac GTPase and Rho GTPase signaling in neurons, we predicted that diminished expression of active Rac1-GTP in ChAT-positive motor neurons in the G93A mSOD1 mouse would correlate with increased expression of active Rho GTPase. Although three isoforms of Rho GTPase (RhoA, RhoB, and RhoC) have been identified, RhoA activity has been widely demonstrated to provoke apoptosis in a variety of neuronal models (Dubreuil et al., 2006; Semenova et al., 2007; Sanno et al., 2010). Thus, we stained lumbar spinal cord sections from end-stage G93A mSOD1 mice (G93A) and their age-matched WT littermates for DAPI, active RhoA-GTP, and ChAT. To our surprise, quantification of RhoA-GTP staining revealed that there was no statistical difference between RhoA-GTP expression in ChAT-positive motors neurons of end-stage G93A mSOD1 mice when compared to WT mice (Fig. 5.9A). These results indicate that increased activation of RhoA GTPase does not contribute to the motor neuronal cell death observed in the G93A mSOD1 mouse model of ALS.

5.4.10 RhoB immunoreactivity is enhanced in motor neuron axons of G93A mSOD1 mice

In addition to RhoA, enhanced RhoB activation has been demonstrated to be an early marker of neuronal death in a murine stroke model (Trapp et al., 2001), and cultured corticohippocampal neurons isolated from RhoB knockout mice are significantly less sensitive than WT neurons to apoptosis induced by staurosporine (Barberan et al., 2011). Therefore, we next stained lumbar spinal cord sections from end-stage G93A mSOD1 mice (G93A) and their age-matched wild type littermates for DAPI, RhoB, and
ChAT. Although the overall expression of RhoB did not appear to be enhanced in motor neurons from end-stage ALS mice, the localization of RhoB was drastically altered. In WT mice, ChAT-positive motor neurons expressed RhoB in a diffuse distribution throughout the nucleus and soma; however, essentially no immunoreactivity was observed in neuronal processes. In marked contrast, motor neurons of end-stage G93A mSOD1 mice demonstrated significant RhoB immunoreactivity within their processes (Fig. 5.9B and 5.9D). To definitively establish the axonal expression of RhoB in motor neurons, spinal cord sections from end-stage G93A mSOD1 mice and WT mice were stained for DAPI, RhoB and the vesicular acetylcholine transporter (VACHT). Our data demonstrate that RhoB expression partially co-localizes with the VACHT in motor neuron axons of end-stage G93A mSOD1, but not WT, mice (Fig. 5.9C). Interestingly, RhoB redistribution appears to occur at a relatively late stage during disease progression as RhoB was consistently localized to the nuclei and soma of both WT and G93A mSOD1 motor neurons at disease onset (~90 days; compare Fig. 5.9D and 5.9E).
Figure 5.9 RhoB immunoreactivity is enhanced in motor neuron axons of G93A mSOD1 mice

A. Spinal cords from end-stage mice (~120 days) harboring the G93A SOD1 mutation (G93A) or wild type (WT) littermates were cryosectioned into 18µM sections. Sections were stained for DAPI, active RhoA-GTP (red), and choline acetyltransferase (ChAT; to label motor neurons). The graph shown represents the average fluorescence intensity (per pixel) per ChAT-positive cell and is presented as the mean ± SEM for 3 paired sets of mice (each pair consisting of an end-stage G93A mSOD1 mouse and its age matched WT littermate). Values are expressed as a percentage of WT. NS denotes not significant compared by a paired t-test.

B. Spinal cords were processed and cryosectioned as described in (A). Sections were stained for DAPI, RhoB (red), and choline acetyltransferase (ChAT; to label motor neurons; green). Note that RhoB immunoreactivity is not observed in neuronal processes of WT motor neurons but brightly labels processes of ChAT-positive cells from G93A mSOD1 mice. Scale bar = 5 micron.

C. Spinal cords were cryosectioned as described in (A). Sections were stained for DAPI, RhoB (red), and vesicular acetylcholine transporter (VAChT; to label motor neuron axons; green). Note that RhoB immunoreactivity is not observed in neuronal processes of WT motor neurons but brightly labels processes of VAChT-positive motor neuron axons from G93A mSOD1 mice. Scale bar = 10 micron.

D. Lumbar spinal cord tissue was stained as described in (A) for 3 paired sets of end-stage mice (~120 days) mice. Note that in each animal set RhoB immunoreactivity is not observed in neuronal processes of WT motor neurons but brightly labels processes of ChAT-positive cells from G93A mSOD1 mice. Arrows indicate RhoB expression in motor neuron processes. Scale bar = 10 micron.

E. Lumbar spinal cord tissue was stained as described in (A) for 3
paired sets of disease onset mice (~90 days) mice. Note that in each 90 day animal set RhoB expression principally localizes to the soma in both WT and G93A ChAT-positive motor neurons. Scale bar = 10 micron.
5.4.11 Proposed model depicting the mechanisms by which dysregulated Rho family GTPase activity may contribute to selective motor neuron degeneration in the G93A mSOD1 mouse model of ALS

We propose in healthy spinal cords (Fig. 5.10A), microglia retain Rac1 in an inactive GDP-bound state while motor neurons promote pro-survival signaling by maintaining Rac1 in an active GTP-bound state. Furthermore, we propose that RhoB localizes principally to the soma of healthy motor neurons. Conversely, in the diseased spinal cords of G93A mSOD1 mice (Fig. 5.10B), we hypothesize that enhanced activation of Rac1-GTP in microglia contributes to the activation of NADPH oxidase and the release of toxic ROS from microglia. Furthermore, loss of Rac1-GTP pro-survival signaling in G93A mSOD1 motor neurons leads to neurite retraction and motor neuron death through deactivation of critical Rac1-dependent pro-survival signaling cascades. Finally, we hypothesize that RhoB redistribution to axons of motor neurons leads to axonal retraction and motor neuron death in G93A mSOD1 mice.
Figure 5.10 Proposed model depicting the mechanism by which dysregulated Rho family GTPase activity may contribute to selective motor neuron degeneration in the G93A mSOD1 mouse model of ALS. A. In wild type spinal cords, active Rac1 (GTP-bound) transmits pro-survival signals in motor neurons. RhoB is localized to the cytosol of motor neurons. In healthy microglia, Rac1 exists primarily in an inactive (GDP-bound) state. B. In G93A mSOD1 spinal cords, decreased Rac1-GTP in motor neurons promotes death via diminished pro-survival signals. The localization of RhoB from the cytosol to motor neuron processes also promotes axonal retraction and motor neuron death. Increased microglia in the spinal cord is indicative of microgliosis. Within reactive microglia, increased Rac1-GTP leads to enhanced activation of NADPH oxidase and the release of toxic reactive oxygen species (ROS). Red indicates increased activation. Blue indicates decreased activation. Green indicates altered localization. I acknowledge Aimee Winter for assistance with creating this figure.
5.5 Discussion

It is well documented that the antagonistic relationship between Rac GTPase and Rho GTPase is a determinant of neuronal survival. Therefore, it is perhaps not surprising that inhibition of Rac GTPase or constitutive activation of Rho GTPase induces cell death and neurite fragmentation/retraction of ESC-derived motor neurons. Indeed, inhibition of Rac GTPase in embryonic rat spinal motor neurons is sufficient to cause suppression of axon outgrowth and induce cell death (Jacquier et al., 2006). Moreover, in NSC34 motor neuron-like cells, constitutively active Rac protects these cells from the toxic expression of ALS-causing SOD1 mutants (Kanekura et al., 2005). Although the involvement of Rho GTPase in regulating motor neuron survival is not as well established, RhoA is activated in vulnerable brain regions following either traumatic brain injury or epileptic insult (Dubreuil et al., 2006), and RhoB null cortical neurons are significantly less sensitive to apoptotic stimuli than wild type neurons (Barberan et al., 2011). Thus, Rac GTPase and Rho GTPase play key roles in a regulating neuronal survival.

Our data indicate that inhibition of Rac GTPase induces neurite fragmentation and motor neuronal cell death through activation of the death executioner of apoptosis, caspase-3, and via deactivation of essential pro-survival kinases, including ERK5 and Akt. These results were anticipated based on our prior findings in primary CGNs demonstrating that selective Rac inhibition with an inhibitor of Tiam1 and Trio induces apoptosis through diminished pro-survival MEK5/ERK5 and Akt signaling (Chapter 3 of this thesis). These findings are consistent with those of Kanekura et al. (2005) who showed that a Rac/PI3K/Akt-dependent pro-survival signaling cascade attenuated mutant SOD1-induced toxicity in the NSC34 motor neuron cell line. Furthermore, our findings in
ESC-derived motor neurons substantiate the critical importance of Rac GTPase in transmitting key pro-survival signaling cascades in terminally differentiated motor neurons.

A critical pro-survival function of active pAkt (S473) is to phosphorylate the pro-apoptotic BH3-only protein Bad to sequester it to 14-3-3 cytosolic scaffolding proteins, effectively diminishing its ability to induce apoptosis at the mitochondria (Zha et al., 1996). Indeed, we have previously shown in NSC23766-treated CGNs that diminished pAkt (S473) induces mitochondrial localization of Bad (Chapter 3 of this thesis). Surprisingly, in the present study, we report that NSC23766 treatment evokes a marked redistribution of the BH3-only protein Bad from a diffuse cytosolic pattern to an exclusively nuclear localization in ESC-derived motor neurons. This result differs from the mitochondrial translocation of Bad observed in CGNs exposed to the Rac inhibitor; however, it is an intriguing finding since nuclear localization of Bad has been suggested to stimulate entry of cells into the cell cycle (Chattopadhyay et al., 2001; Al-Bazz et al., 2009), which is a known trigger of apoptosis in post-mitotic neurons (Folch et al., 2012).

Although our data indicate that inhibition of Rac GTPase induces neurite fragmentation and motor neuronal death, it was surprising to reveal that inclusion of two distinct ROCK inhibitors (i.e. Fasudil, Y-27632) did not significantly protect motor neurons from loss of Rac GTPase activity. Supporting crosstalk between Rac and Rho/ROCK signaling, Rac GTPase has been shown to repress Rho/ROCK signaling in HeLa cells through binding and activating the Rho GAP, p190RhoGAP (Wildenberg et al., 2006). Furthermore, it has been previously demonstrated that Rho/ROCK signaling can antagonize transmission of pro-survival Rac-dependent signaling pathways. In
particular, activation of Rho/ROCK enhances the phospholipid phosphatase activity of PTEN which when coexpressed with RhoA, results in a synergistic reduction in active Akt in human embryonic kidney cells (Li et al., 2005). Nonetheless, given that our data do not support significant involvement of ROCK in mediating death of Rac-inhibited ESC-derived motor neurons, it is possible that unopposed Rho GTPase activation may induce neurite retraction and subsequent apoptosis through an alternative downstream effector. Indeed, Semenova et al. (2007) previously demonstrated that ROCK activity was dispensable in CGNs undergoing Rho-dependent glutamate excitotoxicity as inclusion of Y-27632 did not attenuate nuclear fragmentation. Thus, our data do not exclude the possibility that inhibition of Rac GTPase leads to unopposed pro-apoptotic Rho GTPase signaling that occurs independent of ROCK activation in motor neurons.

Interestingly, although ROCK inhibitors were not protective against selective inhibition of Rac GTPase in ESC-derived motor neurons, both Fasudil and Y-27632 protected against neurite loss and apoptosis elicited via constitutive activation of Rho GTPase with CN03. These data highlight that activation of Rho GTPase may induce cell death through downstream activation of a ROCK/PTEN pathway as PTEN indirectly terminates Akt activation to induce neuronal death (Wu et al., 2012; Lai et al., 2014). Furthermore, a recent report demonstrated that inhibition of ROCK utilizing a metabolite of Fasudil, hydroxyfasudil, reduced G93A mSOD1-induced neurotoxicity in NSC34 motor neuronal-like cells in a manner that was dependent on reducing elevated PTEN levels and restoring diminished levels of active Akt. Moreover, inhibition of Akt abolished the neuroprotective effects afforded by hydroxyfasudil in G93A mSOD1-transfected NSC34 cells (Takata et al., 2013). Collectively, these data predict that
enhanced activation of Rho/ROCK signaling induces selective motor neuronal death through activation of PTEN and subsequent deactivation of the pro-survival protein Akt.

To further investigate whether dysregulated Rho family GTPase activity contributes to the etiology of ALS, we examined the expression of active Rac1 GTPase and RhoA GTPase in the spinal cord of G93A mSOD1 mice. We report that active Rac1-GTP immunoreactivity is significantly decreased in ChAT-positive motor neurons of G93A mSOD1 mice when compared to age-matched littersmates. Significant deficits in active Rac1-GTP were observed in motor neurons of G93A mSOD1 mice at 120 days (end-stage) and 90 days (onset), but not at 60 days (pre-symptomatic). This is consistent with our findings demonstrating an essential role for Rac GTPase in maintaining ESC-derived motor neuron survival and is further supported by studies reporting that expression of Rac1 can antagonize cell death caused by mSOD1 expression in a variety of neuronal models, including primary motor neurons (Kanekura et al., 2005; Jacquier et al., 2006; Pesaresi et al., 2011). A potential mechanism by which G93A mSOD1 may inactivate Rac1 GTPase in diseased motor neurons involves the growth factor adaptor protein p66Shc. Upon oxidative stress, p66Shc is phosphorylated and translocates to the mitochondria where it acts as an oxidoreductase to generate ROS (Giorgio et al., 2005; Gertz and Steegborn, 2010). While G93A mSOD1 expression in SH-SY5Y cells induces cell death consistent with decreased Rac1 GTPase activity, expression of an inactive mutant of p66Shc abrogates this effect. The ability of p66Shc to regulate Rac1 activity occurs via a redox-sensitive mechanism as treatment of SH-SY5Y cells with \( \text{H}_2\text{O}_2 \) led to a proportionate decrease in Rac1 GTPase activity that was attenuated by expression of an inactive p66Shc mutant (Pesaresi et al., 2011). While the precise mechanism by which
active Rac1-GTP is downregulated in spinal motor neurons of G93A mSOD1 mice remains to be determined, these findings highlight loss of Rac1 activity as a significant factor in the selective motor neuron death observed in ALS.

Given the antagonistic relationship exerted between Rac GTPase and Rho GTPase in conjunction with extensive evidence specifically implicating RhoA in neuronal apoptosis, we predicted that decreased active Rac1-GTP immunoreactivity would correlate with enhanced active RhoA-GTP expression in ChAT-positive motor neurons. We were surprised to find that there was no significant difference between RhoA-GTP expression in G93A mSOD1 and WT motor neurons. Instead, we found a marked redistribution of total RhoB expression. While RhoB exclusively localizes to the soma in WT motor neurons, RhoB significantly redistributed to neurites of spinal motor neurons in end-stage, but not disease onset, ALS mice. This is an important finding given substantial evidence demonstrating that RhoB can induce neurite retraction and subsequent neuronal apoptosis (Trapp et al., 2001; Conrad et al., 2005; Barberan et al., 2011). Indeed, in ALS mouse models, analysis of neuromuscular junctions reveals axonal retraction and denervation prior to loss of alpha motor neuron cell bodies in the lumbar spinal cord and associated development of clinical symptoms (Frey et al., 2000; Fischer et al., 2004). However, collectively, our data suggest that loss of active Rac1-GTP may underlie neurite retraction and motor neuron death in early disease stages (disease onset), while RhoB redistribution may contribute to neurite retraction in later disease stages (end-stage).

Contrary to the loss of active Rac1 GTPase observed in spinal cord motor neurons, we show increased Rac1-GTP staining in CD11b-positive microglia of end-
stage G93A mSOD1 mice when compared to WT littermates. In agreement with these findings, a previous report demonstrated that Rac1-GTP is actually upregulated in total spinal cord lysates isolated from G93A mSOD1 mice (Harraz et al., 2008), lending to the hypothesis that Rac1 activation plays a cell type-specific role in the progression of ALS. Indeed, although selective motor neuron degeneration is a hallmark of ALS, several reports indicate that death does not occur strictly via a cell-autonomous mechanism (Ilieva et al., 2009). Importantly, in addition to its role in neuronal development and survival, Rac1 is required to activate both Nox1 and Nox2, catalytic subunits of NADPH oxidase, and activation of this complex may contribute to the death of neighboring motor neurons through the generation of toxic superoxide (O$_2^-$) molecules (Wu et al., 2006; Raz et al., 2010). Nox1, Nox2, and NADPH oxidase activity are upregulated in spinal cord microglia from genetic mouse models of ALS and ALS patients (Wu et al., 2006). Moreover, previous studies have demonstrated that either genetic deletion or chemical inhibition of Nox1 or Nox2 can slow disease progression and improve survival in familial ALS mice (Wu et al., 2006; Harraz et al., 2008). Further substantiating a role for enhanced activation of Rac1 GTPase in microglia as a causative factor underlying the progression of ALS, Harraz et al. (2008) demonstrated that mSOD1 stimulates Rac1-dependent activation of NADPH oxidase in glial cells expressing ALS-causing forms of mutant SOD1. Collectively, these studies indicate that aberrant Rac1 GTPase activation in microglia may enhance NADPH oxidase activation and the release of toxic ROS to elicit motor neuron death in this particular mouse model of ALS.

In addition to activation of NADPH oxidase, expression of G93A mSOD1 in microglia leads to the Rac1-dependent secretion of pro-apoptotic TNFα and subsequent
cell death of neighboring motor neurons in co-culture experiments (Li et al., 2011). Intriguingly, a recent report by Tönges et al. (2014) demonstrated that inhibition of ROCK via oral administration of Fasudil is protective against motor neuron loss in the G93A mSOD1 mouse and this occurs at least partially via reduced secretion of TNFα from microglia. Overall, these data support the notion that balanced signaling between Rac and Rho/ROCK is ultimately disrupted in the spinal cord of G93A mSOD1 mice. One potential mechanism by which G93A mSOD1 may activate Rac1 GTPase in microglia is through the P2X<sub>7</sub> receptor (Apolloni et al., 2013), which is responsible for the release of pro-inflammatory factors from microglia, such as TNFα (Skaper et al., 2010). Although the precise mechanism by which activation of Rac1 in microglia enhances disease progression in the G93A mSOD1 mouse model of ALS requires further investigation; collectively, these data suggest that Rac1 may contribute to neurodegenerative disease in multiple cell type-specific ways; through loss of its pro-survival function in motor neurons and enhancement of its ROS-generating function in glial cells.

Takata et al. (2013) recently administered Fasudil to G93A mSOD1 mice to elucidate the involvement of ROCK activation on the integrity and viability of motor neurons. Indeed, administration of Fasudil reduced motor neuronal loss, slowed disease progression, and extended lifespan of G93A mSOD1 mice. Furthermore, this study confirmed that ROCK inhibition attenuated elevated PTEN activation while restoring diminished Akt activation that is typically observed in this particular familial mouse model of ALS. It is interesting to note that in each study examining ROCK inhibition in vivo, Fasudil was administered at a relatively early stage in disease progression (Takata et
In conjunction with our data implicating RhoB redistribution to neuronal processes in end-stage mice as a potential causative factor underlying disease progression, these data suggest that ROCK inhibition may prove to be an effective treatment even after disease onset. However, it is important to note that the antibody we utilized detected total RhoB expression as an antibody to active RhoB-GTP is not yet commercially available. Nonetheless, given this limitation, our data aid in clarifying the temporal and cell type-specific role that aberrant Rac1 GTPase and/or RhoB GTPase activity may contribute to the underlying pathology in the G93A mSOD1 mouse model of ALS.

In summary, we show that selective inactivation of Rac GTPase or constitutive activation of Rho GTPase is sufficient to evoke neurite retraction and subsequent cell death of ESC-derived motor neurons. Further confirming the pro-survival role of Rac GTPase in motor neurons, we report that Rac1-GTP is significantly reduced in ChAT-positive spinal cord motor neurons of G93A mSOD1 mice, when compared to age-matched WT littermates, at disease onset through end-stage. However, our data indicate that the involvement of dysregulated Rac1 GTPase in contributing to the pathology of ALS is much more complex as Rac1-GTP is upregulated in microglia from end-stage mice harboring the G93A SOD1 mutation. Finally, we highlight that although RhoB localizes principally to the soma of ChAT-positive motor neurons from WT mice, RhoB redistributes to neuronal processes, including motor neuron axons, in end-stage mice harboring the G93A SOD1 mutation. To our knowledge, these data are novel in that they are the first to reveal the temporal and cell type-specific dysregulation of Rho family GTPases in a familial mouse model of ALS.
CHAPTER SIX: DISCUSSION

Accumulating evidence suggests an important and conserved role for Rho family GTPases in mediating neuronal survival and death. Pivotal studies have demonstrated that while Rac GTPase stimulates neurite outgrowth and neuronal survival, Rho GTPase typically provokes neurite retraction and neuronal apoptosis. Nonetheless, the precise pro-survival and pro-apoptotic signaling pathways mediated by Rho family GTPases in neurons remains incompletely understood. We have previously shown that the Rho family GTPase inhibitor ToxB induces CGN apoptosis primarily through deactivation of Rac1 GTPase (Linseman et al., 2001; Le et al., 2005; Loucks et al., 2006). Thus, a principal focus of this thesis was to examine the precise signaling pathways that are regulated by Rac1 GTPase in primary CGNs. Furthermore, given recent evidence suggesting that dysregulation of Rho GTPases may underlie the pathology of motor neurodegenerative diseases such as ALS, an additional focus of this thesis was to examine the effects of dysregulation of Rac GTPase or Rho GTPase in terminally differentiated ESC-derived motor neurons and in an in vivo familial mouse model of ALS.

6.1 Summary of Major Findings

We have previously shown that ToxB, a broad spectrum inhibitor of Rho, Rac, and Cdc42, induces apoptosis of primary CGNs predominantly through deactivation of Rac1 GTPase (Linseman et al., 2001; Le et al., 2005; Loucks et al., 2006). Indeed, in
healthy CGNs, Rac GTPase promotes neuronal survival through activation of a pro-survival MEK1/2/ERK1/2 signaling cascade, acting downstream of PAK, that functions to repress JNK/c-Jun activation of the BH3-only protein Bim, as well as, a pro-apoptotic JAK/STAT signaling cascade (Linseman et al., 2001; Le et al., 2005; Loucks et al., 2006). The aim of Chapter 2 of this thesis was to examine which particular member of the STAT family induced CGN apoptosis downstream of Rac GTPase inhibition. In CGNs treated with ToxB, STAT5 was upregulated, hyper-phosphorylated, and translocated to nuclei to suppress transcription of the pro-survival protein Bcl-xL. These data helped clarify a critical pro-apoptotic pathway that is activated in primary CGNs in response to Rac GTPase inhibition.

Given that ToxB inhibits Rho, Rac, and Cdc42, the aim of Chapter 3 was to examine the effects of a more targeted inhibitor of Rac GTPase in CGNs. NSC23766 is an inhibitor of the Rac-specific GEFs, Tiam1 and Trio, and treatment with this compound elicited apoptosis of CGNs via a mechanism that was distinct from ToxB. While ToxB suppressed a pro-survival MEK1/2/ERK1/2 signaling cascade that led to reduced degradation of the BH3-only protein Bim, the selective Rac inhibitor instead suppressed a distinct pro-survival MEK5/ERK5 pathway that led to increased expression and mitochondrial localization of the BH3-only protein Bad. These data demonstrate that global inhibition of Rho GTPases versus selective inactivation of Rac GTPase induces apoptosis through suppression of unique MAP kinase signaling pathways, which regulate distinct pro-apoptotic members of the BH3-only subfamily of Bcl-2 family proteins.

An intriguing finding revealed in Chapter 4 of this thesis was that treatment of CGNs with LTox or ToxB, which have overlapping specificity for inhibiting Rac
GTPase, induced apoptosis concomitant with reduced expression of the transcriptional corepressors, CtBPs. Thus, Chapter 4 of this thesis aimed to clarify the involvement of CtBP downregulation in neuronal apoptosis. In CGNs undergoing apoptosis, CtBPs appear to be regulated post-transcriptionally and in a mechanism dependent on both caspase activation and intact miRNA biogenesis machinery. Furthermore, MTOB-induced inhibition of CtBPs induced apoptosis that was consistent with upregulation of the CtBP target and BH3-only protein, Noxa. Although the exact mechanism by which Rho GTPases regulate CtBP expression warrants further investigation, these data demonstrate that CtBPs are essential pro-survival factors in CGNs.

Chapter 5 of this thesis aimed to extend our findings elucidating the involvement of Rho GTPases in regulating CGN survival into an in vitro motor neuron model more relevant to the etiology of ALS. Supporting an antagonistic relationship between Rac GTPase and Rho GTPase, we report that inhibition of Rac GTPase or constitutive activation of Rho GTPase was sufficient to induce apoptosis of ESC-derived motor neurons. Similar to our findings in CGNs, selective inhibition of Rac GTPase via NSC23766 evoked apoptosis through suppression of pro-survival kinase pathways involving ERK5 and Akt, as well as, nuclear translocation of the BH3-only protein Bad. Furthermore, we report that Rac1-GTP is diminished in motor neurons and enhanced in microglia in the spinal cords of mice harboring the G93A mSOD1 mutation, when compared to age-matched WT littermates. Interestingly, we also demonstrated a marked redistribution of RhoB from the cell soma of WT motor neurons to neuronal processes of motor neurons in mice carrying the G93A SOD1 mutation. These data are the first to
identify the cell type–specific and temporal dysregulation of Rho family GTPases in a mouse model of familial ALS.

6.2 Elucidating the relationship between Rho GTPases and CtBPs

The downregulation of CtBP1 and CtBP2 in CGNs exposed to the small GTPase inhibitors ToxB and LTox is a novel finding that warrants future investigation. As ToxB (an inhibitor of Rac, Rho, and Cdc42) and LTox (an inhibitor of Rac, Ras, and Rap) have overlapping specificity for inhibiting Rac GTPase, these findings suggests that Rac GTPase may function upstream of CtBPs to regulate their expression and consequently, the transcriptional repression of BH3-only pro-apoptotic proteins, such as Noxa. While the precise mechanism by which CtBPs are downregulated in neurons exposed to small GTPase inhibitors requires further investigation, recent studies have suggested a positive correlation between the expression of CtBPs and the activity of Rho family GTPases in non-neuronal cells. For example, overexpression of CtBP2 enhances Rac-dependent migration of human non-small cell lung carcinoma cells consistent with both PTEN inactivation and Akt activation (Paliwal et al., 2007).

Intriguingly, the CtBP-dependent activation of Rac requires the Rac-specific GEF, Tiam1. Initial studies demonstrated that Tiam1 appears to be regulated by the CtBP binding partner E1A. For instance, E1A expression led to the upregulation of Tiam1 and the formation of Rac-dependent cadherin-based adhesions in mesenchymal V12Ras-transformed Madin-Darby canine kidney II cells (Malliri et al., 2004). More recently, it has been shown that CtBP2 occupies the Tiam1 promoter and enhances its transcription in a Basic Kruppel-like factor (BKLF8)-dependent manner in HCT116 human colon carcinoma cells (Paliwal et al., 2012). Indeed, although the recruitment of CtBPs to
DNA-binding repressors (e.g. E1A, BKLF8) typically results in transcriptional repression, more recent studies have demonstrated that CtBP may also activate transcription in a gene-dependent manner. For instance, *Drosophila* CtBP activates transcription of Wnt target genes following Wnt stimulation, while repressing other Wnt target genes in the absence of Wnt (Fang et al., 2006). Thus, CtBP may enhance transcription of some genes (i.e. Tiam) while repressing others (i.e. PTEN, Noxa).

In conjunction with our own findings, these data suggest that CtBP1/CtBP2 and Rac GTPase may function in a positive feedback loop to regulate the expression and activities of one another (Fig. 6.1). Nonetheless, it is important to consider that our studies demonstrating that CtBPs are regulated downstream of Rac GTPase were conducted in primary neurons, while the CtBP2-dependent upregulation of Tiam1 and subsequent activation of Rac GTPase was shown in H1299 cell lung and HCT116 colon carcinoma cells. Thus, whether or not Rac GTPase lies upstream or downstream of CtBP1 and CtBP2 may simply depend on the cell type. In future studies, it will be important to determine whether CtBP2 similarly triggers Tiam1-dependent activation of Rac GTPase in neurons. Indeed, the link between CtBPs and Rho family GTPases is only recently being elucidated and future studies will be necessary to decipher the precise relationship between CtBPs and Rho family GTPases.
Figure 6.1 Proposed positive feedback loop between CtBPs and Rho GTPases. Through a presently unknown mechanism, Rac GTPase enhances the expression of CtBPs. CtBPs bind to DNA binding proteins (e.g. BKL F8) and occupy the promoter region of the Rac-specific GEF Tiam1 to stimulate transcription. Increased levels of Tiam1 result in enhanced activation of Rac-GTP.
6.3 Inhibition of Rac1 GTPase as a novel in vivo model to study the pathology of ALS

Collectively, the in vitro experiments presented in this thesis highlight a critical function for Rac GTPase in regulating neuronal survival. While Chapter 2 and Chapter 3 demonstrate a crucial function for Rac GTPase in maintaining the survival of primary CGNs; more recently, our work in Chapter 5 has identified a critical function for Rac GTPase in maintaining the survival of terminally differentiated ESC-derived motor neurons. The importance of Rac GTPase in maintaining motor neuron survival is substantiated by the fact that the Rac GEF alsin is mutated in juvenile onset forms of ALS (Yang et al., 2001; Hadano et al., 2007). As previously discussed, alsin activates Rac to maintain survival of embryonic rat spinal motor neurons (Jacquier et al., 2006) and it has also been demonstrated that alsin can antagonize mSOD1-induced cell death of NSC34 motor neuronal-like cells (Kanekura et al., 2005). However, the complexity of whether or not Rac GTPase is responsible for motor neuron specific death associated with alsin loss-of-function in in vivo rodent models is complicated by the fact that alsin loss-of-function models do not fully recapitulate the aspects of the human disease. Indeed, although four groups have independently generated different lines of ALS2 knockout (ALS2−/−) mice, each group found that the loss of alsin does not have a dramatic effect on the survival or function of motor neurons in mice (Cai et al., 2005; Devon et al., 2006; Hadano et al., 2006; Yamanaka et al., 2006). This could be due to the intrinsic differences in organization of the human and mouse motor systems. In addition, the paradox may be explained by compensatory mechanisms due to the fact that gene knockout typically occurs prior to embryonic development (Hadano et al., 2006). Nonetheless,
it is interesting to note that a cross between ALS2−/− mice and mice expressing G93A mSOD1 did not exacerbate G93A mSOD1-associated disease symptoms such as body weight loss and motor dysfunction (Hadano et al., 2010), underscoring the possibility that Rac activity is already compromised in transgenic mice expressing G93A mSOD1.

Furthermore, while we and others have demonstrated that a loss of Rac GTPase activity may contribute to the etiology of disease progression in the G93A mSOD1 mouse model of familial ALS, one caveat of these studies that must be considered is the poor clinical relevance of this particular model to human cases of ALS (reviewed in Wilkins et al., 2011). For instance, the only drug currently approved by the FDA for the treatment of ALS, the anti-glutamateric compound Riluzole, demonstrates signs of preserved motor neuron function and prolongs survival of the G93A mSOD1 mouse by approximately two weeks (Gurney et al., 1996; 1998). Nonetheless, Riluzole has only a minimal effect in human ALS progression and extends lifespan a mere three months (Miller et al., 2007; Brooks, 2009). In addition to Riluzole, a number of compounds targeting different facets of ALS disease progression (e.g. the antioxidant Vitamin E, the antibiotic Minocycline, etc.) have shown no effect in the clinic after demonstrating promising results in the G93A mSOD1 mouse (Wilkins et al., 2011). These data highlight the necessity to develop new rodent models to study the pathogenesis of ALS.

A prominent field of research worthy of future investigation is the design of new in vivo rodent models to study the underlying pathology and progression of ALS. Based on the work presented in this thesis in conjunction with human studies demonstrating that diminished Rac GTPase activity may be a causative factor in both familial and sporadic forms of ALS (Yamanaka et al., 2003; Topp et al., 2004; Kanekura et al., 2005; Jacquier...
et al., 2006; Harraz et al., 2008; Figuera-Romero et al., 2013), development of a Rac GTPase loss-of-function mouse expressing an inducible dominant negative mutant of Rac1 under a motor neuron-specific promoter (e.g. ChAT, HB9) may aid in clarifying the precise consequence that a loss of Rac1 GTPase activity in motor neurons contributes to motor neuron disease. In a similar manner, generation of a mouse model expressing inducible constitutively active Rac1 under a microglia-specific promoter (e.g. CD11b) may aid in clarifying the precise involvement that gain of Rac1 GTPase activity in microglia contributes to the death of neighboring motor neurons. As it stands, while the G93A mSOD1 mouse model of ALS has been helpful in clarifying the molecular mechanisms that underlie ALS disease progression, the fact that mSOD1 research has not led to clinically applicable therapeutic advances indicates the necessity for more therapeutically relevant models.

6.4 Mechanisms and consequences of dysregulated Rho GTPase activity in ALS disease progression

6.4.1 Mechanisms and consequences of dysregulated Rac GTPase activity in ALS disease progression

Supporting a pro-survival function for Rac GTPase, several studies have substantiated an essential role for Rac GTPase in maintaining survival of various different neuronal types, including motor neurons. These data lend to the hypothesis that a loss of Rac GTPase function may underlie the selective motor neuronal death associated with neurodegenerative disorders such as ALS. Nonetheless, this hypothesis has been challenged by recent evidence demonstrating that active Rac1-GTP is actually upregulated in total spinal cord homogenates isolated from mice expressing G93A...
mSOD1 when compared to age-matched littermates (Harraz et al., 2008). These findings are not necessarily mutually exclusive and the data presented in this thesis aid in bridging the gap in current research by demonstrating that Rac1 GTPase activity is diminished in motor neurons and enhanced in microglia of mice harboring the G93A SOD1 mutation. Given the large increase in microglia coincident with the death of substantial numbers of motor neurons in the spinal cord of end-stage ALS mice, our data and those of Harraz et al. seem to be compatible. Moreover, within the diseased spinal cord of transgenic mice expressing G93A mSOD1, recent research indicates that mutant forms of SOD1 may either increase or decrease the activity of Rac1 GTPase in a redox sensitive and cell-type specific manner.

The ability of mutant forms of SOD1 to influence Rac1 GTPase in a redox sensitive manner is particularly interesting given the proposed role of oxidative stress in the disease progression of ALS. For example, several distinct mutant forms of SOD1 form aggregates in the mitochondria of motor neurons and cause a shift in the mitochondrial redox state, ultimately resulting in a more oxidized environment (Ferri et al., 2006). Consistent with our data demonstrating diminished Rac1-GTP expression in motor neurons from G93A mSOD1 mice, a recent study has established the ability of G93A mutant SOD1 to induce cell death via decreased expression of active Rac1 GTPase in a redox sensitive manner in SH-SY5Y neuroblastoma cells. In this case, the growth factor adaptor p66shc, a known regulator of mitochondrial-dependent oxidative balance, diminishes the activity of Rac1 GTPase following adenoviral-driven expression of mutant, but not wild type, SOD1. Intriguingly, p66Shc-mediated downregulation of Rac1 GTPase activity in SH-SY5Y cells expressing G93A mSOD1 appears to occur in a
redox-sensitive manner as increasing concentrations of H$_2$O$_2$ results in a proportional decrease in Rac1 GTPase activity and cell viability that is prevented by expression of an inactive p66Shc mutant. The potential significance of p66shc-induced downregulation of Rac1 GTPase activity is underscored by the fact that G93A mSOD1 mice crossed with p66Shc$^{-/-}$ mice show improved motor performance, delayed disease onset, and increased survival with respect to G93A mSOD1 mice (Pesaresi et al., 2011). While this previous study was pivotal in demonstrating that G93A mSOD1 expression can cause a decrease in Rac1 GTPase activity in SH-SY5Y cells, further studies will need to be conducted to determine whether decreased expression of Rac1 GTPase occurs in a G93A mSOD1- and p66Shc-dependent mechanism in motor neurons in particular.

In addition to G93A mSOD1-mediated downregulation of Rac1 GTPase activity in motor neurons, it is also possible that Rac1 GTPase activity is decreased during the progression of ALS due to alterations in the activity of Rac-specific GEFs. Indeed, as previously discussed, loss-of-function mutations in the GEF alsin underlie early-onset familial forms of ALS (Yang et al., 2001; Yamakana et al., 2003; Hadano et al., 2007). Interestingly, recent evidence also suggests that epigenetic alterations of genes encoding GEFs for Rac GTPase may underlie some sporadic forms of ALS. For example, the Rac GEF, ARHGEF16, is hypermethylated and downregulated in postmortem sporadic ALS spinal cord samples when compared to samples from neurologically normal controls (Figueroa-Romero et al., 2012). These data indicate that loss of Rac activation due to diminished GEF activity may also underlie motor neuron degeneration in both familial and sporadic ALS disease progression.
In a manner dissimilar to motor neurons, several studies indicate that mutant forms of SOD1 associated with the pathology of ALS have an opposing function in microglia, ultimately resulting in enhanced activation of Rac1 GTPase in a redox-sensitive manner. For example, Harraz et al. (2008) demonstrated WT SOD1 binds to Rac1 and inhibits its intrinsic GTPase activity under reducing conditions, ultimately increasing active Rac1 GTPase activity. In the presence of \( \text{H}_2\text{O}_2 \) (oxidizing conditions), the interaction between WT SOD1 and Rac1 GTPase was uncoupled. Intriguingly, ALS-causing mutations in SOD1 prevent the enzyme from redox uncoupling, resulting in enhanced activation of Rac1 GTPase in microglia cells. Inevitably, aberrant Rac1 GTPase activity leads to increased activation of Nox2, a catalytic subunit of NADPH oxidase, resulting in an overproduction of damaging ROS. Indeed, in agreement with Rac1-dependent activation of NADPH oxidase as a contributing factor underlying motor neuron degeneration in the G93A mSOD1 mouse, inhibition of or genetic deletion of Nox2 improves disease progression and increases survival in this particular mouse model of familial ALS (Wu et al., 2006; Marden et al., 2007; Valdmanis et al., 2008).

Furthermore, Apolloni et al., (2013) recently identified extracellular ATP as a potential mechanism by which Rac1 GTPase activity may be enhanced in microglia of G93A mSOD1 mice. In primary microglia cells isolated from G93A mSOD1 mice, stimulation of the P2X\(_7\) receptor by its agonist, 2’-3’-\(O\)-(benzoyl-benzoyl) ATP enhanced Nox2 activation and the production of ROS in a Rac1-dependent manner when compared to microglia derived from non-transgenic animals. Thus, recent experimental evidence suggests that G93A mSOD1 may stimulate Rac1 GTPase activation in a P2X\(_7\)-dependent mechanism to enhance the release of toxic ROS from microglia.
6.4.2 Mechanisms and consequences of dysregulated Rho GTPase activity in ALS disease progression

In contrast to a critical pro-survival role for Rac GTPase, many reports indicate that Rho GTPase has an opposing function and leads to the transmission of pro-apoptotic signals in neurons, including motor neurons. Although three isoforms of Rho GTPase (RhoA, RhoB, and RhoC) have been identified, the involvement of RhoA and RhoB in provoking neuronal apoptosis has been best described. While we predicted that a loss of Rac1-GTP would correlate with increased active RhoA-GTP expression, we did not detect a significant increase in active RhoA-GTP in motor neurons of G93A mSOD1 mice. However, we found a distinct redistribution of total RhoB in end-stage G93A mice when compared to age-matched littermates. While RhoB principally localized to the soma of WT motor neurons, it drastically redistributed to neuronal processes, including motor neuron axons, of end-stage (~120 days), but not disease onset (~90 days), mice. This is an important finding considering the involvement of RhoB GTPase in inducing both neurite retraction and apoptosis (Conrad et al., 2005). Consistent with these functions, we found that constitutive activation of Rho induced both neurite retraction and membrane blebbing indicative of apoptosis in ESC-derived motor neurons. In future studies, it will be important to discern whether RhoB redistribution leads to neurite retraction and/or motor neuron apoptosis in the G93A mSOD1 mouse. Indeed, several studies have highlighted that axonal retraction may occur independent of motor neuron apoptosis in the familial mouse models of ALS. For example, studies indicate that in early stages of the disease the nerve terminals and neuromuscular junctions are disassembling while cell bodies in the spinal cord are relatively intact, leading to the
notion that a “dying back” phenomenon underlies the pathology of ALS (Frey et al., 2000; Fischer et al., 2004). This is somewhat conflicting with our finding that total RhoB redistribution to axons occurs in end-stage, but not disease onset, G93A mSOD1 motor neurons. Future studies will need to be performed to clarify the involvement of RhoB in mediating neurite retraction and motor neuron death in rodent ALS models. Alternatively, the loss of Rac1-GTP observed in motor neurons at disease onset may also contribute to inhibition of axonal outgrowth in early disease stages of ALS progression. Given the antagonistic relationship exerted between Rac GTPase and Rho GTPase, it is likely that dysregulated activity of both GTPases contributes to axonal retraction and motor neuron death observed during the progression of ALS.

**6.5 Pharmacological targeting of Rho GTPase signaling pathways as a therapeutic strategy for the treatment of ALS**

*6.5.1 Nox inhibitors to target aberrant Rac GTPase activation in microglia during ALS disease progression*

Collectively, the data presented in this thesis along with the work of others, has suggested that increased Rac1 GTPase activity in microglia may contribute to the activation of Nox2, the subsequent activation of NADPH oxidase, and generation of toxic ROS. Thus, one potential therapeutic target for the treatment of ALS may be pharmacological inhibition of Nox. Indeed, Marden et al. (2007) reported that homozygous knockout of Nox1 and Nox2 increased the lifespan of B6SJ G93A mSOD1 mice by 33 and 97 days, respectively. In a similar study, it was found that Nox2 deletion extended the survival of C57BL/6J G93A mSOD1 mice by 15 days (Wu et al., 2006). Pharmacological inhibition of Nox with high dose apocynin in B6SJL G93A mSOD1
mice increased survival 113 days (Harraz et al., 2008). Recent efforts have also examined whether diapocynin, the activated metabolite of apocynin, similarly extends survival of G93A mSOD1 mice. While diapocynin and apocynin protected primary motor neurons from nitric oxide-induced death in vitro, Trumbull et al. (2012) did not observe any significant effect of these compounds on survival in B6SJL G93A mSOD1 expressing mice. Although both of these studies treated mice harboring the G93A SOD1 mutation on the same genetic background, the disparity in these reports may have occurred because Trumbull et al. (2012) began apocynin and diapocynin treatment at 21 days of age, while Harraz et al. (2008) began apocynin treatment at 14 days of age. Nonetheless, in the Trumbull et al. (2012) study, mice treated with either apocynin or diapocynin did show a trend toward increased survival. These conflicting studies underscore the need for additional mouse models of ALS that are not related to mutations in SOD1 to further evaluate the potential use of Nox inhibitors as a treatment option for ALS patients.

Apocynin represents an attractive therapeutic compound for the treatment of ALS due to its low toxicity and lack of side effects in humans (Van den Worm et al., 2001; Stefanska and Pawliczak, 2008). Furthermore, diapocynin is also an attractive therapeutic compound as it is predicted to be more permeable to the blood-brain barrier due to its greater hydrophobicity. Indeed, in addition to rodent models of ALS, oral treatment with apocynin is safe and effective in the MPTP marmoset model of Parkinson’s disease (PD). During disease progression, apocynin relieved PD symptoms and increased the number of surviving dopaminergic neurons compared to controls (Philippens et al., 2013). Thus, these prior studies highlight the safety of apocynin for the treatment of devastating neurodegenerative disorders. Apocynin appears to be a relatively safe compound and it is
worthy of consideration for future clinical trials in ALS patients. Nonetheless, further examination is required to determine the safety of long-term use of Nox inhibitors in humans.

6.5.2 ROCK inhibitors to target aberrant Rho GTPase activation in motor neurons during ALS disease progression

The data presented in this thesis suggest that redistribution of RhoB to neuronal processes and potential activation of the downstream effector ROCK may contribute to axonal retraction and motor neuron death in the G93A mSOD1 mouse model of ALS. Thus, inhibition of ROCK represents an attractive therapeutic target for examination in preclinical trials using familial mouse models of ALS. Indeed, we recently conducted preliminary experiments to determine whether treatment with the ROCK inhibitor Fasudil was protective in the G93A mSOD1 mouse model. Mice were administered 100 mg/kg of Fasudil in their drinking water beginning at 30 days of age. FVB G93A mSOD1 mice administered Fasudil showed a delay in disease onset compared to untreated mutant mice as measured by hind limb trembling or failure to extend either hind limb fully when held by its tail (Fig. 6.3A). In addition, utilizing a paw grip endurance (PaGE) test that measures the muscle strength of forelimbs and hindlimbs, G93A mSOD1 mice receiving Fasudil treatment showed a delay in the decline in grip strength when compared to untreated G93A mSOD1 mice (Fig. 6.2A). Fasudil treated mice also displayed a delay in weight loss (Fig. 6.2B). Finally, we noted an increase in survival of G93A mSOD1 mice treated with Fasudil when compared to untreated mutant mice (Fig. 6.3B). Although very preliminary, these results suggest that inhibition of ROCK may prove to be an effective treatment option for ALS patients.
Figure 6.2 Administration of the ROCK inhibitor Fasudil delays latency to fall and weight loss in the G93A mSOD1 mouse. Beginning at 60 days, mice were administered 100 mg/kg Fasudil daily in their drinking water. A. PaGE hanging wire test represented as mean latency to fall at indicated ages. Note the delay in latency to fall in G93A mSOD1 mice when compared to untreated mutant mice (n=2). B. Percent of peak body weight at indicated ages. Note that the weight loss observed in untreated G93A mSOD1 is abrogated by Fasudil treatment (n=2). I would like to acknowledge Aimee Winter for assistance in generating data for this figure.
Figure 6.3 Administration of the ROCK inhibitor Fasudil delays onset and extends survival in the G93A mSOD1 mouse. Beginning at 60 days, mice were administered 100 mg/kg Fasudil daily in their drinking water. A. Onset was determined when a mouse displayed for two consecutive days either hind limb trembling or failure to extend either limb fully when held by its tail (n=2). B. End-stage was determined when mice were unable to right themselves within 15 seconds after being placed on their side (n=2). I would like to acknowledge Aimee Winter for assistance in generating data for this figure.
Consistent with non-cell autonomous death underlying the etiology of ALS, our preliminary data is consistent with two recent reports examining the effect of Fasudil on both microgliosis (Tönges et al., 2014) and motor neurons death (Takata et al., 2013) in G93A mSOD1 mice on a BJSJL background. Tönges et al. (2014) demonstrated that inhibition of ROCK preserved neuromuscular junctions and extended the survival of G93A mSOD1 via a mechanism consistent with reduced microgliosis and decreased release of pro-inflammatory cytokines and chemokines such as TNFα and IL-6. When examining motor neuron death in G93A mSOD1 mice, Takata et al. (2013) demonstrated that administration of Fasudil slowed disease progression and reduced motor neuron loss by a mechanism consistent with reduced activation of ROCK and PTEN and restored activation of the pro-survival kinase Akt. In future studies, it will be important to discern whether the protection afforded by ROCK inhibitors correlates with reduced inhibition of Rac1 GTPase in motor neurons of mice harboring mutations in SOD1. In conjunction with our own work demonstrating that dysregulation of both Rac GTPase and Rho GTPase may contribute to microglial activation and motor neuron death in G93A mutant SOD1 mice, these studies serve to highlight that the intricate balance between Rho GTPases is a critical determinant of motor neuronal survival and may be disrupted in neurodegenerative disease such as ALS.

Collectively, these data highlight ROCK as a promising target for the treatment of ALS patients. Currently, Fasudil is already in clinical use as a vasodilator agent (Zhao et al., 2011) and inhibition of ROCK in patients with cerebral vasospasm does not appear to cause any adverse side effects (Shibuya et al., 1992). Furthermore, Fasudil has not shown any severe side effects or changes in blood pressure and heart rate in patients with
angina or acute ischemic stroke (Shimokawa, 2002; Shibuya et al., 2005; Vicari et al., 2005). Nonetheless, it is important to note that ROCKII knockout mice show embryonic lethality whereas ROCKI knockout mice experience failure of eyelid closure and closure of the ventral wall (Thumkeo et al., 2003; Shimizu et al., 2005), highlighting a critical function for both ROCK isoforms in development. In addition, it was reported that intracerebroventricular administration of the ROCK inhibitor Y-27632 increased anxiety-related behavior in mice (Saitoh et al., 2006). Thus, while inhibition of ROCK offers a promising therapeutic target for the treatment of ALS, further examination is required to determine the safety of long-term use of nonselective inhibitors of ROCKI and ROCKII (such as Fasudil and Y-27632) in humans.

Alternatively, the development of a safe ROCKII inhibitor may offer a viable option for the clinical treatment of ALS. Although Fasudil targets both ROCKI and ROCKII, ROCKII is preferentially expressed in brain and skeletal muscle tissue while ROCKI shows more ubiquitous expression (Nakagawa et al., 1996). The expression pattern of ROCKII is particularly interesting given that current research suggests that motor neuron death in ALS appears to be consistent with a “dying back” mechanism in which the neuromuscular junction (NMJ) begins to disassemble while the motor neuron cell bodies in the spinal cord remain relatively intact (Dadon-Nachum et al., 2011). Given that our data demonstrate a marked redistribution of RhoB to neuronal processes of end-stage G93A mSOD1 mice, aberrant activation of ROCKII downstream of RhoB activation in both motor neuron processes and skeletal muscle may underlie the selective degeneration of NMJs during the progression of ALS. Indeed, Conti et al. (2014) recently demonstrated that ROCKII expression is increased in the skeletal muscle of sporadic
ALS patients. Thus, selective inhibition of ROCKII may prove to be a more direct and effective target for the treatment of ALS.

Interestingly, recent data suggest that unopposed Rho/ROCK signaling may be a common factor underlying additional neurodegenerative diseases. In fact, inhibition of ROCK has been demonstrated to be an effective treatment option in other models of neurodegenerative disease, including Parkinson’s disease (Tönges et al., 2012), Alzheimer’s Disease (Song et al., 2013), and cerebral ischemia (Gibson et al., 2014). Thus, ROCK inhibitors show promise for the treatment of ALS patients as well as those diagnosed with other neurodegenerative diseases.

6.6 Conclusion

In conclusion, this thesis has elucidated the involvement of Rho family GTPases in regulating neuronal survival. In particular, the work presented here has aided in deciphering the precise signaling pathways that are regulated by Rac GTPase in primary CGNs. Consistent with prior studies demonstrating an antagonistic relationship between Rac GTPase and Rho GTPase, we have also demonstrated that inhibition of Rac GTPase or activation of Rho GTPase induces neurite retraction and death of ESC-derived motor neurons. In agreement with a pro-survival function, Rac1 GTPase activation is decreased in spinal motor neurons of mice harboring the G93A mSOD1 mutation. Conversely, RhoB GTPase appears to redistribute to motor neuronal processes in ALS mice. Thus, while the pathology of ALS remains complex and multifaceted, the data presented in this thesis highlight the potential involvement of dysregulated Rho GTPases as a causative factor underlying the etiology of ALS. These data suggest that targeting Rho GTPase
activity, or their downstream effectors, may have beneficial therapeutic effects for ALS patients.
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APPENDIX A: ABBREVIATIONS

5K, 5K- apoptotic medium
6-OHDA, 6-hydroxydopamine
AD, actinomycin D
AD, Alzheimer’s disease
Ad; adenoviral
Ad-CA, adenoviral constitutively active
Ad-DN, adenoviral dominant negative
Ad-GFP, adenoviral green fluorescent protein
Ad-WT, adenoviral wild type
ALS, amyotrophic lateral sclerosis
ALS2, alsin
Anti, morpholino-antisense oligonucleotides
APAF-1, apoptotic protease-activating factor-1
Arp2/3, actin related protein 2/3
Bcl-2, B-cell lymphoma-2
Bcl-3, B-cell lymphoma-3
BDNF, brain-derived neurotrophic factor
BH3, Bcl-2 homology-3 domain
BKLF, basic krüppel-like factor
BKLF8, basic kruppel-like factor 8
BSA, bovine serum albumin
CBP, CREB binding protein
CCD, charge-coupled device
CD11b, cluster of differentiation 11b
CDK, cyclin-dependent kinase
CGN, cerebellar granule neuron
ChAT, choline acetyltransferase
ChIP, chromatin immunoprecipitation
CNTF, ciliary neurotrophic factor
Con, control
CREB, cAMP response-element binding protein
CRIB, Cdc42/Rac-interactive binding
Ct, cycle threshold
CtBP, C-terminal binding protein
CytC, cytochrome C
Cyto, cytosolic
DAPI, 4,6-diamidino-2-phenylindole
dCtBP, drosophila CtBP
DGCR8, DiGeorge critical region 8
DH, DbI homology
Dia, Diaphanous formin
DISC, death-inducing signaling complex
DMEM, Dulbecco’s modified Eagle’s medium
DN, dominant negative
DRG, dorsal root ganglion
EB, embryoid bodies
eGFP, enhanced GFP
EP, endoporter
ERK, extracellular signal-regulated kinase
ESC, embryonic stem cell
FADD, Fas-associated death domain
FBS, fetal bovine serum
GAP, GTPase activating protein
GDI, guanine dissociation inhibitor
GDNF, glial cell line-derived neurotrophic factor
GDP, guanine diphosphate
GEF, guanine nucleotide exchange factor
GITz-short G protein-coupled receptor kinase-interacting protein-z-short
GM-CSF, granulocyte-macrophage colony-stimulating factor
GR, glucocorticoid receptor
GTP, guanine triphosphate
HD, Huntington’s disease
HDAC, histone deacetylases
IB, immunoblot
Inh Pep, inhibitory peptide
Inv, inverse
IP, immunoprecipitate
JAK, Janus kinase
JAK3 Inh, JAK3 Inhibitor
JNK Inh, SP60012
JNK, c- Jun N-terminal kinase
JSI, JSI-124
KO, knockout
LPA, lysophosphatidic acid
LTox, lethal toxin
MAPK, mitogen activated protein kinase
MEFs, murine embryonic fibroblasts
MEK, mitogen activated protein kinase kinase
MEKK, mitogen activated protein kinase kinase kinase
mESCs, mouse embryonic stem cells
miRNA, micro RNA
MLC, myosin II regulatory chain
MLCK, myosin light chain kinase
MLCPPase, myosin light chain phosphatase
MPP\textsuperscript{+}, methyl-4-phenylpyridinium
mSOD1, mutant superoxide dismutase 1
MTOB, 4-methylthio-2-oxobutyric
NADH, nicotinamide adenine dinucleotide
VACHT, vesicular acetylcholine transporter
WASP, Wiskott-Aldrich-syndrome protein
WCL, whole cell lysate
WT, wild type
Chapter 2 entitled “Signal transducer and activator of transcription-5 mediates neuronal apoptosis induced by inhibition of Rac GTPase activity” was published in the Journal of Biological Chemistry. 20:16835-48, 2012.

Chapter 3 entitled “Neuronal apoptosis induced by selective inhibition of Rac GTPase versus global suppression of Rho family GTPases is mediated by alterations in distinct mitogen-activated protein kinase signaling cascades” is submitted to the Journal of Biological Chemistry.

Chapter 4 entitled “C-terminal binding proteins are essential pro-survival factors that undergo caspase-dependent downregulation during neuronal apoptosis” was published in Molecular and Cellular Neuroscience. 56:322-32, 2013.

Chapter 5 entitled “Dysregulation of Rho or Rac elicits death of motor neurons and activation of these GTPases is altered in G93A mutant SOD1 mice” is a manuscript in preparation for submission to the Journal of Neuroscience.