RHOA AND RAC1 PRENYLATION: EFFECTS ON SERINE/THREONINE SIGNALING FOR ACTIN POLYMERIZATION

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DEDICATION

To my mom and dad, for introducing medical science to me, my sisters and niece who have been the support system throughout my career, and Jean for guiding me into the graduate school and all my teachers for having faith in me.

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ABSTRACT

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RHOA AND RAC1 PRENYLATION: EFFECTS ON SERINE/THREONINE SIGNALING FOR ACTIN POLYMERIZATION

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RhoA and Rac1 are small guanosine triphosphates (GTPases) that regulate cytoskeletal rearrangements, cell polarity, axon guidance, and signaling pathways. Alteration of the subcellular localization or activation of Rho GTPases is implicated in several neurological conditions. Rho GTPases are activated by binding to guanosine triphosphate (GTP) and is thought to require translocation to the plasma membrane by the addition of an isoprenoid moiety (geranylgeranyl) to the protein. However, previous experiments indicate that RhoA or Rac1 can be activated in the cytosol without translocation to the plasma membrane. Based on this, and evidence the Rho GTPases are centrally located in signaling pathways regulating actin dynamics, it was hypothesized that overexpressing non-geranylgeranylatable RhoA or Rac1 would decrease the actin filament content in neuronal growth cones by altering the location of actin regulating molecules. In particular, it was hypothesized that non-geranylgeranylatable RhoA or Rac1 would decrease the activity of actin filament promoters (i.e., association of WAVE with the ARP2/3 complex and activation of JNK and ERK), and inactivation of the actin-depolymerizing protein cofilin. Overexpressing non-geranylgeranylatable (EmGFP-RhoA^{C190A}), but not wild-type RhoA (EmGFP-RhoA), increased activation of ERK in the

cytosol and increased association of WAVE with the ARP2/3 complex at the membrane, compared to cells overexpressing only the empty vector (EmGFP). However, overexpressing neither RhoA construct affected actin dynamics. Overexpressing wild-type Rac1 (EmGFP-Rac1), but not non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}), increased the actin filaments content in growth cones compared to neurons expressing only EmGFP, concomitant with an increase in JNK activation. Overexpression of EmGFP-RacC^{189A} decreased JNK activation and increased WAVE/ARP2/3 complexing, compared to cells expressing wild-type Rac1 or EmGFP alone. Studies with signaling molecule inhibitors indicated significant cross-talk between signaling pathways, which is altered by overexpressing wild-type or non-geranylgeranylatable forms of RhoA or Rac1. The results suggest that altering the subcellular localization of RhoA or Rac1 changes the activation of signaling molecules that regulate actin dynamics in neuronal growth cones. Elucidating the signaling cascades of the active GTPases may identify the distinct functions of these GTPases in the cytosol and can be used as novel targets to facilitate axon regeneration in neurodegenerative and neurological conditions.

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CHAPTER I

INTRODUCTION

Understanding the molecular mechanisms regulating axon guidance and the molecules implicated in the process of neuronal development is very important for defining the mechanisms involved in the onset of neurological disorders. The Rho family of small guanosine triphosphatases (GTPases), their regulators, and their involvement in the downstream signaling pathways of axon guidance are very important in regulating neuronal development and process regeneration. Clinical and preclinical research indicates that abnormal patterns in various intracellular signaling molecules occurs during the onset of several neurodegenerative diseases, such as Alzheimer's diseases (AD) or Parkinson diseases (PD), as well as neuropsychiatric and developmental diseases, such as schizophrenia and autism spectrum disorder [1-4]. Understanding the pathways involved in actin polymerization leading to neurite extensions may ultimately novel therapeutic targets for treating neurological disorders.

Rho GTPases are major regulators of signaling molecules involved in actin dynamics at the tips of extending neuronal processes (growth cones) [5, 6]. Rho GTPases are the molecular switches that cycle between active guanosine triphosphate (GTP)- or inactive guanosine diphosphate (GDP)-bound forms [7-9]. Rho GTPase activation is accomplished by guanine nucleotide exchange factors (GEFs), [10-12]. GEFs catalyze the release of GDP allowing GTP to bind, whereas GTPase activating proteins (GAPs) initiate

a return to the GDP-bound state. In addition, Rho GTPases can be sequestered in the cytosol in an inactive form through the action of guanosine dissociation inhibitors (GDIs) [1, 13, 14]. When Rho GTPases are GTP-bound, they are active and perform a plethora of functions from cell migration and differentiation, cell polarity, vesicular trafficking, cell adhesion, proliferation, and regulation of the actin cytoskeleton [7, 9, 10, 15]. Rho GTPases also interact with and activate downstream effector proteins to initiate different signaling cascades and networks when bound to GTP [16-18].

Among the 20 members of the Rho GTPases family, the Ras homolog gene family member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) are the most studies Rho GTPases [7, 9, 19]. RhoA and Rac1 are crucial for axon guidance, dendrite formation, and spine morphogenesis in the growth cones of extending neurons. RhoA and Rac1 perform these functions by regulating the actin cytoskeleton in growth cones actin-based substructure lamellipodia and filopodia; whereas, in non-neuronal cells, activation of RhoA, Rac1 and Cdc42 results in the formation of stress fibers [15, 20]. A growth cone is a large actin-supported extension of a developing or regenerating neurite that consists of a peripheral (P) domain and a central (C) domain. The P domain contains long finger-like projections called filopodia and sheet-like structures called lamellipodia (see Fig. 1). Filopodia actin dynamics depend on several environmental factors for guidance cues, while lamellipodial dynamics are associated with directional growth cone movement. Actin filaments are branched in the lamellipodia whereas actin longitudinally aligns and bundles in the filopodia (see Fig. 1) [18, 21].

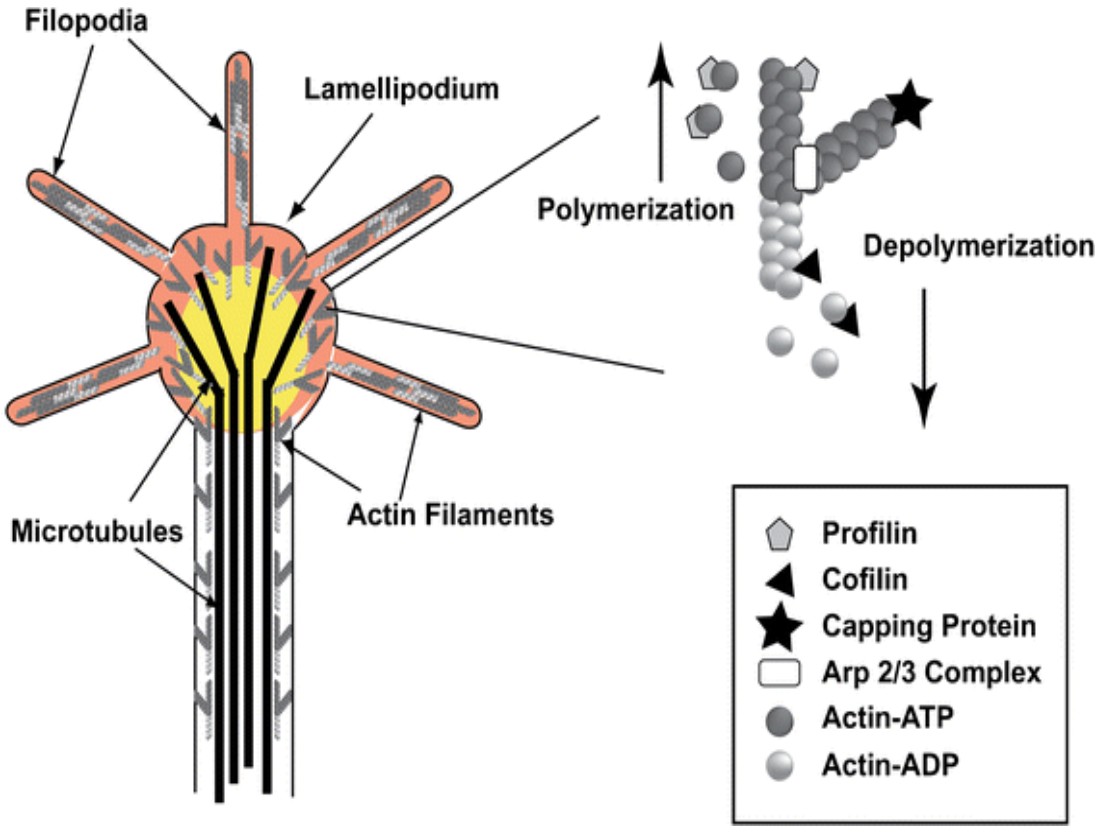

Fig 1.1. Growth cone cytoskeletal structure. Growth cones are composed of an actin-based peripheral (P) domain (*in orange*) and a microtubule-rich central (C) domain (*in yellow*). The P domain consists of finger-like filopodia with linearly arranged actin filaments and sheet-like lamellipodia that have branched actin filaments. Actin dynamics including polymerization, depolymerization, severing, and capping are controlled by specific actin-binding proteins including profilin, cofilin, capping protein and the Arp2/3 complex. (adapted from [21])

The Importance of Prenylation

Rho GTPases need to be GTP bound to be active and perform their functions by activating various downstream effectors [6, 7, 12]. RhoA and Rac1 need to be translocated to the membrane by post-translational modification (PTMs) such as prenylation [22-25], which is an irreversible lipid modification where the isoprenoid intermediates farnesyl (15-carbon) or geranylgeranyl (20-carbon) pyrophosphates are added to the C-terminus of

proteins [26]. These intermediates are attached to the protein by thioether linkages at a C-terminal cysteine residue [27]. The sequence CAAX (C—cysteine, A—aliphatic amino acid, X—any amino acid) at the C-terminus determines whether a protein is farnesylated or geranylgeranylated [22, 26]. All Rho GTPases are geranylgeranylated and not farnesylated. Prenylation is followed by truncation where a peptidase cleaves off the last three -AAX amino acids and methylation where the free carboxyl group is methylated and the lipid moiety makes the C-terminus hydrophobic that allows them to be targeted to the membrane [28]. This membrane translocation allows the proteins to interact with regulatory and effector proteins [18, 22]. The activation of downstream effectors is important to attain Rho GTPases function [6, 17, 29]. After membrane localization, RhoA facilitates the formation of stress fibers in the growth cone. On the other hand, Rac1 facilitates neurite outgrowth and extension by the formation of filopodia recruiting different effectors in different compartments of the cell [6, 16, 30]. Inhibition of prenylation leads to cessation of the translocation of Rho GTPases and in turn activation of these effectors [31]. The most accepted theory of Rho GTPases is that Rho GTPases need to GTP-bound via membrane-localized GEFs and only then can Rho GTPases activates their signaling cascades [11, 14, 32]. However, previous work from our lab has reported an active pool of Rho GTPases in the cytosol [16]. The pathways via which these Rho GTPases are active in other compartments rather than just in the membrane are not yet known and needs to be investigated.

Signaling Pathways regulated by the Rho GTPases RhoA and Rac1

RhoA activates Rho-associated kinase (ROCK) that, in turn, activates LIM-kinase **(**LIMK). LIMK is a kinase that phosphorylates and inactivates cofilin, which severs and depolymerizes actin when active [29, 33, 34]. RhoA also regulates the actin cytoskeleton via mitogen-activated protein kinase (MAPK) pathways by indirectly promoting phosphorylation and activation of extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK). Phosphorylation of ERK and JNK allows the polymerization of actin at the growing end of the neurites [35, 36]. RhoA regulates ERK via ROCK; whereas, it affects JNK activity via linking to the MEKK 1/MEKK 4-dependent cascade and then, activating JNK [35-39]. RhoA also works via a second isoform of ROCK, ROCK-2, leading to activation of actin remodeling via the actin-binding protein moesin that works via Wiskott-Alsdrich syndrome protein (WASP)-family verprolin homologous protein (WAVE1) [40].

Similar to RhoA, Rac1 also triggers the actin dynamics by activating downstream effectors [6, 29]. Rac1 promotes actin polymerization via the actin-related protein (ARP) pathway [41, 42]. The Arp2/3 complex nucleates branched actin polymers; however, being a poor actin nucleator, it needs to be activated by nucleation promoting factors (NPFs) like WAVE, which is activated through membrane recruitment by Rac1 [42-45]. Rac1 also activates PAK, which activates LIMK that phosphorylates and inactivates cofilin [40, 46, 47]. Rac1 acts upstream of ERK to activate it and allows actin reorganization that is eotaxin-induced [48]. JNK is also indirectly activated by Rac1 via a MAPK that leads to the polymerization of the actin [49-51]. Any change in the signaling pathways mediated by

either RhoA or Rac1, which are thought to require the GTPase to be prenylated and localized to the plasma membrane where it can interact with membrane-associated GEFs, could lead to abnormal actin de/polymerization. Such dysregulation has been one of the factors implicated in the onset of many neurodegenerative, neurodevelopmental, neuropsychiatric, and neurotraumatic disorders, collectively known as neurological disorders [4, 52-55].

Aberrant Prenylation of RhoA and Rac1 is Implicated in Neurological Disorders

Alternations in signaling cascades initiated by RhoA and Rac1 are implicated in a variety of neurological conditions [4, 52, 56]. Rho GTPases regulate actin dynamics through initiating signaling cascades that involve serine/threonine kinases [6, 29]. Different forms of dementia, including AD-related dementia, have displayed decreased levels of geranylgeranylated Rho GTPases [57]. The loss of dendritic spines observed in the brains of AD patients [57, 58] suggests changes in RhoA signaling may be occurring. RhoA activates downstream serine/threonine kinases that mediate effects on the actin cytoskeleton [6, 29, 59]. The ROCK pathway has been associated with several neurodegenerative diseases and is strongly linked to AD as therapies like statins and nonsteroidal anti-inflammatory drugs (NSAIDs) work through the RhoA/ROCK signaling pathway [57]. Also, RhoA can colocalize with hyperphosphorylated tau [60], suggesting that altered subcellular targeting of RhoA is related to neurodegeneration. Rac1 does not associate with hyperphosphorylated tau [61]. Thus, RhoA may be a possible therapeutic target to reduce neurodegeneration in AD. The RhoA/ROCK pathway inactivates the actin depolymerizing factor (ADF) cofilin-mediated actin depolymerization in AD by

phosphorylating LIMK [62, 63]. Activation of ERK is increased in cells expressing a mutated huntingtin (Htt) gene, possibly contributing to the pathology of HD [64]. ADF/cofilin rods are present in the cytosol of cells in neurological conditions indicating that subcellular localization of these GTPases contribute to actin rearrangement resulting in rods [65]. These studies indicate that RhoA-mediated inactivation of cofilin and its activation of MAP kinases allows actin polymerization and may prevent neurodegeneration.

Rac1 is upregulated in the brains of AD patients, compared to the age-matched control brains, but has decreased activity and signaling to PAK [57, 66]. Decreases in Rac1 activity lead to tauopathy, which is pathological aggregation of tau protein in neurofibrillary or gliofibrillary tangles in the human brain and is associated with memory deficits in ischemia mice. Furthermore, aberrant Rac1 signaling is implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) [57, 67]. When bound to GTP, Rac1 can interact with and directly activate PAK, which activates LIMK that phosphorylates and inactivates cofilin [68]. Rac1 also interacts with plenty of SH3s (POSH), a scaffold protein that binds to and regulates JNK following cerebral ischemia and the MLK3-JNK kinase signaling pathway can promote apoptosis following cerebral ischemia [57]. Further, Rac1 activation can lead to increased ERKl/2 activity, a process that counteracts neuronal degenerative processes in the retina [58]. Together, these studies indicate that Rac1 mediated inactivation of the actin severing protein cofilin, activation of the MAP kinases via ERK 1/2 and JNK, and its activation of the actin nucleator WAVE that allows actin polymerization.

Hypothesis

Emerging evidence indicates that alterations in the level and function of some small GTPases may contribute to the pathogenesis of all these neurological diseases [65, 69, 70]. Our work has focused on teasing apart the pathways involved in actin polymerization and uncovering the role of small GTPase prenylation, particularly for RhoA and Rac1. Our overarching hypothesis is overexpressing non-geranylgeranylatable RhoA or Rac1 decreases the actin content in different subcellular locations by affecting the MAPK and WAVE/ARP2/3 pathways. This refutes the concept that Rho GTPases need to be membrane-bound to be functional. Traditionally, the Rho GTPase function has been studied by expressing mutant forms that mimic GTP-bound GTPases (constitutively active) or prevent interaction with effectors, thus mimicking the GDP-bound state (dominant negative). The idea that altering the subcellular localization of Rho GTPases affects their signaling and promotes degeneration is emerging as a potential site of therapeutic intervention. While some groups are currently exploring the correlation of Rho GTPase geranylgeranylation with different disease states [71], to our knowledge no one has investigated how translocating RhoA or Rac1 to the cytosol affects signaling from these GTPases. In this dissertation, the hypothesis was tested across the following three Specific Aims:

Specific Aim 1: To determine if expressing non-geranylgeranylatable RhoA or Rac1 decreases the actin filament content at the tips of extending axons (growth cones, lamellipodia and filopodia).

Specific Aim 2: To determine if expressing non-geranylgeranylatable RhoA or Rac1 activates actin-depolymerizing (cofilin), or inactivates actin polymerizing (ERK1/2, JNK) serine/threonine kinases.

Specific Aim 3: To determine how changes in serine/threonine kinases lead to decreased actin nucleation.

Overall, the research is attempting to uncover a new area of exploration that may reveal novel molecular targets to better treat neurodegenerative conditions. Ultimately, our findings aim to inform on the possible role of aberrant RhoA and/or Rac1 signaling in the pathogenesis of different neurological conditions and hence address for the therapeutic approach to mitigate these neurodegeneration.

CHAPTER II

DECREASING GERANYLGERANYLATION OF RHOA ACTIVATES WAVE/ARP2/3 AND MAPK PATHWAYS.

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Abstract

Abnormal cytoskeletal organization of actin is one of the common features of many neurodegenerative diseases like Alzheimer's disease (AD), Parkinson diseases (PD) and Huntington's disease (HD). Actin polymerization and depolymerization are controlled in large part by Rho guanosine triphosphatases (GTPases). Like all Rho guanine triphosphatases (GTPases), RhoA is targeted to the membrane by the addition of a geranylgeranyl moiety, an action thought to result in Rac1 guanosine triphosphate (GTP) binding. However, the role that RhoA localization plays in its activation (GTP loading) and subsequent activation of effectors is not thoroughly investigated. Here, we report that expressing non-geranylgeranylatable RhoA in different compartments of the cells increases active extracellular signal-regulated kinases (ERK) in the cytosol and growth cones. It also increased the WASP-family verprolin homologous protein (WAVE) and actin related protein (ARP) association in the membrane and growth cones. Elucidating the role of aberrantly-localized active Rho GTPases and their downstream effectors on actin

may identify novel targets to facilitate axon regeneration in traumatic or degenerative neurological conditions.

2.1. Introduction

Neurodegenerative diseases, neurodevelopmental, or neuropsychiatric disorders possess aberrant signaling pathways [4, 53, 59, 62, 72, 73]. These signaling pathways are remotely regulated by RhoA, one of the most studied small guanosine triphosphatases (GTPases) [74, 75]. RhoA plays an important role in cell polarity, microtubule dynamics, membrane transport pathways, transcription factor activity and regulation of actin cytoskeleton [7].

RhoA, being a member of small GTPases family, acts like a molecular switch that is active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphates (GDP) [7, 29, 76, 77]. This differential distribution of Rho GTPases regulates their functions by localizing them to different compartments [78]. The intracellular localization of Rho GTPases is regulated by post-translational modifications (PTMs), such as isoprenylation which provides a membrane attachment [18]. Prenylation is an irreversible lipid modification where the isoprenoid intermediates farnesyl (15-carbon) or geranylgeranyl (20-carbon) pyrophosphates are added to the C-terminus of proteins [18, 26, 30, 79]. The sequence CAAX (C—cysteine, A—aliphatic amino acid, X—any amino acid) at the C-terminus determines whether a protein is farnesylated or geranylgeranylated. Prenylation is followed by truncation and methylation [28] and the lipid moiety makes and helps the protein to get attached to the membranes. Membrane

translocation allows the protein to interact with regulatory and effector proteins and initiate various signaling cascades [13, 30].

RhoA stimulates the actin cytoskeleton by many pathways [33, 51, 80, 81]. RhoA alters the actin arrangement via Isl-1 and Mec-3 kinase (LIMK), which in turn regulate cofilin[33]. Cofilin is an actin nucleator that contributes to the actin depolymerization when phosphorylated [33]. The LIMK/cofilin pathway is important as cytoplasmic rod-shaped bundles of filaments i.e., actin-depolymerizing factor (ADF)/cofilin-actin are increased in AD brain [62]; whereas, active cofilin was able to restore movement of actin introduced by alpha-synuclein, a cytosolic protein involved in PD [82].

Another important pathway regulated by RhoA that aids in actin polymerization is the mitogen-activated protein kinases (MAPK) pathway [83]. The migration of human mobile cells, Th17 cells was achieved by activating the RhoA, and its effector Rho-associated kinase (ROCK) and the MAPK/ERK pathways [84]. A player of MAPK that is implicated in many neurological disorder is C-Jun N terminal kinase (JNK). The proper balance between cytoprotective JNK and cytotoxic p38 signaling maintains the phenotypic outcome to TAR DNA-binding protein (TDP-43), a protein implicated in ALS [85] and the inhibitors that disrupt the association of JNKs with the mitochondria may be useful neuroprotective agents for the treatment of PD [86].

Another MAPK member, extracellular signal-regulated kinases (ERK) is involved in proper neurite growth promoted by the Rac1/MAPK/ERK pathway that leads to actin polymerization by phosphorylating ERK [58]. The aberrant signaling in ERK pathway

leads to neuronal death by forming actin rods that get sequestered in different fractions of the cell, which is a major phenomenon in neurodegenerative diseases like PD [87].

Another important structural component of neuronal remodeling is spine density. The formation of spines in neurons is dependent on actin remodeling by the WASP-family verprolin homologous (WAVE1) protein, which controls actin polymerization through the actin-related protein (Arp)-2/3 complex [40]. WAVE has also been found to be activated by 17β-estradiol (E2) recruiting a $G\alpha_{13}$-dependent pathway that involves RhoA and leads to actin remodeling via the actin-binding protein, moesin [40]. These data provide evidence that WAVE might be one of the players in the RhoA pathway leading to the actin polymerization.

Besides playing a role in actin polymerization and spine density, RhoA also plays a pivotal role in the formation of stress fibers (actin-myosin filaments) in motile cells and focal adhesions at the ends of the extending neurons [6]. RhoA also activates and plays a pivotal role in signal transduction pathways that link cell surface receptors to a variety of intracellular responses including the assembly and disassembly of the actin cytoskeleton [9]. How the pathways that are downstream of the prenylatable form of RhoA in differential locations allow actin polymerization still remain unclear. So with this work, we focus on the pathways that allow actin polymerization in differential locations of the cell.

2.2.Materials and Methods

2.2.1. Constructions of the expression vectors

We have made mammalian expression vectors containing wild-type RhoA that is N-terminally tagged with emerald green fluorescence protein, referred to as EmGFP-RhoA. This construct can be prenylated and was achieved by inserting the open reading frame of RhoA (Open Biosystems, Houston, TX) into a mammalian expression vector that is fused with EmGFP at the N-terminus of RhoA (Invitrogen, Carlsbad, CA). RhoA mutant construct was achieved using site-directed mutagenesis (Kit from Invitrogen, Carlsbad, CA) to change the cysteine at the CaaX box (C = cysteine, a = any aliphatic amino acid, X = terminal amino acid) to an alanine, making this construct non-prenylatable (referred as EmGFP-RhoA^{C190A}).

2.2.2. Cell Culture

Rat neuroblastoma B35 cells (CRL: 2754, ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM; 12634010, ThermoFisher) containing 10% fetal bovine serum (FBS; 12103C Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were passaged when 85-90% confluent using 0.25% trypsin (9002-07-7, Sigma-Aldrich). Cells were seeded in 6 well- plates at 20,000 cells/cm^2 for western blotting, and on 12 mm glass coverslips in 24-well tissue culture plates at 5,000 cells/cm^2 for immunocytochemistry (ICC).

Primary cortical neurons were extracted from postnatal day one rat pups. Pups were anesthetized on ice and decapitated. An incision was made on the dorsal side of the head to have a clear view of the skull. The skull was removed using scissors and forceps and the

brain was removed with the help of a sterile spatula. Brains were then sliced coronally into 1-3 mm sections using sterile scalpels. The remaining meninges and blood vessels were removed carefully using forceps. The cortices were dissected from the rest of the brain tissues. The tissues obtained were then dissociated using 2 mg/mL papain (9001-73-4, Sigma). The obtained cells were plated on poly-D-Lysine (PDL) coated glass coverslips in at the density of 3000 cells/cm^2 in 24 well-plates in neurobasal media (21103049, Gibco) supplemented with B27 (10%; A3582801, Gibco) for seven days before transfection. After 48 hours, cells were fixed using 4% paraformaldehyde for 20 minutes and subjected to ICC as explained below.

All the work in animals was approved by the TWU Institutional animal care and use committee (IACUC).

2.2.3. Transfection

Cells were transfected with EmGFP-RhoA, EmGFP-$RhoA^{C190A}$ or EmGFP using Lipofectamine 2000 or 3000 (L3000015, ThermoFisher), according to the manufacturer's instructions. 3 µg and 0.8 µg of plasmid were used for transfection for western blotting and immunocytochemistry respectively. A control with untransfected cells was maintained in serum-free medium (SFM). After 48 hrs, cells were either fixed in 4.0% paraformaldehyde for immunolabeling, for immunocytochemistry, or were lysed for western blotting as described below. Transfection efficiency was assessed by the cells expressing EmGFP only.

2.2.4. Inhibitor studies

Untransfected and transfected cells were treated with 0.125 µM of CK-869, i.e., actin-related protein (ARP) inhibitor (4984, EMD Millipore), 40 µM of c-Jun N-terminal kinase (JNK) inhibitor-II (420119, EMD Millipore) or 25 µM of extracellular signal-regulated kinase (ERK) inhibitor (328006, EMD Millipore) for 1 hr and with 20 µM of LIMKi-3 (435930, Sigma-Aldrich) for 4 hrs and observed regularly for viability. Afterward, lysates were collected, and the samples were run on SDS-PAGE gel as described above.

2.2.5. Subcellular Fractionation

Untransfected and transfected cells were fractionated using the S-PEK Proteolysis kit (Pierce, 539790) into cytosolic (fraction 1), membrane (fraction 2), nuclear (fraction 3) and cytoskeletal (fraction 4) fractions using the manufacturer's instructions. For confirmation of membrane and cytosolic fractionation, western blots were performed as described below and probed with antibodies against a membrane protein, anti-$GABA_A\beta2$ (ab72445, Abcam), and for a cytosolic protein anti-calnexin with 1:1000 (ab108400, Abcam) and the nuclear-associated protein histone 1.1 with 1:1000; (ab17584, Abcam) respectively.

2.2.6. Co-immunoprecipitation

Lysis buffer containing cell lysates with 1:1000 mouse anti- WASP-family verprolin homologous protein 1(WAVE) (ab211427, Abcam) and purified mouse serum as the control corresponding to the primary antibody were rocked overnight at 4ºC. Goat anti-rabbit IgG agarose magnetic beads, magnabind was washed 3 X 10 min with lysis buffer

containing protease inhibitor cocktail (PIC) (21356, ThermoScientific). Twenty µL of washed magnabind were added to the overnight rocked samples and control serum and left on the rocker for 3 hours at room temperature. The beads were precipitated three times with washing in lysis buffer using a magnet that applies high magnetic field and allows the separation of beads from lysate. The sample buffer was added to the collected beads' samples and subjected to SDS-PAGE as described below.

2.2.7. Western blotting

After transfection, cells were lysed in lysis buffer containing 25 mM tris-HCl (pH = 7.4; 15506017, ThermoFisher), 150 mM NaCl (S7653-250G, Sigma) 1.5 mM EDTA (AM9260G, ThermoFisher) and 1.0% IGEPAL CA-630 (85124, ThermoFisher). 10–20 µg of each protein sample were subject to electrophoretic molecular weight separation on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) gels at 120V for 90 minutes and electrotransferred to nitrocellulose (0.2 µm, BioRad, Temecula, CA) at 400mA for 60 minutes. Nonspecific binding was blocked by incubation with 5% nonfat dry milk in phosphate-buffered saline for 2 hrs. Blots were probed with 1:1000 in TBS only with mouse anti-cofilin (ab42824, Abcam), rabbit anti cofilin phospho S3 (ab12866, Abcam), rabbit anti-JNK1 (ab124956, Abcam), rabbit anti-JNK phosphoT183 (ab 47337, Abcam), rabbit anti-ERK (ab180163, Abcam), or rabbit anti-ERK phosphoY204 (ab 194770, Abcam) primary antibodies for 4 hrs at room temperature on rocker. Blots were washed 3 X 10 minutes with 1X Tris-buffered saline with 0.001% Tween (P1379-25ML,Sigma; TBST) and incubated in blocking buffer containing 1:10,000 goat anti-mouse IgG DyLight 650 (ab97018, Abcam) or goat anti-rabbit IgG with 1:10,000

(ab216773, Abcam) for 1 hr in the dark at room temperature on rocker. Finally, the blots were washed 3 X 10 minutes and immunoreactive bands were visualized by near-infrared detection using Li-COR odyssey system.

2.2.8. Immunocytochemistry

Transfected and untransfected cells fixed in 4.0% paraformaldehyde were incubated for 10 minutes with PBS containing 1.5 % pre-immune secondary serum, 0.1% bovine serum albumin (A215350G, Sigma, St. Louis, MO and 0.1 % Triton-X 100 (94343, BioChemika). Cells were then incubated with primary antibodies for 2 hrs at 37 ºC. Cells were washed 3 X 5 minutes on the rocker, and then incubated with 1:10000 appropriate secondary antibodies conjugated with respective Alexa fluor 488, 555 or 647 (Jackson immunolabs) for 1 hr at 37 ºC. Cells were then washed 3 X 5 minutes with PBS and coverslips were mounted on slides 3 µL mounting media containing DAPI (A-1000, Vectashield). Images were captured through 40X or 60X objectives using a Nikon A1 confocal microscope equipped with NIS element software.

2.2.9. Phalloidin Staining

Coverslips containing cells fixed in 4.0 % paraformaldehyde were incubated in 0.5% Triton X-100 in PBS (94343, BioChemika) for 10 minutes to permeabilize. Cells were then washed 1 X 5 minutes with PBS at room temperature. After wash, 200 µl of 100 nM of phalloidin (Cat. # PHDH1-A) was added. The cells were incubated at room temperature in the dark for 30 min. Cells were then washed 3 X 5 minutes each and coverslips were mounted on slides 3 µL mounting media containing DAPI (A-1000, Vectashield). The area of interest, growth cones were identified for imaging. Images were

captured through 40X or 60X objectives using a Nikon A1 confocal microscope equipped with NIS element software.

2.2.10. Statistics

Data for all the experiments approximated a normal distribution and had similar variances across the experimental conditions, and hence, were analyzed for differences across group's analyses of variance (ANOVA) and appropriate posthoc test (Tukey). All the statistical analyses were run in GraphPad Prism software, version 8.1. For all the analyses, *p*-value was as indicated in individual figures.

2.3.Results

2.3.1. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not have any effect on actin-depolymerizing (cofilin) pathway.

Axonal retraction involves RhoA signaling [33, 54], activation of its downstream effector cofilin (a direct binding protein of actin filaments), and regulation of actin de/polymerization by RhoA-LIMK-cofilin signaling [33]. Hence, to determine the role of RhoA in the actin depolymerization by cofilin, we assessed the cofilin and phosphocofilin (pcofilin) levels in whole-cell lysates of transfected neuroblastoma cells by western blotting. The ratio of p-cofilin and cofilin was determined to estimate the level of active cofilin. There was no significant difference in cofilin activity from WCLs of cells transfected with EmGFP-RhoA or EmGFP-RhoA^{C190A} (see Figures 2.3.1. A and B). Cofilin-1 is a major regulator of actin dynamics in dendritic spines, and is subject to phospho-regulation by different pathways, including the Rho-associated protein kinase (ROCK) pathway [88]. Cytoplasmic cofilin-actin bundles (rods), present in axons and

dendrites of stressed neurons, leads to disrupted synapses and impaired cognitive functions in dementia [65, 70, 89]. Hence, we then investigated the total cofilin in cytosol and membrane/organelle fractions of the transfected neuroblastoma cells. There was no significant change in the levels of the activated cofilin in the cytosol (see Figures 2.3.1. C and D) or membrane fractions between (see Figures 2.3.1. E and F) EmGFP-RhoA or EmGFP-RhoA^{C190A} expressing cells compared to cells expressing only EmGFP.

2.3.2. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not affect the actin-depolymerizing (cofilin) pathway in the growth cones.

In neuronal development, cofilin plays a critical role in dynamic rearrangement of actin that regulates the promotion of the axonal growth cone extension and spatiotemporal translation of local mRNAs in response to guidance cues [90]. Thus, to determine the effect of overexpression of non-geranylgeranylatable RhoA, we performed ICC. The transfected neuroblastoma cells were immunostained for cofilin (AF555, red) and p-cofilin (AF647, cyan). Growth cones were defined and there was no significant difference in the ratio of pcofilin/cofilin (intensities) in EmGFP-RhoA and EmGFP-RhoA^{C190A} (see Figures 2.3.2. A and B).

2.3.3. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not affect active JNK.

JNK is activated downstream of RhoA and activates signaling pathways in addition to those directly controlled by activated RhoA. [13-16]. Hence, to determine the role of RhoA involved in the actin de/polymerization by MAPK members, JNK, and p-JNK level in whole-cell lysates of transfected neuroblastoma cells by western blotting. The ratio of p-JNK and JNK was determined to estimate the level of active JNK. There was no

significant difference in the levels of activated JNK between WCLs of neuroblastoma cells transfected with any of the constructs (see Figures A and B). RhoA has been found to localize in cytosol, membrane, and nuclear fractions [13], and the activation of RhoA, localized in a different compartment is very important in transducing the signals for actin cytoskeletal dynamics and JNK translocation [14]. MAPKs are known to move from the cytosol to the nucleus upon activation [15, 16]. MAPKs can, in turn, be activated by Rho GTPases [17, 18]. To determine the role of prenylated RhoA in regulating the MAPK JNK, we examined the JNK and p-JNK in cytosol and membrane/organelle fractions of the transfected neuroblastoma cells. There was no significant change in the expression of the JNK in cytosolic fractions (see Figures 2.3.3. A and B) or membrane/organelle fractions (see Figures 2.3.3. A and B) of EmGFP-RhoA and EmGFP-RhoA^{C190A}, compared to cells expressing EmGFP alone.

2.3.4. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not change the JNK pathway in the growth cones.

RhoA GTPases seem to have anti-apoptotic properties promoting neuronal survival acting through JNK [5], so we next checked the effect of overexpressing non-geranylgeranylatable RhoA on active JNK in transfected neuroblastoma cells. The transfected cells were immunostained for JNK (AF555, red) and p-JNK (AF647, cyan). There was no significant difference in the co-localization of the JNK and p-JNK in EmGFP-RhoA and EmGFP-RhoA^{C190A}, compared to cells expressing EmGFP alone (see Figures 2.3.4. A and B) in the defined region of growth cones.

2.3.5. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} increased the active ERK in the cytosol.

ERK is downstream of RhoA and plays an important role in activating signaling pathways in addition to those directly controlled by activated RhoA [13-16]. To determine the role of RhoA in the actin de/polymerization by MAPK members, we assessed the ERK and phosphor-ERK (p-ERK) in transfected neuroblastoma cells by western blotting. The ratio of ERK and p-ERK was determined to estimate the level of active ERK. There was no significant difference in the levels of activated ERK in WCLs of cells transfected with any of the constructs (see Figures 2.3.5.A and B). To determine whether the proteins were sequestered in other cell compartments, we examined membrane/organelle and cytosol fractions separately. There was significant increase in ERK activation in cells expressing EmGFP-RhoA^{C190A}, but not in cells expressing wild-type RhoA in the cytosolic fraction, compared to cells transfected with EmGFP alone (see Figures 2.3.5.C and D). There was no significant difference in the levels of ERK activation in membrane/organelle fraction of cells overexpressing any of the constructs (see Figures 2.3.5. E and F)**.**

2.3.6. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} increased active ERK in the growth cones.

Axonal regeneration has been addressed to be influenced by the ERK pathway that was initiated by apolipoprotein E (ApoE3) [37]. Also, ERK, along with many other effectors, is associated with the formation of long-lasting neuronal plasticity [91]. To further analyze the effect of prenylation on ERK, we performed ICC. The transfected neuroblastoma cells were immunostained for ERK (AF555, red) and p-ERK (AF647, cyan). Images were taken at 60X using the Nikon A1 microscope and growth cones were

identified as regions of interest for the analysis. There was no significant difference in the ratio of p-ERK/ERK (average fluorescent intensities) for cells overexpressing EmGFP-RhoA or EmGFP-RhoA^{C190A} compared to cells expressing EmGFP alone (see Figures 2.3.6. A and B).

2.3.7. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} increased WAVE and ARP2/3 association in the cytosol.

The WASP and WAVE family proteins are fundamental organizers of the actin cytoskeleton [92] that enable them to associate with the Arp2/3 complex, leading to nucleation and formation of a branched filament meshwork [93]. RhoA activity is increased in WAVE knockdown cells suggesting that there might be some cross-talk in-between WAVE and RhoA [94]. To determine whether prenylated RhoA contributes to the association of ARP2/3 via WAVE, we performed co-IP on transfected neuroblastoma cells. Anti-WAVE antibodies were used to pull down WAVE and associated proteins and resulting precipitates were assessed for association of ARP3 with WAVE in WCLs. There was no change in the amount of ARP2/3 that associated with WAVE in cells overexpressing EmGFP-RhoA or EmGFP-RhoA^{C190A} compared to cells expressing only EmGFP (see Figures 2.3.7. A and B) when analyzed by western blotting. Also, subcellular localization and the potential role of WAVE has been identified in the regulation of cell migration [95]. We then examined the co-localization of the WAVE and ARP2/3 in different fractions of the transfected B35 cells, and there was a significant difference in the cytosolic fractions (see Figures 2.3.7. C and D) between EmGFP-RhoA and EmGFP-RhoA^{C190A} and no difference in the membrane fractions (see Figures 2.3.7. E and F).

2.3.8. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} increased the WAVE and ARP2/3 association in the growth cones.

Western blot analyses showed there was an increased association of WAVE and ARP3 by overexpressing EmGFP-RhoA^{C190A} compared to cells expressing EmGFP alone; hence, to see if there is any difference in the growth cone, we performed co-localization studies. The transfected neuroblastoma cells were immunostained for WAVE (AF555, red) and ARP2/3 (AF647, cyan) and analyzed from images captured through a 60X objective. The growth cones were identified and co-localization was assessed using the Pearson coefficient. The analysis by one-way Anova showed there was significant difference in WAVE and ARP2/3 association by overexpressing non-geranylgeranylatable RhoA (see Figure 2.3.8. B).

2.3.9. Overexpressing non-geranylgeranylated EmGFP-RhoA^{C190A} allows the cross-talk between JNK and ARP.

ERK and JNK both need to be phosphorylated to be active and perform their specific functions [96, 97]. The phosphorylation happens on serine, threonine and tyrosine residues [84, 98]. Also, the ARP2/3 complex is the major actin nucleator involved in the initiation of polymerization of the actin, that needs to be activated by the activator, WAVE [42]. Hence, to check if there was any other interplay between the pathways, we did inhibitor studies. ERK inhibition decreased active JNK in cells overexpressing wild-type RhoA (see Figures 2.3.9. A and B) and decreased WAVE and ARP3 association (see Figures 2.3.9. A and C). JNK inhibition decreased active ERK in cells overexpressing wild-type and mutant RhoA (see Figures 2.3.9. D and E) and there was no effect on WAVE and ARP2/3 association (see Figures 2.3.9. D and F). Similarly,

ARP inhibition decreased the active ERK by overexpressing wild-type and mutant RhoA (see Figures 2.3.9. G and H) and decreased active JNK by overexpressing mutant RhoA (see Figures 2.3.9. G and I).

2.3.10. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not show any change in the actin filament content in the cytoskeleton of neuroblastoma cells.

Rho GTPases regulate a wide range of cellular responses, including changes to the cytoskeleton [6, 59, 99]. The ongoing researches has made it apparent that Rho GTPases are regulated by post-translational modifications [6, 16, 21], and hence, to analyze the effects of expressing non-geranylgeranylatable EmGFP-Rac1^{C190A} on actin, we assessed the total amount of actin in transfected whole cell lysates (WCL) by western blotting. There was no significant difference in the expression of the actin content in WCL of EmGFP-RhoA and EmGFP-RhoA^{C190A} (see Figures 2.3.10. A and B). RhoA needs to be efficiently present in subcellular locations to enable cells to move in response to different effectors and requires activation of different signaling pathways [29]. We next analyzed whether the location of RhoA affects the actin content in different subcellular locations. For this, we separated transfected cells into cytosol, and cytoskeleton fractions and determined the actin content in each fraction. The expression of non-geranylgeranylatable RhoA did not change the actin content in the cytosolic or cytoskeleton fractions compared to cells expressing EmGFP alone or wild-type RhoA (see Figures 2.3.10. C and D).

2.3.11. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} didn't show any change in the actin filament at the tips of extending axons (growth cones, lamellipodia, and filopodia) in the neuroblastoma cells.

RhoA is implicated in the assembly of focal adhesions in growth cones and it inhibits neuronal outgrowth by promoting growth cone collapse [5, 100]. To determine the effect of overexpressing RhoA on actin, we compared the actin filament content in the growth cones of the transfected neuroblastoma cells. For this purpose, we counted the growth cones stained with phalloidin. There was no significant difference in the actin filament content in growth cone in cells with overexpression of the non-geranylgeranylatable RhoA compared to cells overexpressing EmGFP (see Figures 2.3.11. A and B).

2.3.12. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} fails to show any change in the actin filament at the tips of extending axons in the rat cortical neurons.

RhoA is a negative regulator of axon formation in neurons, through its downstream effector Rho-kinase (ROCK) [6, 101]. Therefore, we next determined the effect of overexpressing non-geranylgeranylated RhoA in rat cortical neurons. The growth cones were identified after phalloidin staining. There was no significant difference in the actin content in the growth cones of transfected rat cortical neurons (see Figures 2.3.12. A and B) when compared to the control neurons expressing EmGFP.

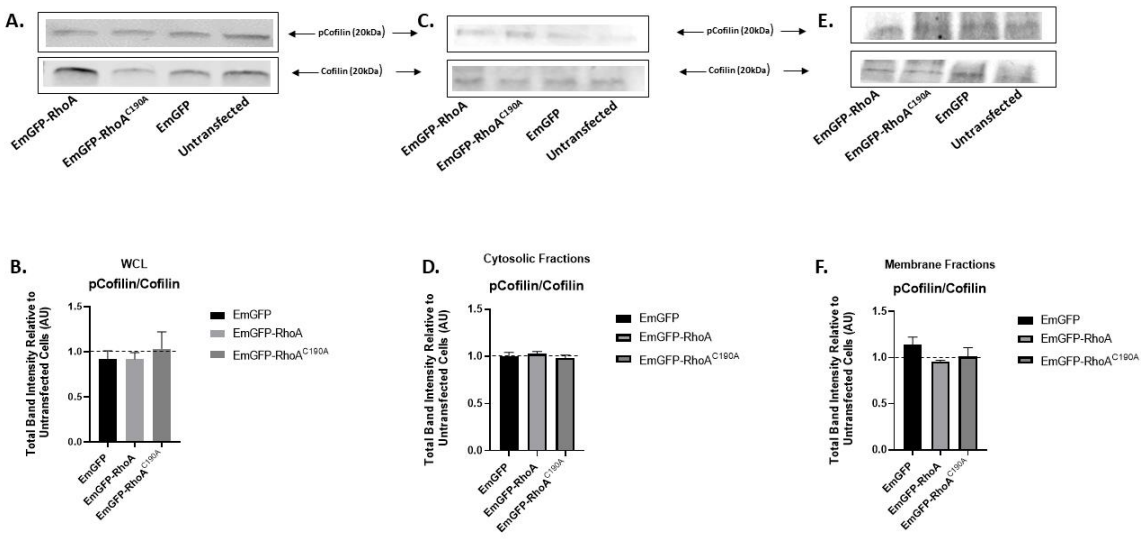


Figure 2.3.1.: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not have any affect on the actin-depolymerizing (cofilin) pathway. B35 cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable RhoA for 48 h at 37 °C. (A) Cells were collected and subjected to western blotting and probed against pcofilin (on top) and cofilin (on bottom). (B) Analysis of pcofilin/cofilin showed no significant difference in the active cofilin in whole-cell lysates by overexpressing geranylgeranylatable RhoA. Also, cells were fractionated into membrane/organelle and cytosolic fractions and subjected to western blotting and probed for pcofilin and cofilin (C). Representative blot of pcofilin (on top) and Cofilin (on bottom) (D) Analysis of pcofilin/cofilin showed there was no significant difference in the active cofilin in the cytosol by overexpressing geranylgeranylatable RhoA. (E) Representative blot of pcofilin (on top) and Cofilin (on bottom) (D). Analysis of pcofilin/cofilin showed there was no significant difference in the active cofilin in the membrane by overexpressing geranylgeranylatable RhoA. (D and F) Analysis of pcofilin/cofilin showed there was no difference in the active cofilin in the cytosol or membrane fractions by overexpressing geranylgeranylatable RhoA when compared to EmGFP. Quantification was performed on three blots from three sets of experiments. Analysis was done by using one way ANOVA. Data in B, D and F are expressed as means ± s.e.m.

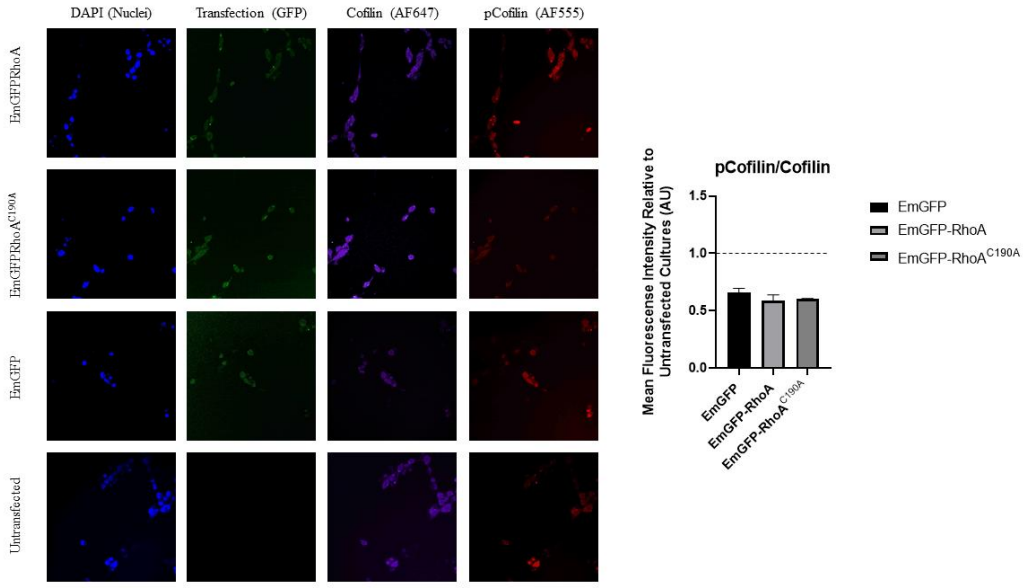


Figure 2.3.2.: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not affect the actin-depolymerizing (cofilin) in the growth cones. B35 cells transfected with EmGFP-RhoA or EmGFP-RhoA^{C190A} or EmGFP didn't show any significant difference for the active cofilin of the growth cones. The area of interest (growth cone) was identified and intensity for cofilin (cyan) and pcofilin (red) was used to find the ratio of pcofilin and cofilin. A. (a, e, i and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k and o) represent the transfected cells immunoblotted for cofilin and (d, h, I and p) represent the transfected cells immunoblotted for pcofilin. B. Quantification for pcofilin/cofilin represents there was no significant difference in the active cofilin by overexpressing EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Data in B are expressed as means ± s.e.m. The scale bar in the image (d) indicates 20 µm and is valid for all the images.

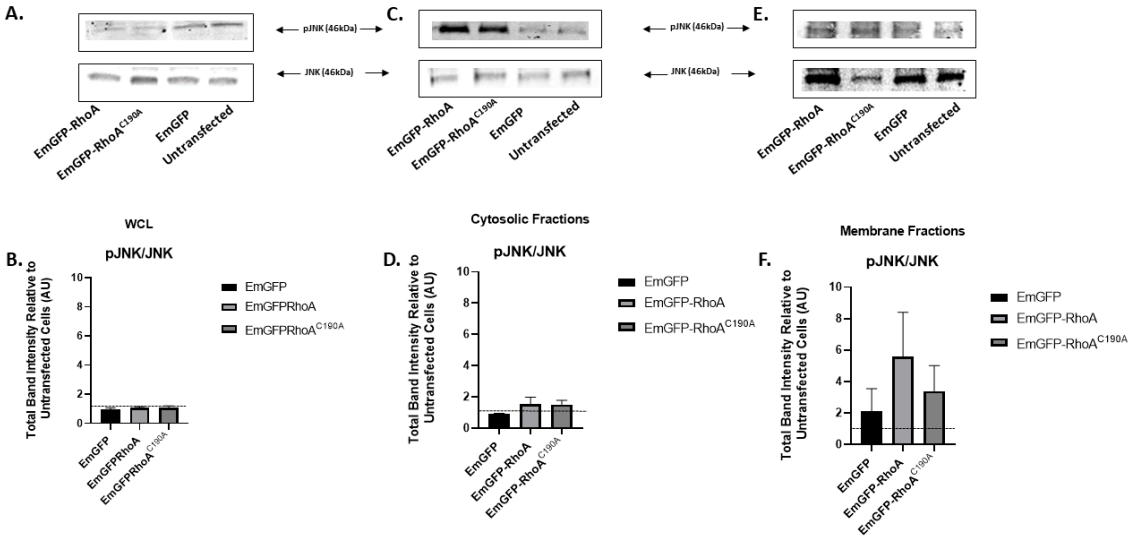


Figure 2.3.3.: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not affect the active JNK. (A) Cells from the transfected conditions, i.e., EmGFP, EmGFP-RhoA, and EmGFP-RhoA^{C190A} were collected and subjected to western blot and probed for pJNK (on the top) and JNK (at the bottom) in the sample. (C)The cytosolic fractions were also probed for pJNK (on the top) and JNK (at the bottom) and (E) The membrane/organelle fractions were probed for pJNK (on the top) and JNK (at the bottom) (B) Analysis of pJNK/JNK showed there was no significant difference in the active JNK in EmGFP-Rac1 or EmGFP-Rac1^{C189A} when compared to EmGFP. in WCL. (B) in the cytosol (D) or in the membrane fractions (F). Data in (B), (D) and (F) are expressed as means ± s.e.m. and were analyzed by one way ANOVA using the three western blots from three sets of experiments.

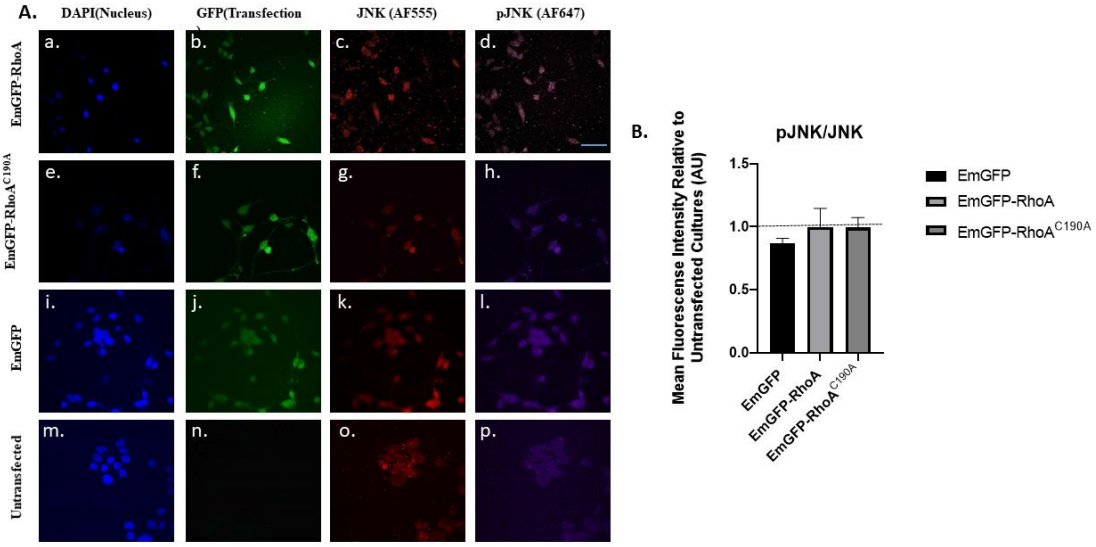


Figure 2.3.4.: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} did not change the JNK pathway in the growth cones. The area of interest (growth cone) was identified and intensity for JNK (AF555, red) and pJNK (AF647, cyan) was used to find the ratio of pJNK and JNK. A. (a, e, i and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k and o) represent the transfected cells immunoblotted for JNK and (d, h, I and p) represent the transfected cells immunoblotted for pJNK. B. Quantification for pJNK/JNK represents there was no significant difference in the active JNK by overexpressing EmGFP-RhoA^{C190A} when compared with the control, i.e., EmGFP. Analysis was done by one-way ANOVA. Quantification was done on nine images that were derived from three sets of experiments that were in triplicates for all conditions. Data in B are expressed as means ± s.e.m. The scale bar in the image (d) indicates 20 μm and is valid for all the images.

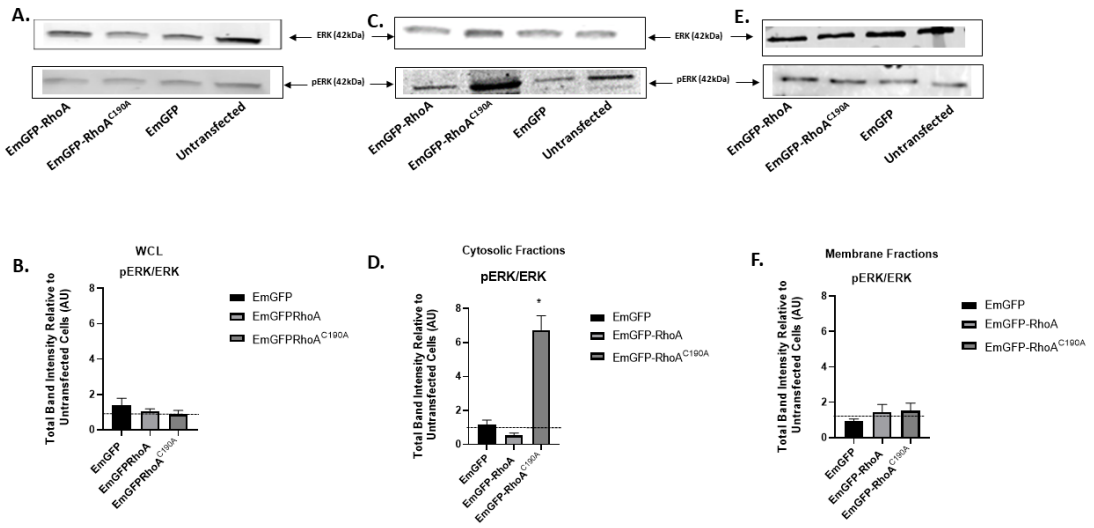


Figure 2.3.5.: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} increased active ERK in the cytosol. (A) Cells transfected with EmGFP, EmGFP-RhoA and EmGFP-RhoA^{C190A} were collected and subjected to western blotting and probed for pERK (on top) and ERK (on bottom) in the sample. (B). Analysis of pERK/ERK by one-ways ANOVA showed no significant difference in the active ERK in whole-cell lysates by overexpressing non-geranylgeranylatable Rac1. Also, cells were fractionated into membrane/organelle and cytosol fractions and subjected to western blotting and probed for pERK and ERK. (C) Representative blot of pERK (on top) and ERK (on bottom) (D) Analysis of pERK/ERK by ANOVA showed there was a significant difference in the active ERK in the cytosol by overexpressing non-geranylgeranylatable RhoA and "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). (E.) Representative blot of pERK (on top) and ERK (on bottom) (D) Quantification of pERK/ERK by one-way ANOVA showed there was no significant difference in the active ERK in the membrane by overexpressing non-geranylgeranylatable RhoA. (D and F) Data in B, D, and F are expressed as means ± s.e.m. All the experiments were performed in three times.

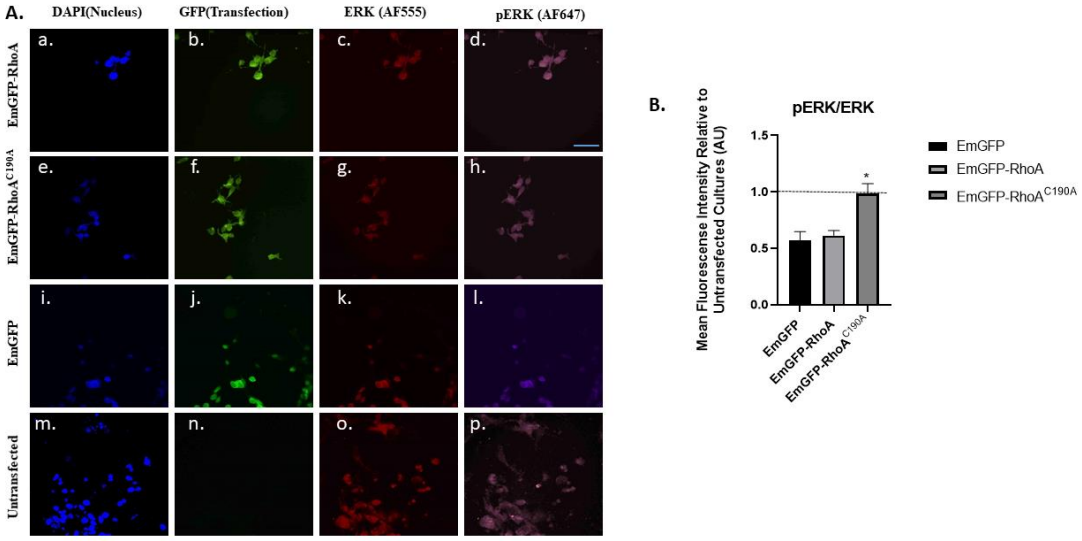


Figure 2.3.6.: Overexpressing non- geranylgeranylatable EmGFP-RhoA^{C190A} increased the active ERK in the growth cones. B35 cells transfected with EmGFP-RhoA and EmGFP-RhoA^{C190A} or EmGFP didn't show any significant difference for the active ERK in the growth cones. The area of interest (growth cone) was identified and intensity for ERK (Af555, red) and pERK (AF647, cyan) was used to find the ratio of pERK and ERK. A. (a, e, I and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k and o) represent the transfected cells immunoblotted for ERK and (d, h, I and p) represent the transfected cells immunoblotted for pERK. B. Analysis of pERK/ERK by one-way ANOVA showed there was a significant difference in the active ERK by overexpressing EmGFP EmGFP-RhoA and EmGFP-RhoA^{C190A}, "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). Data in B are expressed as means ± s.e.m. The scale bar in the image (d) indicates 20 μm and is valid for all the images.

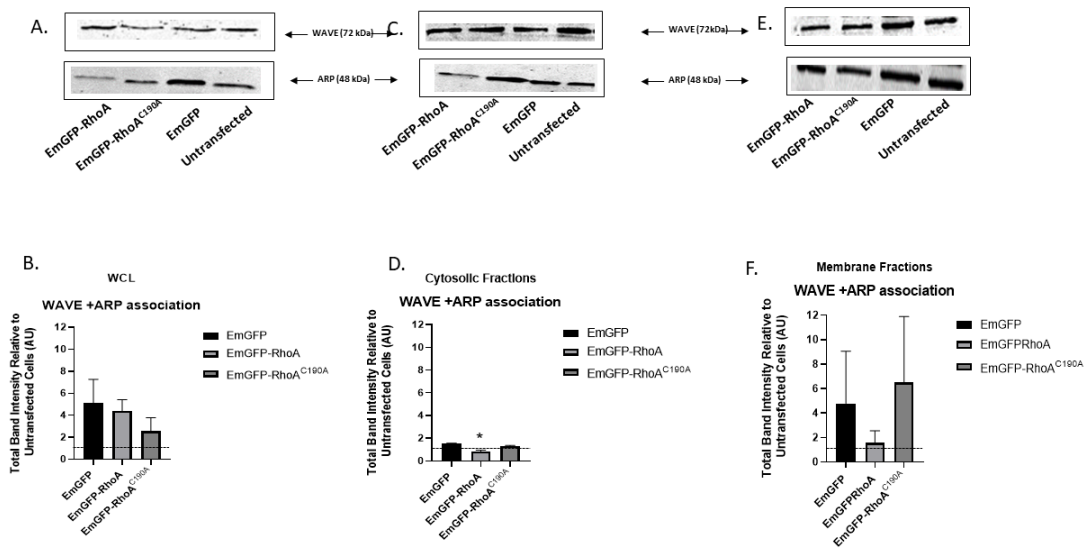


Figure 2.3.7.: Overexpressing geranylgeranylatable RhoA increased WAVE and ARP association in the cytosol: B35 cells transfected with or EmGFP, EmGFP-RhoA and EmGFP-RhoA^{C190A} and cells were extracted for co-immunoprecipitation. (A) Immunoprecipitation for ARP2/3 was performed in the WCL (at the bottom). Western blots on the eluate for WAVE in the eluate were consistent (on the top). (B). Analysis of the association of WAVE and ARP showed no significant difference in the whole-cell lysates from the overexpression of EmGFP-RhoA. (C) Immunoprecipitation for ARP2/3 was performed in the cytosolic fraction (at the bottom) and western blots on the eluate for WAVE in the eluate were consistent (on the top). (D). Analysis of the association of WAVE and ARP2/3 in the cytosolic fractions showed significant difference. (E) Immunoprecipitation for ARP2/3 was performed in the membrane fraction (at the bottom) and western blots on the eluate for WAVE in the eluate were consistent (on the top). (F) Analysis of the association of WAVE and ARP in the membrane showed no significant difference in the membrane fractions. "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). Data in B, D, and F are expressed as means ± s.e.m.

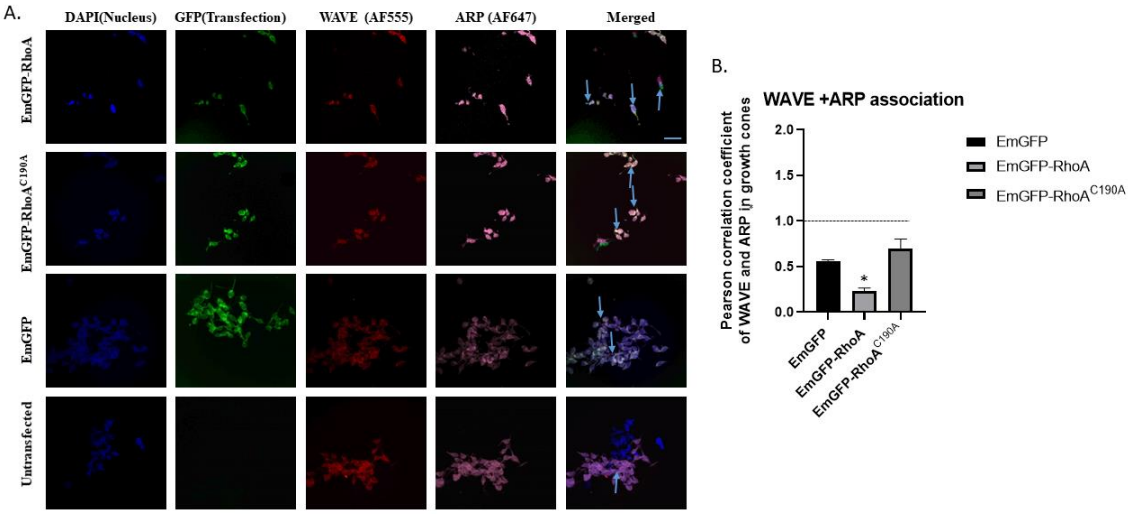
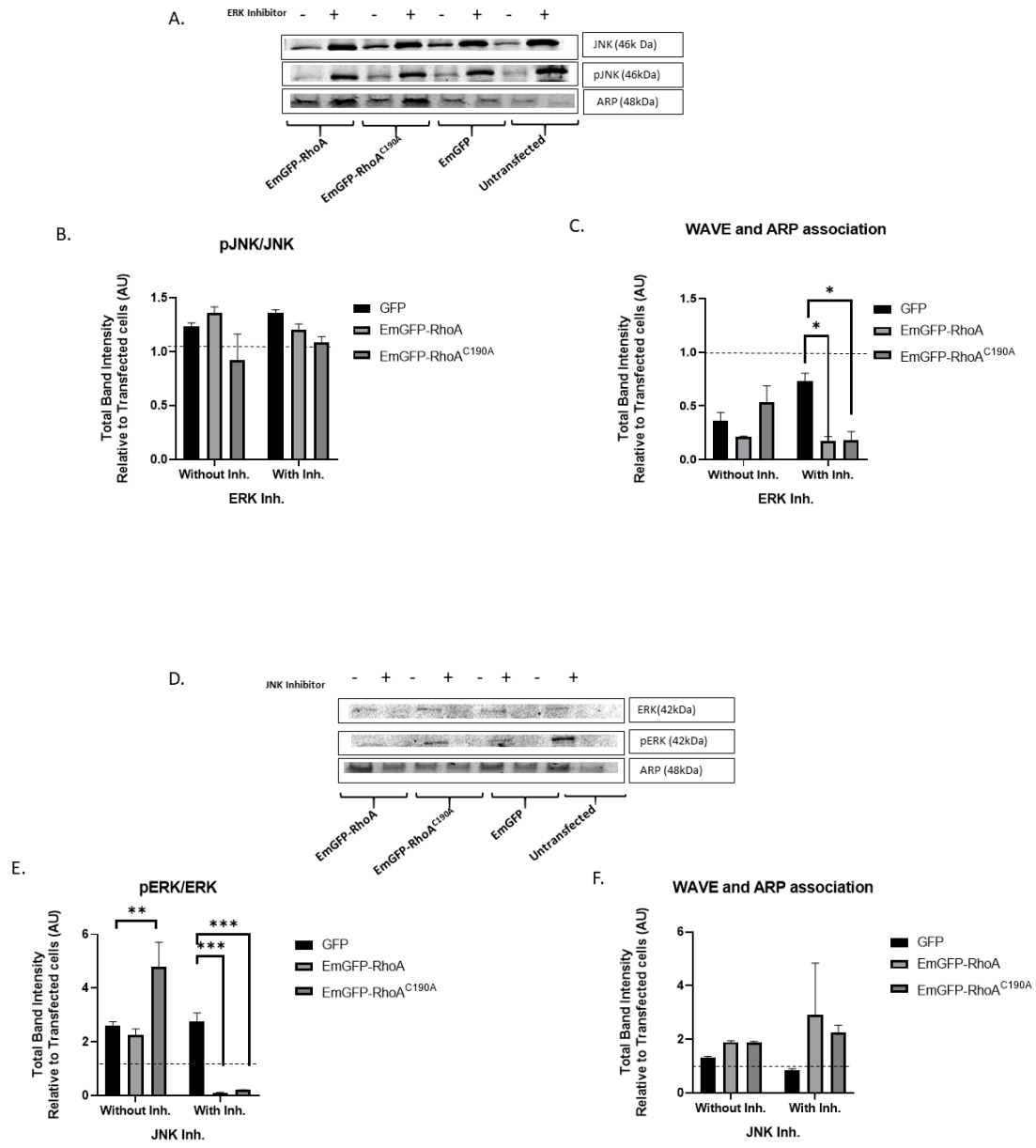


Figure 2.3.8.: Overexpressing geranylgeranylatable RhoA increased the WAVE and ARP association in the growth cones. B35 cells transfected with EmGFP-RhoA and EmGFP-RhoA^{C190A} or EmGFP showed a significant difference for the WAVE and ARP association in the growth cones. The area of interest (growth cone) was identified and the co-localization of WAVE (AF555, red) and ARP (AF647, cyan) was performed using A1 confocal software and Pearson coefficient was calculated. A. (a, f, k and p) represent the nuclei, (b, g, l and q) represent transfected cells, (c, h, m, and r) represent the transfected cells immunoblotted for WAVE, (d, I, n and s) represent the transfected cells immunoblotted for ARP and (e, j, o and t) represent the merged images. (B.) Quantification of WAVE and ARP association showed there was a significant difference in the association by overexpressing non-geranylgeranylatable RhoA when compared to EmGFP. Data in B are expressed as means ± s.e.m. and, "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). The scale bar in image (e) indicates 20 μm and is valid for all the images. The arrows refer to the co-localized section.


A.
ERK Inhibitor - + - + - + - +
JNK (46k Da)
pJNK (46kDa)
ARP (48kDa)
EmGFP-RhoA
EmGFP-RhoA C190A
EmGFP
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B.
pJNK/JNK
Total Band Intensity Relative to Transfected cells (AU)
1.5
1.0
0.5
0.0
Without Inh.
With Inh.
ERK Inh.
GFP
EmGFP-RhoA
EmGFP-RhoA C190A
C.
WAVE and ARP association
Total Band Intensity Relative to Transfected cells (AU)
*
*
1.5
1.0
0.5
0.0
Without Inh.
With Inh.
ERK Inh.
GFP
EmGFP-RhoA
EmGFP-RhoA C190A
D.
JNK Inhibitor - + - + - + - +
ERK(42kDa)
pERK (42kDa)
ARP (48kDa)
EmGFP-RhoA
EmGFP-RhoA C190A
EmGFP
Untransfected
E.
pERK/ERK
Total Band Intensity Relative to Transfected cells (AU)
**
***
***
6
4
2
0
Without Inh.
With Inh.
JNK Inh.
GFP
EmGFP-RhoA
EmGFP-RhoA C190A
F.
WAVE and ARP association
Total Band Intensity Relative to Transfected cells (AU)
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2
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Without Inh.
With Inh.
JNK Inh.
GFP
EmGFP-RhoA
EmGFP-RhoA C190A

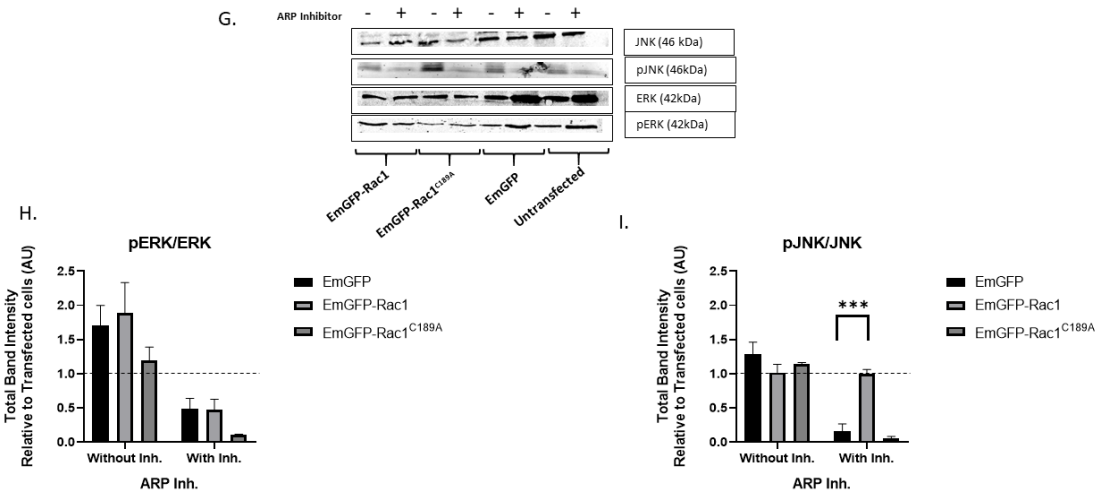

Figure 2.3.9. Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} allows the cross-talk between ARP and JNK. B35 cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable RhoA for 48 h at 37 °C and then treated with 0.125µM of CK-869, 40µM of JNK inhibitor, 25µM of ERK inhibitor-I 1 hrs for 4 hrs respectively. (A) Cells treated with ERK inhibitor were collected and probed for JNK (on the top), pJNK (in middle) and ARP (at the bottom). (D) Cells treated with JNK inhibitor were collected and probed for ERK (on the top), pERK (in middle) and ARP (at the bottom). (G) Cells treated with ARP inhibitor were collected and probed for JNK (on the top), pJNK (2nd from the top), ERK (2nd from the bottom) and pERK (at the bottom). + represent cells treated with inhibitor and – represent cells were not treated with inhibitor. The ERK inhibition decreased the active JNK by overexpressing non-geranylgeranylatable RhoA (B.), decreased the association of WAVE and ARP by overexpressing wild type and mutant RhoA (C). JNK inhibition decreased the active ERK by overexpressing wild type and mutant RhoA when treated with inhibitor (E.) and didn't affect the association of WAVE and ARP (F) on the other hand, ARP inhibition did not change the active ERK by overexpressing wild type and mutant RhoA (H.), and decreased the active JNK by overexpressing wild type RhoA (I). All the analysis were done by two-way ANOVA from three western blots.

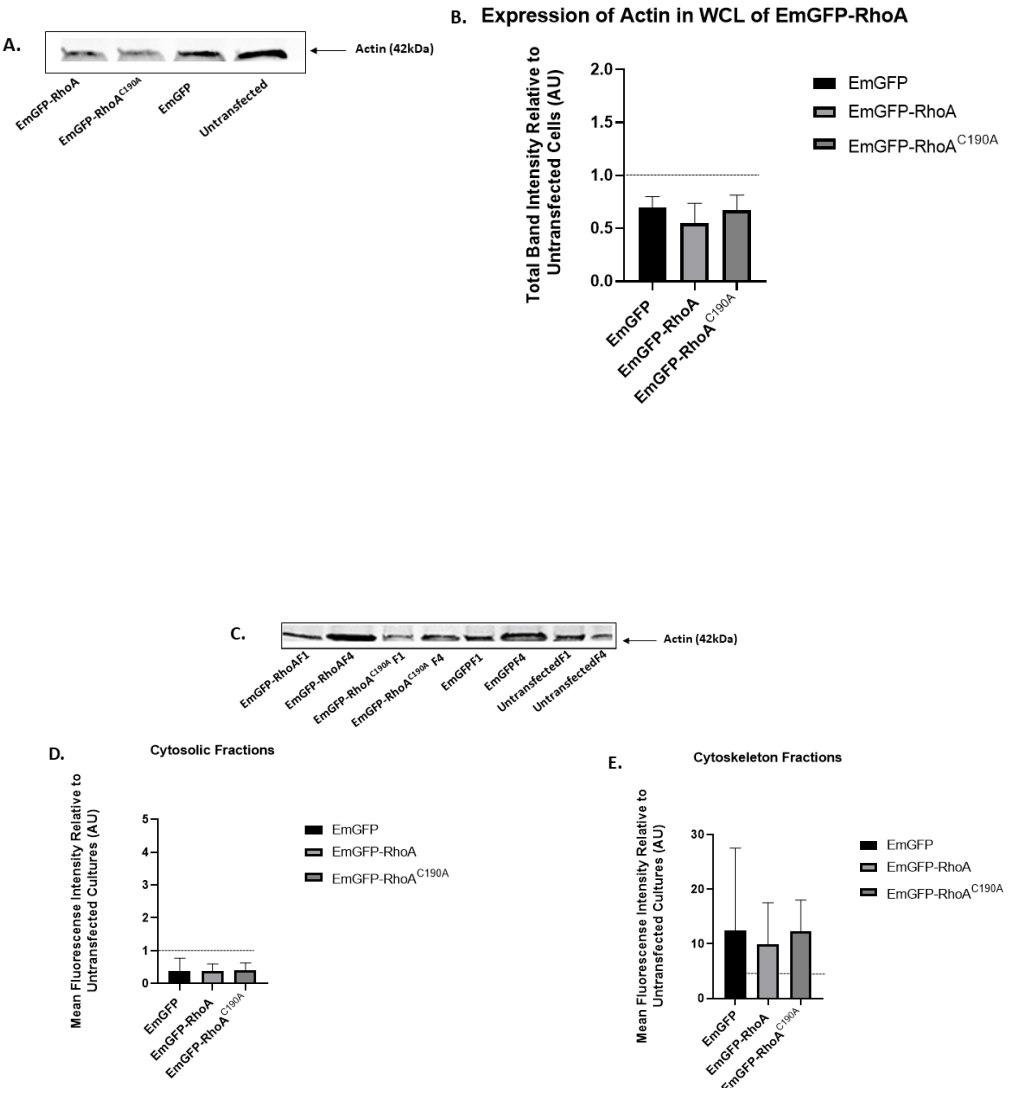

Figure 2.3.10: Overexpressing non-geranylgeranylatable EmGFP-RhoA^{C190A} didn't show any change in the actin filament content in the cytosol and cytoskeleton of neuroblastoma cells. Neuroblastoma (B35) cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable RhoA for 48 h at 37 °C. (A) Cells were collected and subjected to western blotting and probed for actin. (B.) Quantification of actin showed there was no significant difference in the actin content in whole-cell lysates by overexpressing geranylgeranylatable RhoA. (C) Cells were fractionated into cytosol and cytoskeleton fractions and subjected to western blotting and probed for actin. (D) Quantification of actin showed there was no significant difference in the actin content in the cytosol by overexpressing geranylgeranylatable Rac1. (E) Quantification of actin in the cytoskeleton fractions showed no significant difference. All the quantification were by one way ANOVA. Data in B, D and E are expressed as means ± s.e.m.

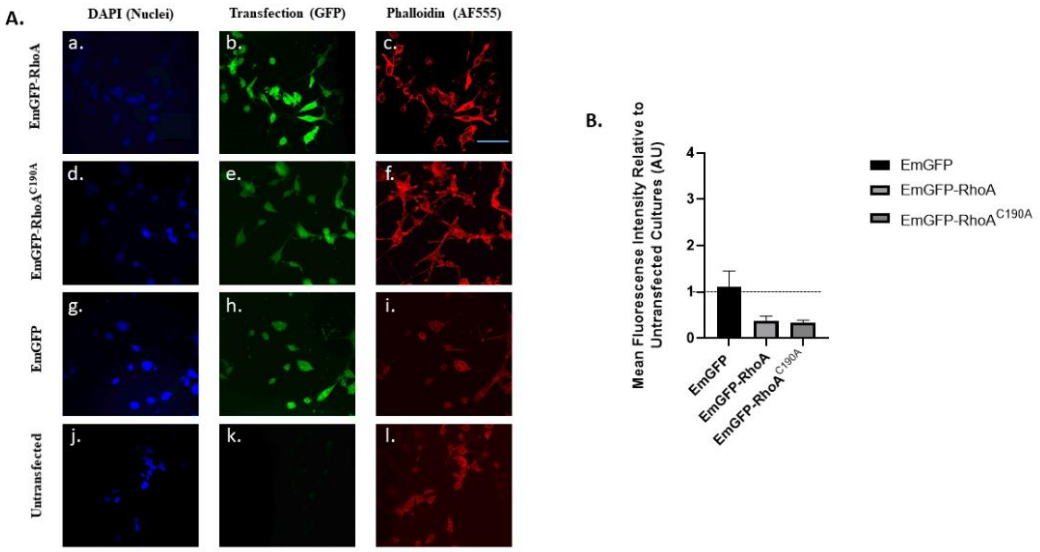

Figure 2.3.11.: Overexpressing geranylgeranylatable RhoA did not show any change in the actin filament at the tips of extending axons (growth cones, lamellipodia, and filopodia) in the neuroblastoma cells. (A.) Growth cones were identified in all the images captured. (a, d, g and j) represent the nuclei stained for Dapi, (b, e, h, and K) represent transfected cells and (c, f, I and l) represent average fluorescence intensities for Alexa fluor 555 conjugated phalloidin labeled actin filaments and were determined from corresponding images captured through an A1 confocal microscope. (B.) Analysis of the phalloidin staining actin filaments form the identified growth cone regions was done by one way ANOVA and are expressed as means ± s.e.m. for 3 replicate cultures in each condition, normalized to the overall mean intensity for untransfected control in each region of interest. There was no significant difference between mean fluorescence by overexpressing RhoA when compared to controls EmGFP condition in each experiment. The scale bar in the image (c) indicates 20 μm and is valid for all the images. 16-20 growth cones were identified in each image.

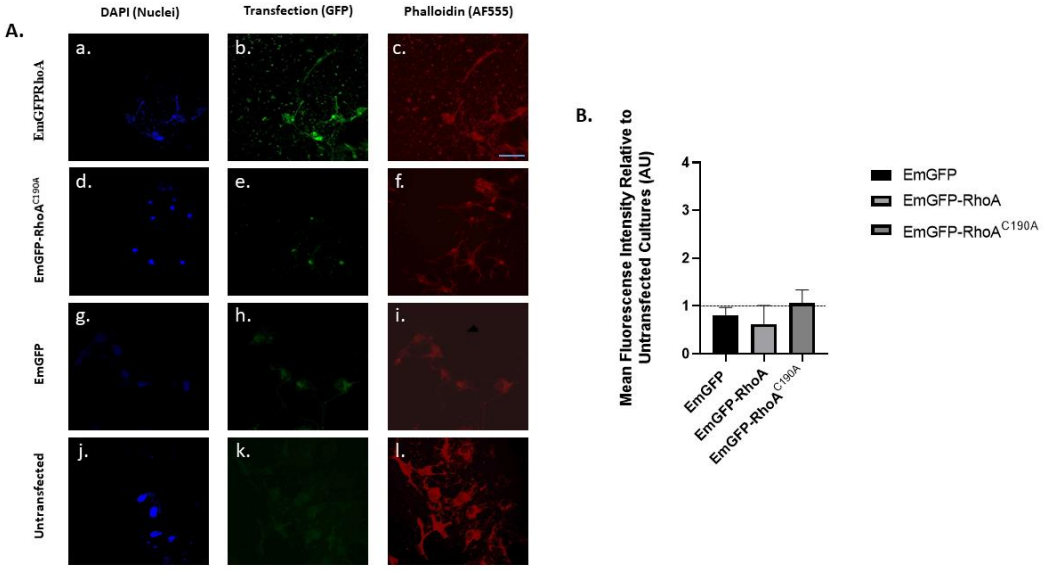

Figure 2.3.12.: Overexpressing non-geranylgeranylatable RhoA fails to show any change in the actin filament content at the tips of extending axons in the rat cortical neurons. (A.) Growth cones were identified from representative phase-contrast images, (a, d, g and j) represent the nuclei stained for dapi, (b, e, h and K) represent transfected cells and (c, f, I and l) average fluorescence intensities for Alexa fluor 555 conjugated phalloidin labeled actin filaments were determined from corresponding images captured through an A1 confocal microscope. (B.) Quantification of the phalloidin staining actin filaments form the identified growth cone regions were performed by one way ANOVA and are expressed as means ± s.e.m. for 3 replicate cultures in each condition, normalized to the overall mean intensity for untransfected control in each region of interest and compared to EmGFP(control in each experiments). The scale bar in the image (c) indicates 20 μm and is valid for all the images.

2.4. Discussion

The main focus of the study was to see if over-expressing non-geranylgeranylatable RhoA leads to the differential signaling pathways. Overexpressing non-geranylgeranylatable RhoA increased the active ERK in the growth cone. This suggests that there is active ERK in the growth cone by overexpressing of mutant RhoA (EmGFP-RhoA^{C190A}). This was also confirmed by immunoblotting for ERK in the cytosolic fractions of the transfected cells. Our data concurs with previous studies reporting that there is active pool of RhoA in the cytosol [13, 102]. We found that overexpressing non-geranylgeranylatable RhoA mutant (EmGFP-RhoA^{C190A}) in neuroblastoma cells failed to increase the actin content in the cytosol or cytoskeleton, compared to cells expressing EmGFP or wild-type RhoA (EmGFP-RhoA). The similar findings in rat cortical neurons also strengthen the fact that these RhoA GTPases functions in the same way in the primary cell line. The actin present in the different compartments of the cell might have canceled the effect of each other; hence, we did not see any difference in the actin content in whole-cell lysates by overexpressing geranylgeranylatable or non-geranylgeranylatable RhoA compared to cells overexpress only EmGFP.

Also, the actin nucleator ARP needs to be activated by WAVE and is one of the important players in polymerizing actin. By overexpressing mutant RhoA, that is non-geranylgeranylatable, it allows the significant association of WAVE and ARP in the membrane fraction. When analyzed in the identified growth cones of the extending cells, a similar pattern was visible. Immunocytochemistry (ICC) focused on the growth cones and gave an idea on by overexpressing RhoA the association of WAVE and ARP increased in

the growth cones. Similarly, subcellular fractions provided the evidence that there was increased WAVE and ARP association in the membrane by overexpressing non geranylgeranylatable RhoA. Both techniques supported our finding that WAVE and ARP association was increased by overexpressing non-geranylgeranylatable RhoA. The WAVE and ARP association in the membrane was expected as the larger pool of RhoA is present in the membrane [7-9, 103]. Rho GTPases are the molecular switches that remain active by GTP binding and inactive when GDP bonded [12, 44]. This GTP binding is achieved by the guanine nucleotide exchange factor (GEF) [104]. Hence, there is a possibility that this activated RhoA in the cytosol might be working under the influence of the unknown GEF present in the cytosol. Also, the active RhoA in the membrane may be under the influence of the GEF and the identification of this particular GEF will shed some light on the upstream regulators in these pathways.

The inhibitor studies revealed that the ARP inhibition also decreased the total JNK and total ERK by overexpressing mutant RhoA. These data indicate cross-talk is occurring between these pathways. JNK pathway signaling is associated with many neurodegenerative diseases like in PD and the inhibitors for the JNK pathways are main targets for novel therapeutic approaches for PD [105-107]. Manipulation of the JNK pathway by inhibiting ARP could also provide a novel therapy via activating the ARP pathway leading to actin polymerization.

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CHAPTER III

DECREASING GERANYLGERANYLATION OF RAC1 DECREASES ACTIN CONTENT BY WAVE/ARP AND MAPK PATHWAYS.

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Abstract

Encouraging axon regeneration after traumatic lesions to the central nervous system (CNS) or the onset of neurodegenerative conditions like Alzheimer's disease (AD) may increase the functional recovery. Unfortunately, there is no effective treatment that promotes axon regeneration or synaptic plasticity. Both regeneration and synapse formation involve dynamic rearrangements of the growth cone actin cytoskeleton. For process extension, actin monomers polymerize to filaments near the leading edge and monomers are removed at the transition to the axon. Actin polymerization and depolymerization are controlled in large part by Rho guanosine triphosphatases (GTPases). These proteins are active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP). Rho GTPases are targeted to the plasma membrane by the addition of 20-carbon lipophilic geranylgeranyl isoprene. It is not known

how Rho and RhoA geranylgeranylation affects the location and activity of downstream effectors to facilitate either polymerization or depolymerization of actin. We used non-geranylgeranylatable Rac1 constructs to test how inhibiting geranylgeranylation affects localization and activation of Rac1 cell signaling pathways. Expressing non-geranylgeranylatable Rac1 decreased the actin filament content and activates WASP-family verprolin-homologous protein (WAVE) and actin related protein ARP association in the cytosol and JNK pathway in the cytosol. With emerging evidence of differential activation of these Rho GTPases based on their subcellular localization, elucidating the signaling cascades of the active GTPases may identify novel targets to facilitate axon regeneration in traumatic or degenerative neurological conditions.

3.1.Introduction

Abnormal cytoskeletal organization of actin is one of the common features of many neurodegenerative diseases (ND) like Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [69, 73]. Actin polymerization and depolymerization are controlled in large part by Rac1, a member of the Ras superfamily of small GTPases [15, 20, 103, 108]. Rac1 dysfunction has been reported in the case of NDs like AD, PD and HD [56, 69, 73].

Rac1 functions as a guanosine diphosphate (GDP)/ guanosine triphosphate (GTP)-regulated switch [15, 20, 103]. This switching of GDP/GTP cycling is regulated by guanine nucleotide exchange factors that promote the formation of the active GTP-bound form [12] and GTPase-activating proteins (GAP) that catalyze the intrinsic GTPase activity and

promote the formation of inactive GDP-bound [12]. It is accepted that proteins present in membrane-bound states are activated through GTP loading and the protein pools that are present in the cytosol and nucleus are inactive being GDP-bound [30, 109]. All this is facilitated by the post-translational modification of the GTPases by prenylation [22, 110].

Prenylation is accomplished by the addition of a 15-carbon (farnesyl isoprenoid) or a 20-carbon (geranylgeranyl isoprenoid) lipid moiety to the protein of interest in an irreversible process. The isoprenoids are added to the C-terminus of the protein after the post-translational modification of the CAAX terminus (where C represents a cysteine, A represents any aliphatic amino acid and X represents a specific amino acid) by prenyltransferases and prior to the cleavage of the –AAX is subsequently cleaved by a peptidase [22, 111]. This lipid modification eventually leads to the targeting of these prenylated proteins to membranes where GTP loading and activation occur [21, 30]. Active GTP-bound Rac1 then bind to their downstream effectors, stimulating diverse signaling cascades that regulate cellular functions like polarity, motility, adhesion and membrane trafficking and actin reorganization[32]. Here, we assess how expressing EmGFP-$Rac1^{C189A}$ in neuroblastoma cells and primary cortical neurons affects actin polymerization and depolymerization. The second thing we wanted to test were pathways involved in actin polymerization in differential locations of the cells by overexpressing RhoA and Rac1 as it is still unclear on how these modifications to GTPases affect neurite extension [16, 30].

Rac1 allows actin de/polymerization by many pathways like ARP, JNK or cofilin, which are downstream of Rac1 [44, 56, 68, 99]. Rac1 promotes actin nucleation through activation of IRSp53 [43], which activates the Wiskott-Aldrich syndrome protein (WASP)

family verprolin-homologous protein (WAVE) allowing it to interact with the actin-related protein (ARP2/3) complex to nucleate actin side chains and produce branched actin meshwork's [14]. WAVE has been found to co-aggregate with both hyperphosphorylated tau neurofibrillary tangles and abnormal neurites of the AD brain [111] [112].

Rac1 also activates mitogen associated protein (MAP) kinases, like extracellular signal-regulated kinase (ERKl/2) or Jun N-terminal kinases (JNK), through direct activation of MAP or ERK kinase (MEKKI), which leads to actin nucleation [113, 114] . The ERK signaling pathway has been found to ameliorate learning deficits [83, 115], identifying a novel pathway through which protects against AD-related cognitive impairments [38, 81]. Together, these reports shed light on a feasible therapeutic approach to control the progression of AD [58]. JNK has been defined as a novel therapeutic target for AD and PD [116, 117]. Further, studies indicate that JNK could be a target to prevent cell loss [105]. Together, these studies indicate that Rac1 and the identified MAP kinases are part of a complex, intracellular cascade that mediates axon growth and cell survival [51]. It remains unclear whether downstream regulation by prenylated Rac1 activates these pathways for neuronal guidance.

Another important pathway regulated by Rac1 is via PAK, which activates LIMK that phosphorylates and inactivates cofilin [68]. Cofilin, under stress, undergoes dephosphorylation (activation) and forms rod-shaped actin bundles in neurons [62, 70]. Recent research supports the link between neurite transport defects in the very early stages of many neurodegenerative diseases and alterations in the organization and dynamics of

the actin cytoskeleton initiated by proteins in the actin-depolymerizing factor (ADF)/cofilin family[62].

Recent work from our lab has reported that Rac1 mutant (EmGFP-Rac1^{C189A}), or expressed proteins that cannot be geranylgeranylated, are present in the cytosol [16]. This is fascinating as Rho GTPases are believed to be active only in membrane and inactive in any other compartments of the cell. This is important as it will help to find out the pathways that is downstream of these GTPases when differentially localized. These mutants were created by mutating the cysteine in the CAAX motif to an alanine that decreased Rac1 membrane association [16]. Here, we assess how expressing EmGFP-Rac1^{C189A} in neuroblastoma cells and primary cortical neurons affects actin polymerization and depolymerization and the possible pathways involved as it is still unclear on how these modifications to GTPases affect neurite extension or how subcellular localization alters signaling from the classical Rho GTPases [30].

3.2. Material and Methods

3.2.1. Constructions of the expression vectors

We have made mammalian expression vectors containing wild-type Rac1 that is N-terminally tagged with Emerald green fluorescence protein referred to as EmGFP-Rac1 [16]. This construct can be prenylated and was achieved by inserting the open reading frame of Rac1 (Open Biosystems, Houston, TX) into a mammalian expression vector that is fused with EmGFP to the N-terminus of Rac1 (Invitrogen, Carlsbad, CA). RhoA mutant construct was achieved using site-directed mutagenesis (Kit from Invitrogen, Carlsbad,

CA) to change the cysteine at the CaaX box (C = cysteine, a = any aliphatic amino acid, X = terminal amino acid) to an alanine, making this construct non-prenylatable (referred as EmGFP-Rac1^{C189A}).

3.2.2. Cell Culture

Rat neuroblastoma B35 cells (CRL: 2754, ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle medium (DMEM; 12634010, ThermoFisher) supplemented with 10% fetal bovine serum (FBS; 12103C Sigma, St. Louis, MO) with 5% carbon dioxide at 37℃ in the incubator. Cells were passaged when 85-90% confluent using 3 mL Trypsin (9002-07-7, Sigma-Aldrich) in a T-75 flask. Cells were seeded in 6-well plates at 20,000 cells/cm2 for western blotting, and on 12 mm glass coverslips in 24-well tissue culture plates at 5,000 cells/cm2 for immunocytochemistry (ICC). Primary cortical neurons were extracted from rats within 24 hours of birth. Pups were anesthetized on ice and decapitated. An incision was made on the dorsal side of the head to have a clear view of the skull. The skull was removed using scissors and forceps and the brain was removed with the help of a sterile spatula. Brains were then sliced into 1 mm-3 mm sections using sterile scalpels. The remaining meninges and blood vessels were removed carefully using forceps. The cortices were dissected from the rest of the brain tissues. The tissues obtained were then dissociated using papain (2 mg/mL; 9001-73-4, Sigma). The obtained cells were then plated and allowed to stabilize on poly-D-Lysine (PDL) coated glass coverslips in 6-well plates at a density of 3000 cells/cm2 for 24-well plates in neurobasal media (21103049, Gibco) supplemented with B27 (10%; A3582801, Gibco) for 7 days before transfection.

After 48 hrs, cells were fixed using 4% paraformaldehyde for 20 minutes and subjected to ICC as explained below.

All the work in animals was approved by the TWU Institutional animal care and use committee (IACUC).

3.2.3. Transfection

Cells were transfected with Emerald green fluorescent protein (EmGFP), EmGFP-Rac1 or EmGFP-Rac1^{C189A} using Lipofectamine 2000 or 3000 (L3000015, ThermoFisher), according to the manufacturer's instructions. 3 µg and 0.8 µg of plasmid were used for transfection for western blotting and immunocytochemistry respectively. A control with untransfected cells was maintained in serum-free medium (SFM). After 48 hrs, cells were either fixed in 4.0% paraformaldehyde for immunolabeling for immunocytochemistry or were lysed for western blotting as described below. Transfection efficiency was assessed by the number of cells expressing GFP only.

3.2.4. Inhibitor studies

Untransfected and transfected cells were treated with 0.125 µM of CK-869, i.e., actin-related protein (ARP) inhibitor (4984, EMD Millipore), 40 µM of c-Jun N-terminal kinase (JNK) inhibitor-II (420119, EMD Millipore), 25 µM of extracellular signal-regulated kinase (ERK) inhibitor (328006, EMD Millipore) for 1 hr and with 20 µM of LIMKi-3 (435930, Sigma-Aldrich) for 4 hrs and observed regularly for viability. Afterward, the lysate was collected, and the samples were run on SDS gel as described above

3.2.5. Subcellular Fractionation

Untransfected and transfected cells were fractionated using the S-PEK Proteolysis kit (Pierce, 539790) into cytosolic (fraction 1), membrane (fraction 2), nuclear (fraction 3) and cytoskeletal (fraction 4) fractions using the manufacturer's instructions. Efficient separation of cell fractions was routinely assessed by western blotting for the membrane-associated protein using rabbit anti-GABA-Aβ2 (1:1000; ab72445, Abcam), for cytosol associated protein using antibody rabbit anti-calnexin (1:1000; ab108400, Abcam), and the nuclear-associated protein using rabbit anti- histone 1.1 (1:1000; ab17584, Abcam). Fractions were then run on a 12% SDS/PAGE gel and western blots were analyzed following the procedure listed below.

3.2.6. Co-immunoprecipitation

Lysis buffer containing cell lysates was treated with mouse anti-WASP-family verprolin homologous protein 1 (WAVE; 1:1000; ab211427, Abcam) and purified mouse serum as the control corresponding to the primary antibody were rocked overnight at 4ºC. Goat anti-rabbit IgG agarose magnetic beads, also called as magnabind (31289, ThermoScientific) was washed 3 X with lysis buffer containing protease inhibitor cocktail (PIC; 21356, ThermoScientific). 20 µL of washed magnabind were added to the overnight rocked samples and control serum and left on the rocker for 3 hours at room temperature. The beads were washed 3 X using a magnet that applies a high magnetic field and allows the separation of beads from the lysate. The sample buffer was added to the collected beads' samples and subjected to SDS-PAGE as described below.

3.2.7. Western blotting

After transfection, cells were lysed in lysis buffer containing 25 mM tris-HCl (pH=7.4; 15506017, ThermoFisher), 150 mM NaCl (S7653-250G, Sigma), 1.5 mM EDTA (AM9260G, ThermoFisher) and 1.0% IGEPAL CA-630 (85124, ThermoFisher). Lysates were electrophoresed through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) gels at 120V for 90 minutes and electrotransferred to nitrocellulose (0.2 µm, BioRad, Temecula, CA) at 400 mA for 60 minutes. Membranes were then blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 hrs. Blots were probed with primary antibodies mouse anti-cofilin (1:1000; ab42824, Abcam), rabbit anti-cofilin phospho S3 (ab12866, Abcam), rabbit anti-JNK1 (ab124956, Abcam), rabbit anti-JNK phosphoT183 (ab 47337, Abcam), rabbit anti-ERK (ab180163, Abcam) or rabbit anti-ERK phosphoY204 (ab 194770, Abcam) in TBS only for 4 hrs at room temperature on rocker. Blots were then washed 3 X 10 minutes with 1X Tris-buffered saline with 0.001% Tween (TBST; P1379, Sigma) and incubated in blocking buffer containing goat anti-mouse IgG DyLight 650 (1:10,000; ab97018, Abcam) or goat anti-rabbit IgG (1:10,000 ab216773, Abcam) for 1 hr in the dark at room temperature on rocker. Finally, the blots were washed 3 X 10 minutes and immunoreactive bands were visualized by near-infrared detection using a Li-COR Odyssey system.

3.2.8. Immunocytochemistry

Cells fixed in 4.0% paraformaldehyde were incubated for 10 min with PBS containing 1.5% pre-immune secondary serum (manufacturer info missing), 0.1% bovine

serum albumin (A215350G, Sigma, St. Louis, MO) and 0.1% Triton-X 100 (94343, BioChemika). Cells were then incubated with primary antibodies mouse anti-cofilin (1:1000; ab42824, Abcam), rabbit anti-cofilin phospho S3 (ab12866, Abcam), rabbit anti-JNK1 (ab124956, Abcam), rabbit anti-JNK phosphoT183 (ab 47337, Abcam), rabbit anti-ERK (ab180163, Abcam) or rabbit anti-ERK phosphoY204 (ab 194770, Abcam) for 2 hrs at 37 ºC in the incubator. Cells were washed 3 X 5 minutes on a rocker and then incubated with appropriate secondary antibodies conjugated with respective donkey anti-rabbit Alexa Fluor 488 (1:10,000 128-605-003, Jackson Immunolabs), goat anti-rabbit 555 (1:10,000, 128-605-0053, Jackson Immunolabs) and donkey anti-mouse 647(1:10,000, 128-605-120, Jackson immunolabs) for 1 hour at 37 ºC. Cells were then washed 3 X 5 minutes each and coverslips were mounted on slides using 3 µL mounting media containing DAPI (A-1000, Vectashield). Images were captured through 40X or 60X objectives using a Nikon A1 confocal microscope equipped with NIS element software.

3.2.9. Phalloidin Staining

Coverslips containing cells fixed in 4.0 % paraformaldehyde were incubated in 0.5 % Triton X-100 in PBS (94343, BioChemika) for 10 minutes to permeabilize. Cells were then washed 1 X 5 minutes with PBS at room temperature. After wash, 200 µl of 100 nM of phalloidin (Cat. # PHDH1-A) was added. The cells were incubated at room temperature in the dark for 30 min. Cells were then washed 3 X 5 minutes each and coverslips were mounted on slides 3 µL mounting media containing DAPI (A-1000, Vectashield). The area of interest, growth cones were identified for imaging. Images were captured through 40X

or 60X objectives using a Nikon A1 confocal microscope equipped with NIS element software.

3.2.10. Statistics

Data for all the experiments approximated a normal distribution and had similar variances across the experimental conditions, and hence, were analyzed for differences across groups using univariant analyses of variance (ANOVA) and Tukey posthoc test. The p value was set at $\alpha = 0.05$. All the statistical analyses were run in GraphPad Prism software, version 8.1.

3.3. Results

3.3.1. Overexpressing geranylgeranylatable Rac1 increased the actin filament content in the cytoskeleton of neuroblastoma cells.

Actin polymerization is guided by Rac1 [29], which in turn guides the formation of lamellipodia and filopodia at the extending axons [99]. Hence to analyze the effects of expressing non-geranylgeranylatable EmGFP-Rac1^{C189A} on actin, we assessed the total amount of actin in transfected whole cell lysates (WCL) of neuroblastoma (B35) by western blotting. There was no significant difference in the expression of the actin content in WCL of EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.1. A and B). Subcellular localization of the GTPases also plays an important role in regulating actin content in the particular compartment for the cell [78], so we next analyzed whether the location of Rac1 affects the actin content. For this, we separated transfected cells into cytosol, and cytoskeleton fractions and determined the actin content in each fractions. Overexpressing

non-geranylgeranylatable Rac1 did not have any effect on the actin content in the cytosol (see Figures 3.3.1. C and D) but increased the actin content in cytoskeleton fractions (see Figures 3.3.1. C and E).

3.3.2. Overexpressing geranylgeranylatable Rac1 increased the actin filament at the tips of extending axons (growth cones, lamellipodia, and filopodia) in the neuroblastoma cells.

External guidance cues bind to growth cone receptors and trigger signaling activities involving Rho GTPases [118]; hence we, then compared the actin in the growth cone of the transfected neuroblastoma cells. For this purpose, we counted the growth cones stained with phalloidin of the transfected cells. We found increased actin content in the growth cone of the overexpressed non-geranylgeranylatable Rac1 (see Figures 3.3.2. A and B).

3.3.3. Overexpressing geranylgeranylatable Rac1 increased the actin filament at the tips of extending axons in the rat cortical neurons.

Rho GTPases transduce extracellular stimuli into structural changes such as filopodia and lamellipodia, and it is generally accepted that Rac1 are positive regulators of neurite outgrowth [98], so we analyzed the growth cones of transfected rat cortical neurons. We found there was increased actin content in the over-expressed non-geranylgeranylated Rac1 (see Figures 3.3.3. A and B).

3.3.4. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} did not activate the actin-depolymerizing (cofilin) pathway.

Rac1 regulates cytoskeletal reorganization through cofilin cascade which is best known as a regulator of actin filament [68, 119]; hence, to determine the role of Rac1

involved in the actin de/polymerization of cofilin, we assessed the cofilin and phosphocofilin (p-cofilin) level in whole-cell lysates of transfected neuroblastoma cells by western blotting. The ratio of p-cofilin and cofilin was determined to estimate the level of active cofilin. There was no significant difference in WCL of EmGFP-Rac1 and EmGFP-Rac1^{C189A} transfected neuroblastoma cells (see Figures 3.3.4. A and B). Actin rods are increased in the cytosol of the Alzheimer's brain [62], hence we then investigated the total cofilin in cytosol and membrane/organelle fractions of the transfected neuroblastoma cells. There was no significant change in the expression of the cofilin in the cytosol (see Figures 3.3.4. C and D) or membrane fractions between EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.4. E and F).

3.3.5. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} did not activate the actin-depolymerizing factor (cofilin) pathway in the growth cones.

We then located the active cofilin by ICC analysis. The transfected neuroblastoma cells were immunostained with cofilin (AF555, red) and p-cofilin (AF647, cyan) and analyzed at 60X using Nikon A1 microscope. The ratio of p-Cofilin to cofilin was calculated to determine the total active cofilin. There was no significant difference in the active cofilin in EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.5. A and B).

3.3.6. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} activate JNK pathway in the cytosol.

Rac1 activates mitogen associated protein (MAP) kinases, like extracellular signal-regulated kinase (ERKl/2) or Jun N-terminal kinases (JNK), through direct activation of MAP or ERK kinase (MEKKI), which leads to actin nucleation [106, 120]. JNK is the key player in regulating actin dynamics and activating different signaling pathways in the cell

[38, 106, 115]. Hence to determine the role of prenylated Rac1 regulating MAP kinase effectors, i.e., JNK, we examined the JNK and phosphoJNK (p-JNK) level in whole-cell lysates of transfected neuroblastoma cells by western blotting. The ratio of p-JNK and JNK was determined to estimate the level of active JNK. There was no significant difference in WCL of EmGFP-Rac1 and EmGFP-Rac1^{C189A} transfected neuroblastoma cells for JNK (see Figures 3.3.6. A and B). We then examined the total JNK and total in cytosol and membrane/organelle fractions of the transfected neuroblastoma cells. There was no significant change in the expression of the JNK in membrane fractions of EmGFP-Rac1 and EmGFP-Rac1^{C189A} cells (see Figures 3.3.6. E and F), but there was a significant difference in the expression of total JNK in the cytosolic fractions of the EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.6. C and D).

3.3.7. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} activate JNK pathway in the growth cones.

We then examined the total JNK by ICC analysis. The transfected neuroblastoma cells were immunostained with JNK (AF555, red) and p-JNK (AF647, cyano) and analyzed at 60X using the Nikon A1 microscope (see Figures 3.3.7. A and B). There was a significant difference in the active JNK between EmGFP-Rac1 and EmGFP-Rac1^{C189A}.

3.3.8. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} did not affect ERK pathway.

Rac1 plays an important role in the activation of the mitogen associated protein (MAP) kinases, like extracellular signal-regulated kinase (ERKl/2) through direct activation of MAP or ERK kinase (MEKKI), which leads to actin nucleation [83]. Hence to analyze the effect of Rac1 on ERK activation, we examined the ERK and phosphoERK

(p-ERK) level in whole-cell lysates of transfected neuroblastoma cells by western blotting. The ratio of p-ERK and ERK was determined to estimate the level of active ERK. There was no significant difference in WCL of EmGFP-Rac1 and EmGFP-Rac1^{C189A} transfected neuroblastoma cells for ERK (see Figures 3.3.8. A and B). We then examined the total ERK in the cytosol and membrane/organelle fractions of the transfected neuroblastoma cells. There was no significant change in the expression of the ERK in cytosolic fractions of EmGFP-Rac1 and EmGFP-Rac1^{C189A} cells (see Figures 3.3.8. C and D), but there was a significant difference in the expression of total JNK in the membrane fractions of the EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.8. E and F).

3.3.9. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} did not affect the ERK pathway in the growth cones.

We checked the total ERK in the growth cones of the transfected neuroblastoma cells by ICC analysis. The transfected neuroblastoma cells were immunostained with ERK (AF555, red) and p-ERK (AF647, cyan) and analyzed at 60X using the Nikon A1 microscope. (see Figures 3.3.9. A and B). There was a significant difference in the active ERK between EmGFP-Rac1 and EmGFP-Rac1^{C189A}.

3.3.10. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} increased WAVE and ARP2/3 association in the WCL.

Rac1 promote actin nucleation through activation of IRSp53, which activates the Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous protein (WAVE) allowing it to interact with the actin-related protein (ARP2/3) complex to nucleate actin side chains and produce branched actin meshwork's [121-123], to see how prenylated Rac1 contribute to the association of Arp2/3 via the WASP, we performed the

co-IP on transfected neuroblastoma cells. The WAVE was used to pull down ARP in WCL and there was no significant difference in the WAVE and ARP association in EmGFP-Rac1 and EmGFP-Rac1^{C189A} (see Figures 3.3.10. A and B) when analyzed by western blotting. Cytosolic ARP has been found to be the major actin nucleator enhancing the sensory neuronal growth in the larva of the drosophila [42]; hence, we also checked the effect of association of WAVE and ARP in the different fractions of transfected cells by co-IP. Overexpressing EmGFP-Rac1^{C189A} did not any effect on the association of the WAVE and ARP in the cytosol (see Figures 3.3.10. C and D) or in the membrane fractions (see Figures 3.3.10. E and F).

3.3.11. Overexpressing non-geranylgeranylatable EmGFP-Rac1^{C189A} did not have any effect on WAVE and ARP2/3 association in the growth cones.

We also analyzed the effect of overexpressing non-geranylgeranylated Rac1 in the growth cones of the transfected cells and did not find any significant difference in the association of the WAVE and ARP. The co-localization of WAVE (AF555, red) and ARP (AF647, cyan) was performed to calculate the Pearson's coefficient (see Figures 3.3.11. A and B).

3.3.12. Overexpressing non-geranylgeranylated EmGFP-Rac1^{C189A}allows the cross-talk between JNK and ARP2/3.

To determine the cross-talk between the different effectors, we performed the inhibitor studies. By inhibiting the ERK, there was no significant difference in the JNK (see Figure 3.3.12 B) but decreased association of WAVE and ARP (see Figure 3.3.12. C) in the mutant transfected cells. By inhibiting JNK, the active ERK decreased (Figure 3.3.12. E) by overexpressing wild-type Rac1 and increased association of WAVE and ARP

by overexpressing wild-type Rac1 (see Figure 3.3.12. F). Similarly, by inhibiting ARP, there was no change in the active ERK by overexpressing wild-type or mutant Rac1 (see Figure 3.3.12. H) but the WAVE and ARP association increased by overexpressing wild-0type Rac1 (see Figure 3.3.12. I).

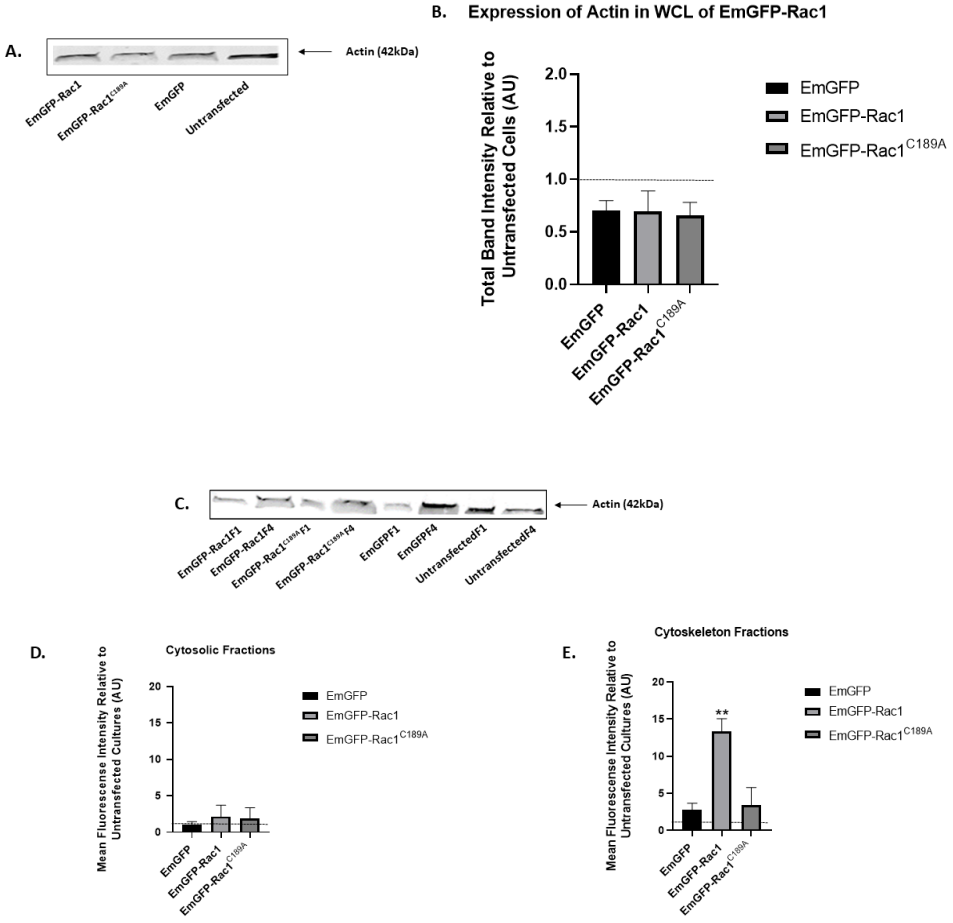


Figure 3.3.1.: Overexpressing geranylgeranylatable Rac1 (EmGFP-Rac1) increased the actin filament content in the cytoskeleton of Neuroblastoma (B35): cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable Rac1 for 48 h at 37 °C. (A) Cells were collected and subjected to western blotting and probed for actin. (B) Analysis of actin using the densitometry method showed there was no significant difference in the actin content in whole-cell lysates by overexpressing geranylgeranylatable Rac1. (C) Cells were fractionated into cytosol and cytoskeleton fractions and subjected to western blotting for actin. (D) Analysis of actin showed there was no significant difference in the actin content in the cytosol by overexpressing geranylgeranylatable Rac1. (E) Analysis of actin in the cytoskeleton fractions showed a significant difference. Data in B, D and E are expressed as means ± s.e.m. and "* *" in (E) indicates significant difference from EmGFP controls at $p < 0.05$ (ANOVA and Tukey post hoc).

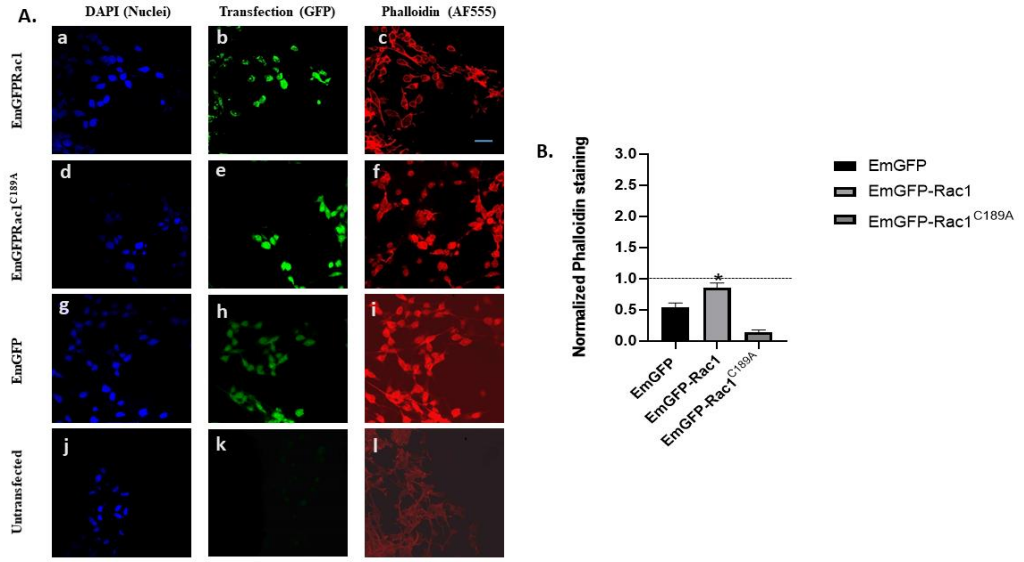


Figure 3.3.2. Overexpressing geranylgeranylatable Rac1 (EmGFP-Rac1) increased the actin filament at the tips of extending axons (growth cones, lamellipodia, and filopodia) in the neuroblastoma cells. (A) From growth cones identified from representative phase-contrast images, (a, d, g and j) represent the nuclei stained for Dapi, (b, e, h and K) represent transfected cells and (c, f, I and l) represent average fluorescence intensities for Alexa fluor 555 conjugated phalloidin labeled actin filaments and were determined from corresponding images captured through an A1 confocal microscope. (B) Data in (B) are expressed as means ± s.e.m. ranges for 3 replicate cultures in each condition, normalized to the overall mean intensity for untransfected control in each region of interest. * indicates significant difference from controls (EmGFP condition in each experiment) and * indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). Scale bar in the image (c) indicates 20 μm and is

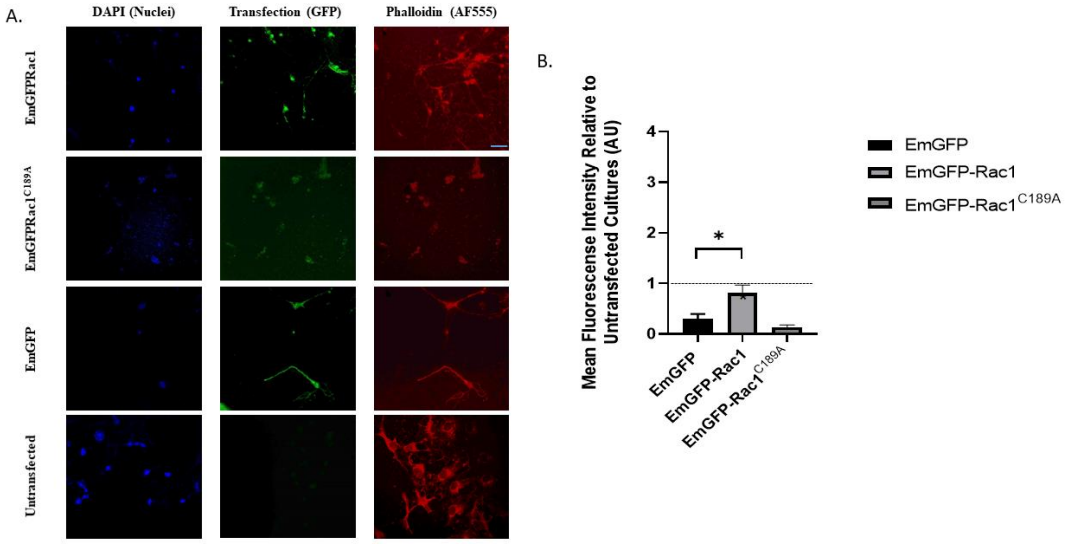


Figure 3.3.3.: Overexpressing geranylgeranylatable Rac1 (EmGFP-Rac1) increased the actin filament content in the growth cones of rat cortical neurons. (A) Growth cones were identified from representative phase-contrast images, (a, d, g and j) represent the nuclei stained for dapi, (b, e, h and K) represent transfected cells and (c, f, I and l) average fluorescence intensities for Alexa fluor 555 conjugated phalloidin labeled actin filaments were determined from corresponding images captured through an A1 confocal microscope. (B) Data in (B) are expressed as means ± s.e.m. ranges for 3 replicate cultures in each condition, normalized to the overall mean intensity for untransfected control in each region of interest. * indicates significant difference from controls (EmGFP condition in each experiment) and * indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). Scale bar in the image (c) indicates 20 µm and is valid for all the images.

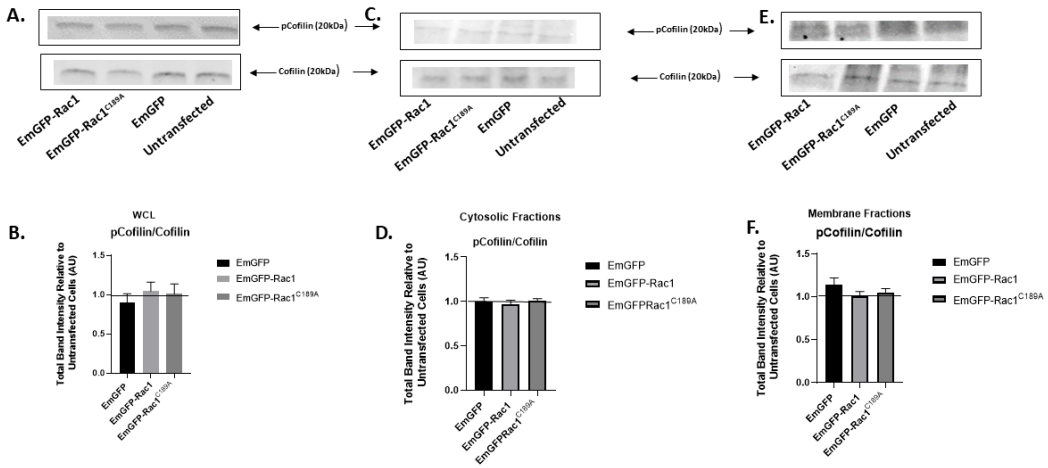


Figure 3.3.4.: Overexpressing non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) did not activate the actin-polymerizing (cofilin) pathway. B35 cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable Rac1 for 48 h at 37 °C. (A) Cells were collected and subjected to western blotting and probed for pcofilin (on top) and cofilin (on bottom) in the sample. (B) Analysis of pcofilin/cofilin showed no significant difference in the active cofilin in whole-cell lysates by overexpressing geranylgeranylatable Rac1. Also, cells were fractionated into membrane/organelle and cytosol fractions and subjected to western blotting for pcofilin and cofilin. (C) Representative blot of pcofilin (on top) and Cofilin (on bottom) (D). Analysis of pcofilin/cofilin showed there was no significant difference in the active cofilin in the cytosol by overexpressing geranylgeranylatable Rac1. (E) Representative blot of pcofilin (on top) and Cofilin (on bottom) (D) Analysis of pcofilin/cofilin showed there was no significant difference in the active cofilin in the membrane by overexpressing geranylgeranylatable Rac1. (D and F) Analysis of pcofilin/cofilin showed there was no difference in the active cofilin in the cytosol or membrane fractions. Data in B, D and F are expressed as means ± s.e.m.

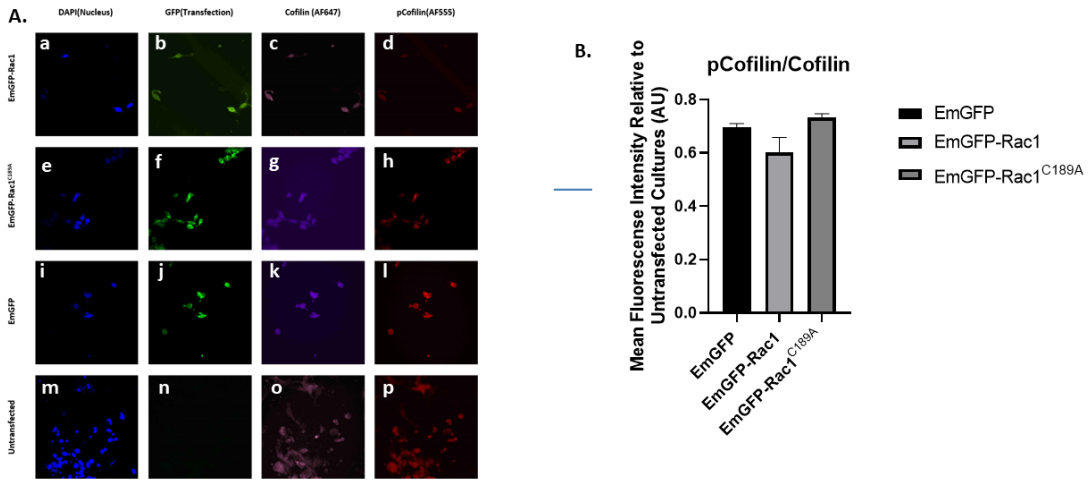


Figure 3.3.5.: Overexpressing non-geranylgeranylatable Rac1(EmGFP-Rac1^{C189A}) did not activate the actin depolymerizing (cofilin) pathway in the growth cones: B35 cells transfected with EmGFP-Rac1 or EmGFP-Rac1^{C189A} or EmGFP didn't show any significant difference for the active cofilin of the growth cones. The area of interest (growth cone) was identified and intensity for cofilin (cyan) and pcofilin (red) was used to find the ratio of pcofilin and cofilin. (A) (a, e, I and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k, and o) represent the transfected cells immunoblotted for cofilin and (d, h, I and p) represent the transfected cells immunoblotted for pcofilin. (B) Analysis for pcofilin/cofilin represents there was no significant difference in the active cofilin by overexpressing EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Data in B are expressed as means ± s.e.m. The scale bar in an image (d) indicates 20 µm and is valid for all the images.

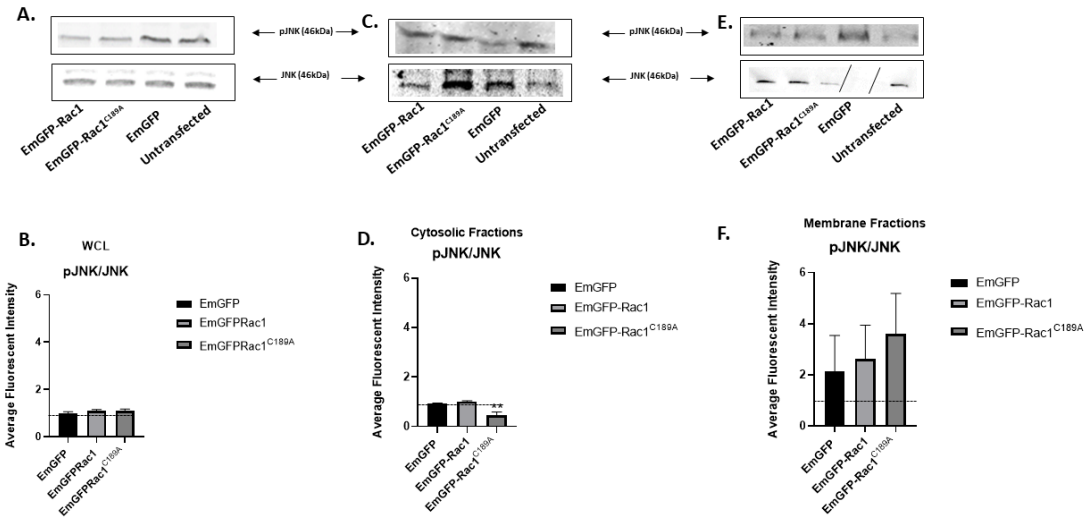


Figure 3.3.6: Overexpressing non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) activate JNK pathway in the cytosol. (A) Cells from the transfected cells were subjected to western blot and probed for pJNK (on the top) and JNK (at the bottom). (C)The cytosolic fractions were also probed for pJNK (on the top) and JNK (at the bottom) and (E) The membrane/organelle fractions were probed for pJNK (on the top) and JNK (at the bottom) (B.) Analysis of pJNK/JNK showed there was no significant difference in the active JNK in between EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} in the WCL. (D) Analysis of pJNK/JNK in the cytosol showed the significant difference between EmGFP and EmGFP-Rac1^{C189A} and "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). (F) Analysis of pJNK/JNK in the membrane fractions did not show any significant difference between any conditions. Data in (B.), (D) and (F) are expressed as means ± s.e.m.

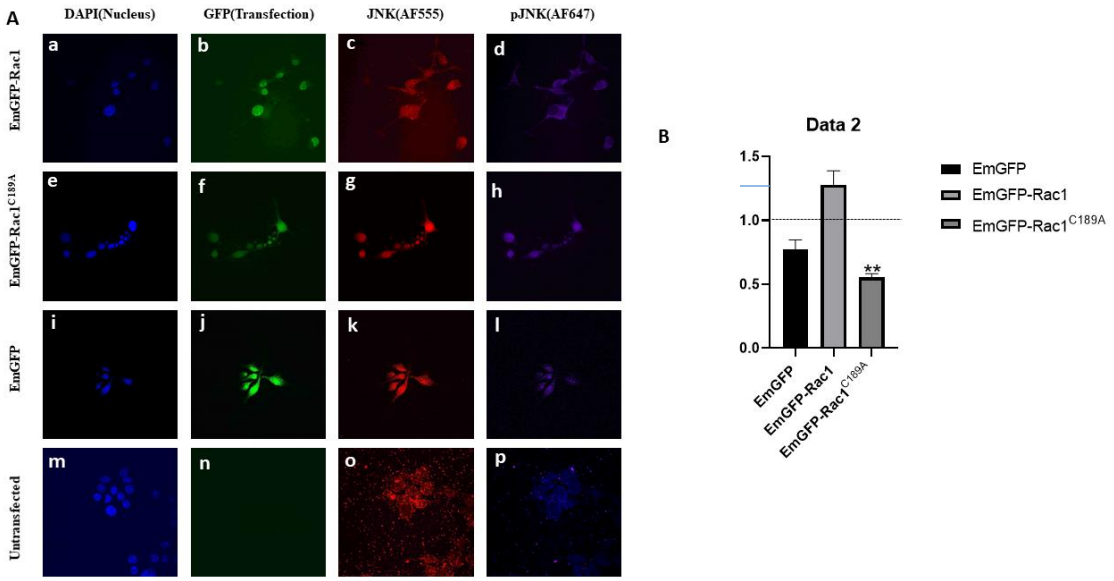


Figure 3.3.7: Overexpressing non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) activates the JNK pathway in the growth cones. The area of interest (growth cone) was identified and intensity for JNK (AF555, red) and pJNK (AF647, cyan) was used to find the ratio of pJNK and JNK. A. (a, e, i and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k and o) represent the transfected cells immunoblotted for JNK and (d, h, I and p) represent the transfected cells immunoblotted for pJNK. B. Analysis for pJNK/JNK represents there was a significant difference in the active JNK by overexpressing EmGFP-Rac1^{C189A} when compared with the control, i.e., EmGFP. Data in B are expressed as means ± s.e.m and "**" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). The scale bar in the image (d) indicates 20 μm and is valid for all the images.

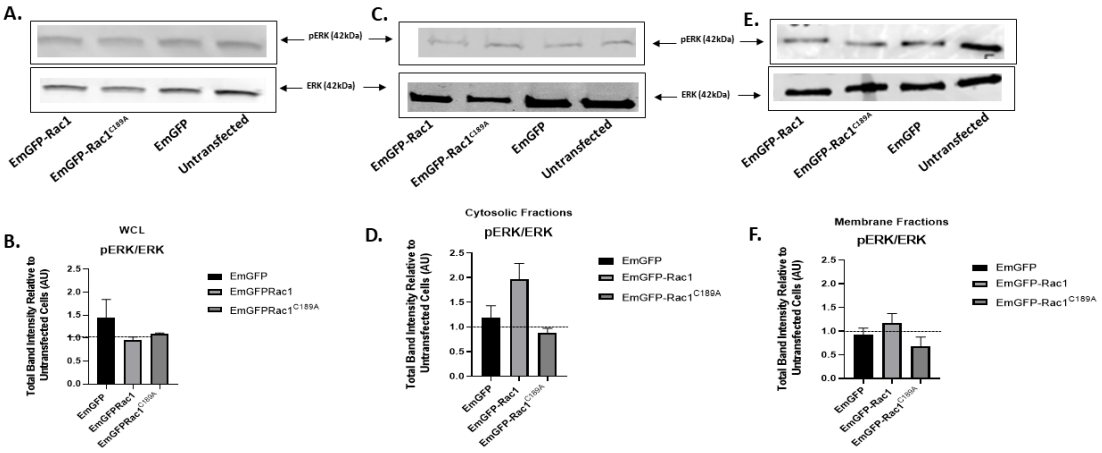


Figure 3.3.8.: Overexpressing geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) did not affect the ERK pathway. (A) Cells transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} were collected and subjected to western blotting and probed for pERK (on top) and ERK (on bottom). (B) Analysis of pERK/ERK showed no significant difference in the active ERK in whole-cell lysates by overexpressing geranylgeranylatable Rac1. Also, cells were fractionated into membrane/organelle and cytosol fractions and subjected to western blotting for pERK and ERK. (C) Representative blot of pERK (on top) and ERK (on bottom) (D) Analysis of pERK/ERK showed there was no significant difference in the active ERK in the cytosol by overexpressing geranylgeranylatable Rac1. (E) Representative blot of pERK (on top) and ERK (on bottom) (D) Analysis of pERK/ERK showed there was no significant difference in the active ERK in the membrane by overexpressing geranylgeranylatable Rac1. (D and F) Analysis of pERK/ERK showed there was no difference in the active ERK in the cytosol or membrane fractions. Data in B, D, and F are expressed as means ± s.e.m.

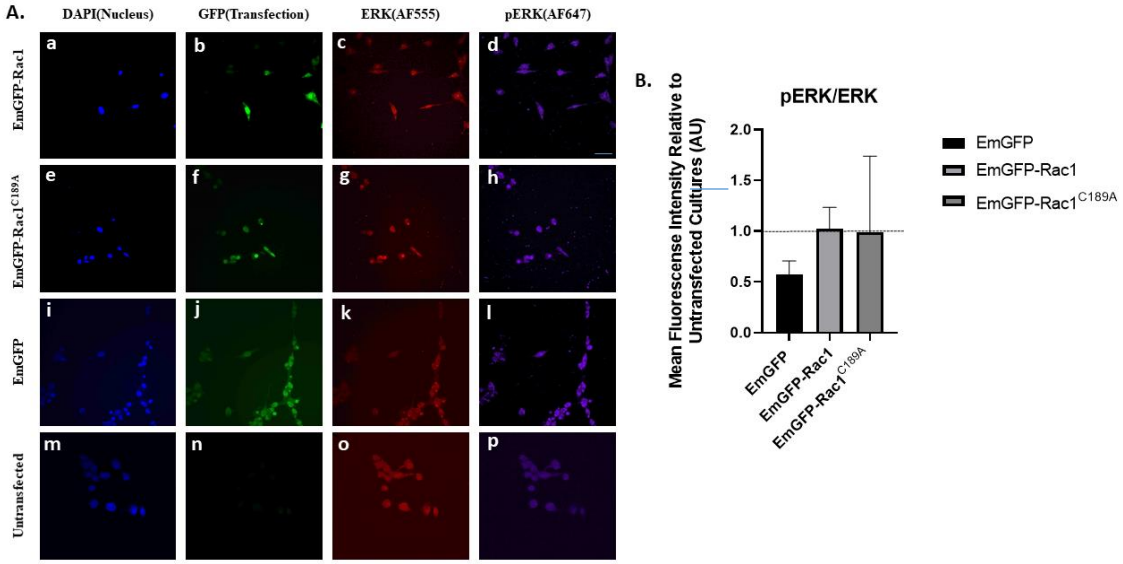


Figure 3.3.9.: Overexpressing geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) did not affect the ERK pathway in the growth cones. B35 cells transfected with EmGFP-Rac1 or EmGFP-Rac1C189A or EmGFP didn't show any significant difference for the active ERK of the growth cones. The area of interest (growth cone) was identified and intensity for ERK (Af555, red) and pERK (AF647, cyan) was used to find the ratio of pERK and ERK. A. (a, e, I and m) represent the nuclei, (b, f, j, and n) represent transfected cells, (c, g, k and o) represent the transfected cells immunoblotted for ERK and (d, h, I and p) represent the transfected cells immunoblotted for pERK. B. Analysis of pERK/ERK showed there was no significant difference in the active ERK by overexpressing EmGFP, EmGFP-Rac1 or EmGFP-Rac1C189A. Data in B are expressed as means ± s.e.m. The scale bar in the image (d) indicates 20 μm and is valid for all the images.

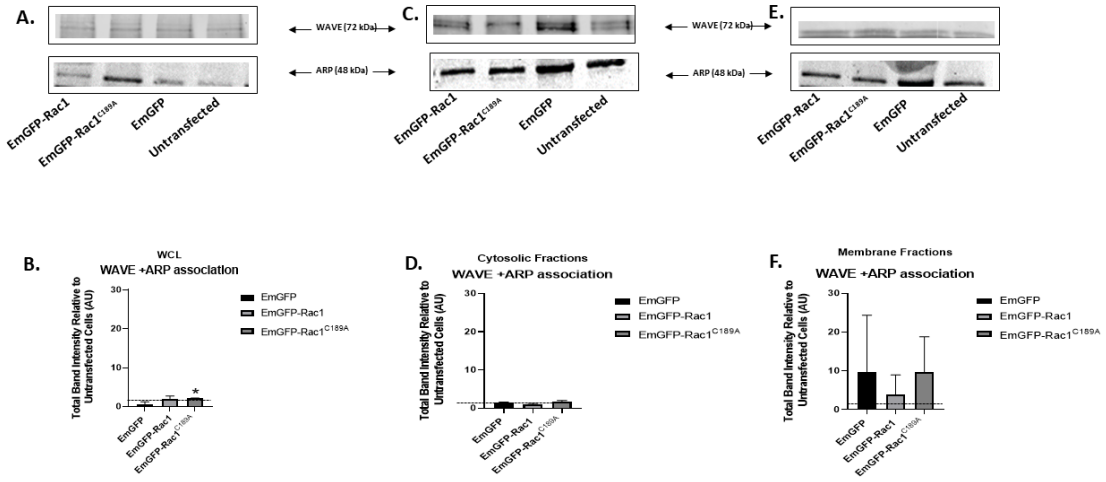


Figure 3.3.10.: Overexpressing non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) increased WAVE and ARP association in the WCL. B35 cells transfected with EmGFP-Rac1 or EmGFP-Rac1^{C189A} or EmGFP and cells were extracted for co-immunoprecipitation. (A) Immunoprecipitation for ARP was performed in the WCL (at the bottom). Western blots on the eluate for WAVE in the eluate were consistent (on the top). (B) Analysis of the association of WAVE and ARP showed significant difference in the whole-cell lysates from the overexpression of EmGFP-Rac1^{C189A}. "*" indicates significant difference from EmGFP at $p < 0.05$ (Tukey post hoc). (C) Immunoprecipitation for ARP was performed in the cytosolic fraction (at the bottom) and western blots on the eluate for WAVE in the eluate were consistent (on the top). (E) Immunoprecipitation for ARP was performed in the membrane fraction (at the bottom) and western blots on the eluate for WAVE in the eluate were consistent (on the top). (D and F) Analysis of the association of WAVE and ARP in the cytosolic fractions and membrane fractions showed there was no significant difference by overexpressing EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Data in B D and F is expressed as means ± s.e.m

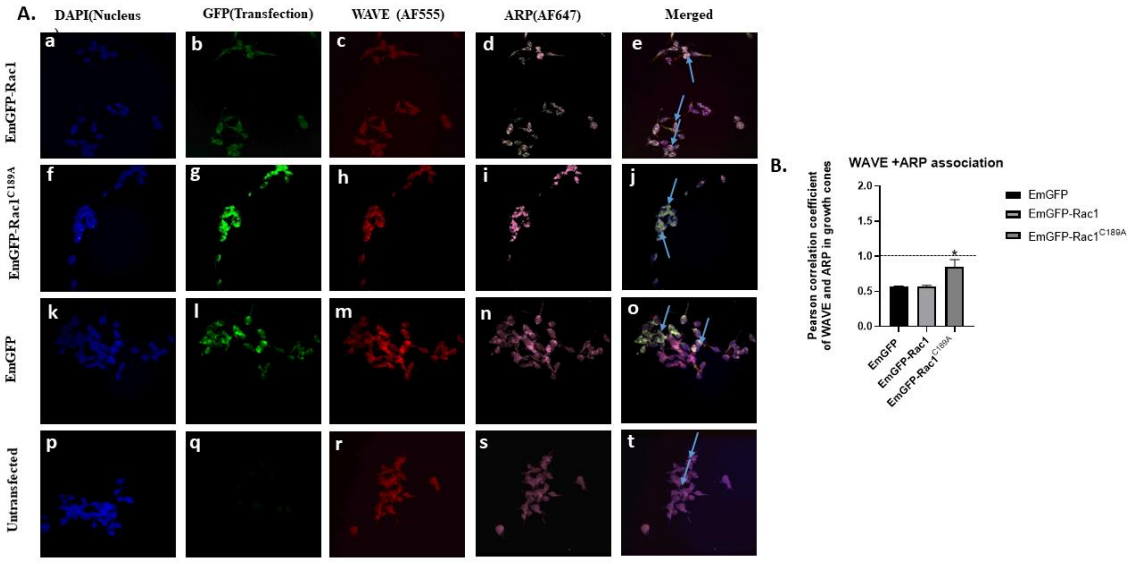
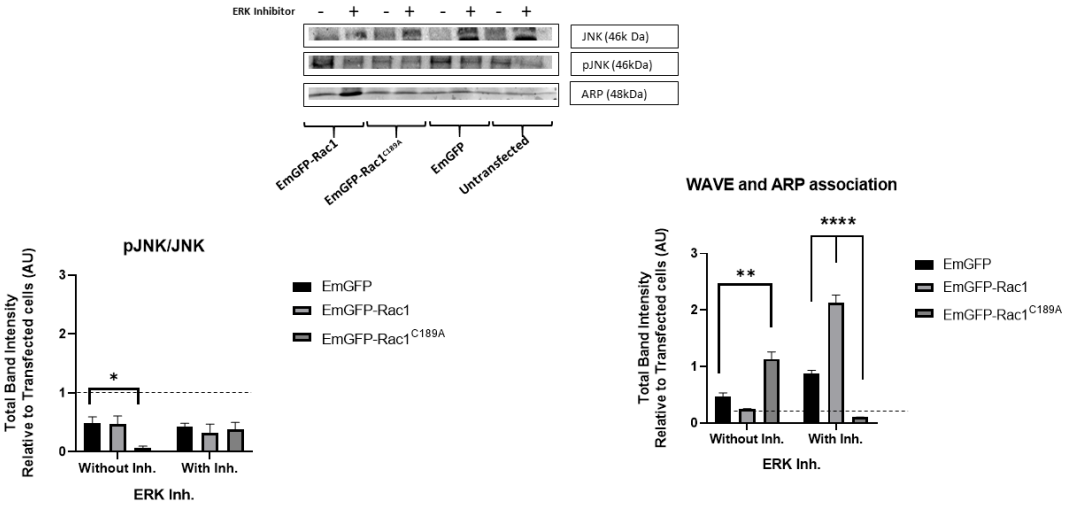
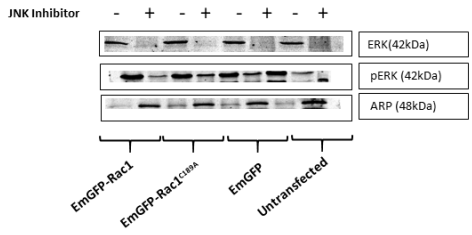


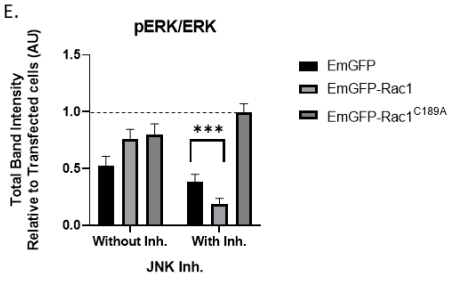
Figure 3.3.11: Overexpressing non-geranylgeranylatable Rac1 (EmGFP-Rac1^{C189A}) increased the WAVE and ARP association in the growth cones. B35 cells transfected with EmGFP-Rac1 or EmGFP-Rac1^{C189A} or EmGFP showed any significant difference for the WAVE and ARP association in the growth cones. The area of interest (growth cone) was identified and the co-localization of WAVE (AF555, red) and ARP (AF647, cyan) was performed using A1 confocal software and Pearson coefficient was calculated. A. (a, f, k and p) represent the nuclei, (b, g, l and q) represent transfected cells, (c, h, m, and r) represent the transfected cells immunoblotted for WAVE, (d, I, n and s) represent the transfected cells immunoblotted for ARP and (e, j, o and t) represent the merged images. B. Analysis of WAVE and ARP association showed there was a significant difference in the association by overexpressing EmGFP-Rac1^{C189A}. Data in B are expressed as means ± s.e.m. The scale bar in image (e) indicates 20 μm and is valid for all the images. The arrows refer to the co-localized section.


ERK Inhibitor - + - + - + - +
JNK (46k Da)
pJNK (46kDa)
ARP (48kDa)
EmGFP-Rac1
EmGFP-Rac1C189A
EmGFP
Untransfected
pJNK/JNK
Total Band Intensity Relative to Transfected cells (AU)
EmGFP
EmGFP-Rac1
EmGFP-Rac1C189A
Without Inh.
With Inh.
ERK Inh.
WAVE and ARP association
Total Band Intensity Relative to Transfected cells (AU)
EmGFP
EmGFP-Rac1
EmGFP-Rac1C189A
Without Inh.
With Inh.
ERK Inh.

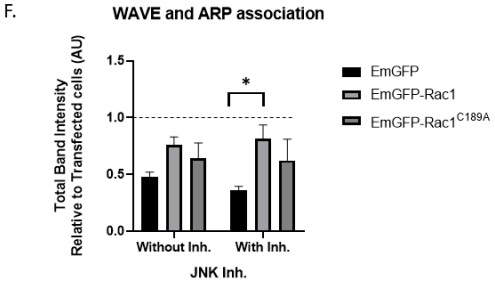
D.


JNK Inhibitor - + - + - + - +
ERK(42kDa)
pERK (42kDa)
ARP (48kDa)
EmGFP-Rac1
EmGFP-Rac1C189A
EmGFP
Untransfected

E.


pERK/ERK
Total Band Intensity Relative to Transfected cells (AU)
EmGFP
EmGFP-Rac1
EmGFP-Rac1C189A
Without Inh.
With Inh.
JNK Inh.

F.


WAVE and ARP association
Total Band Intensity Relative to Transfected cells (AU)
EmGFP
EmGFP-Rac1
EmGFP-Rac1C189A
Without Inh.
With Inh.
JNK Inh.

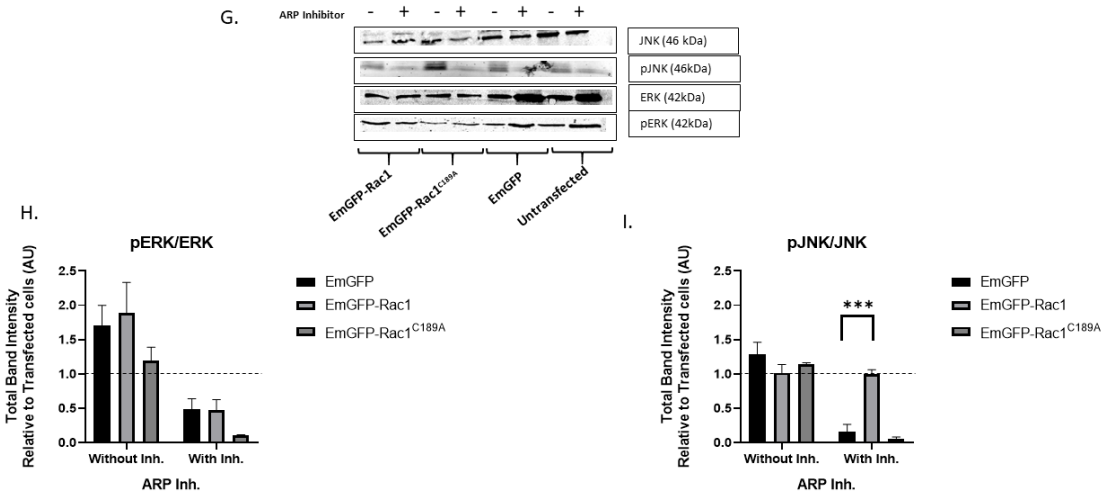


Figure 3.3.12. Overexpressing non-geranylgeranylated EmGFP-Rac1^{C189} allows the cross-talk between MAPK and ARP. B35 cells were transfected with EmGFP, geranylgeranylatable or non-geranylgeranylatable Rac1 for 48 h at 37 °C and then treated with 0.125µM of CK-869, 40µM of JNK inhibitor, 25µM of ERK inhibitor-I 1 hrs for 4 hrs respectively. (A) Cells treated with ERK inhibitor were collected and probed for JNK (on the top), pJNK (in middle) and ARP (at the bottom). (D) Cells treated with JNK inhibitor were collected and probed for ERK (on the top), pERK (in middle) and ARP (at the bottom). (E) Cells treated with ARP inhibitor were collected and probed for JNK (on the top), pJNK (2nd from the top), ERK (2nd from the bottom) and pERK (at the bottom). The analysis of ERK inhibition showed no change in the active JNK (B.), increased association of WAVE and ARP for wild type Rac1 (D). JNK inhibition decreased ERK by overexpressing wild type Rac1 (E) and increased association of WAVE and ARP by overexpressing wild type Rac1 (F). ARP inhibition did not change the active ERK (H) by overexpressing wild type or mutant Rac1 (H), and increased active JNK by overexpressing wild type Rac1 (I). + represent cells treated with inhibitor and – represent cells were not treated with inhibitor. The analysis showed that the ARP inhibition affected the JNK pathway and vice-versa.

3.4. Discussion

The focus of the present study was to determine whether over-expressing non-geranylgeranylatable Rac1 decreases the actin filament in the cytosol and cytoskeleton. Another objective was to determine whether over-expressing non-geranylgeranylatable Rac1 activates certain signaling pathways in distinct cellular compartments. We found that expressing a non-geranylgeranylatable Rac1 mutant (EmGFP-Rac1^{C189A}) in neuroblastoma cells increased the actin content in the cytoskeleton, compared to cells expressing EmGFP or wild-type Rac1 (EmGFP-Rac1). Expression of actin filament in the cytoskeleton also correlated with a pool of active Rac1 in the cytoskeleton, whereas Rac1 is usually associated with the membrane. Similar findings were observed in rat cortical neurons (see Figure 3.3.3.) supporting the observation in the primary cell line. Also, overexpressing non- geranylgeranylatable increased the active JNK in the cytosol and overexpressing non-geranylgeranylatable Rac1 supported WAVE and ARP association in the WCL indicating that these geranylgeranylatable and non- geranylgeranylatable pool of Rac1 might be operating via JNK and ARP pathways. Our results also indicate that overexpressing Rac1 works by JNK pathway by increasing the active JNK in the cytosol. This concurs with our previous reports observing an active pool of Rac1 in the cytosol [16] as Rac1 needs to be active to activate JNK. Similarly, expressing non-geranylgeranylatable Rac1, allowed WAVE and ARP in the WCL of the non-geranylgeranylatable Rac1, which indicates the involvement of WAVE and ARP pathway

Rho GTPases are the molecular switches that remain active by GTP binding and are inactive when GDP bound [12, 44]. This GTP binding is achieved by the guanine

nucleotide exchange factor (GEF) [104]. Hence, there is a possibility that this activated Rac1 is due to the undefined GEF present in the cytosol. There was no significant difference in the association of WAVE and ARP in the cytosol and membrane fractions suggesting that the significant difference present in the WCL might be coming from the nuclear or cytoskeleton fractions. The mechanisms through which cytosol-localized Rac1 can be loaded with GTP and activated are unknown[12]. Our data suggest that the WAVE/ARP and JNK pathways might be involved in regulating the actin polymerization and hence the axon guidance.

The inhibitor studies demonstrated that ARP inhibition decreased the total JNK indicating there is cross-talking going in between these pathways. The association of WAVE with JNK has been found to control molecular complexes that include cytoskeletal and mitogenic events [41]. Together these data suggest that cross-talk is occurring between these two pathways contributing to the formation of networks that lead to the arrangement of cytoskeleton. The JNK signaling pathway is critical for the induction of neuronal apoptosis [80] whereas inhibiting JNK facilitates and protects against cognitive decline in a mouse model of AD [124]. Also, autophagy of dopaminergic neurons happens through JNK signaling [125]. Hence, our data inform that geranylgeranylated proteins is required for polymerization that finding the effective target inhibiting these polymerization could help ameliorate complications in other neurodegenerative disorders.

As a pool of the geranylgeranylatable form of Rac1 has been reported in the cytosol, our data concurs with this finding and contributes findings that overexpressing mutant Rac1 that is non-geranylgeranylatable decreases the actin content via WAVE/ARP and

JNK pathways that will provide an additional support to characterizes the molecule that will act on these pathways and hence in turn guide the axon growth and guidance.

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CHAPTER IV

DISCUSSION

Abnormal actin in the extending axon cues is one of the identifying features of many neurological disorders that is largely influenced by RhoA and Rac1 [7, 8, 20]. The importance of axon guidance cues is illustrated by reports that nucleotide polymorphisms (SNPs) and mutations in the genes encoding these proteins occur in patients with neurodegenerative diseases such as PD, AD, Mirror movement disorder (MMD), dyslexia, Kallmann's syndrome, Hirschsprung's disease, autism spectrum disorders (ASD), epilepsy, and Amyotrophic lateral sclerosis[126, 127].

The main focus of this dissertation was to determine how inhibiting prenylation affects the actin content and subcellular activation of effectors downstream of RhoA and Rac1 in B35 neuroblastoma cells and, in certain experiments, rat cortical neurons. The main purpose of focusing on the Rho guanosine triphosphatases (GTPases) RhoA and Rac1 is that these small GTPases are implicated in many neurological conditions, including AD, PD, schizophrenia, and the ASD [4, 53, 57, 62, 66]. Despite modern efforts to identify the effect of various molecules involved in the mevalonic pathway on these neurological conditions, it remains unclear how inhibiting prenylation affects the subcellular localization and actin content [71, 128, 129]. To determine how actin content is modulated by the Rho GTPases and their pathways, specifically within distinct compartments of the neuron, we tested the hypothesis by the following experiments:

1. Overexpressing geranylgeranylatable RhoA and Rac1 would decrease the actin content in the growth cones, lamellipodia, and filopodia of the extending neurons;
2. Overexpressing geranylgeranylatable RhoA and Rac1 would activate actin-depolymerizing (cofilin) or deactivate actin polymerizing (ERK, JNK) serine/threonine kinases; and
3. Overexpressing geranylgeranylatable RhoA and Rac1 lead to decreased actin nucleation via serine/threonine pathway.

We tested these hypotheses in two neural model systems, B35 neuroblastoma cells and rat cortical neurons. B35 cells mimic neurons when left in Dulbecco's modified eagle medium (DMEM) with the withdrawal of serum and no addition of any exogenous growth factors. Hence, they provide an optimal screening system with high transfection efficacy to test our hypotheses. The average transfection efficiency for B35 neurons is 75-80%, while the transfection efficacy in primary neuron cultures is typically 10-20%. Utilization of the immortal cell line helped to optimize the transfection technique before testing our hypotheses in primary cortical neurons; thus initial data in B35 neurons guided the work in the rat cortical neurons. Compared to the B35 cell line, rat cortical neurons are more physiologically similar to human neurons, so testing the effects observed in the B35 cells in primary neuron cultures as well was critical to provide a translatable understanding of neuronal guidance in human neurons.

In the first round of experiments, overexpressing wild-type RhoA (EmGFP-RhoA) or and mutant RhoA (EmGFP-RhoA^{C190A}) did not change the actin content in cytosol or in cytoskeleton fractions. On the other hand, overexpressing Rac1 (or EmGFP-Rac1^{C189A})

decreased the actin content in the cytoskeleton of the cell. Our data indicates that WAVE/ARP2/3, JNK, and ERK are involved in signaling initiated by RhoA and Rac1 that leads to actin polymerization. Prenylation is important as it translocate RhoA and Rac1 to membrane to activate WAVE/ARP2/3, ERK, and JNK. Also, the differential localization provides evidence that these Rho GTPases are also present and active in other compartments than membrane[16]. Interestingly, these novel signaling events initiated by GTPases are also functional in the cytosol, occurring not only when the GTPases are attached to the membrane. This concurs with previous findings [16], while in contrast with traditional hypotheses of how GTPases function [7, 9]. Overexpressing wild-type RhoA or mutant RhoA did not change actin content in the cytosol, cytoskeleton, (see Chapter II, Figure 2.3.10) or the growth cones, lamellipodia, or filopodia of the extending neurons (see Chapter II, Figures 2.3.11. and 2.3.12.). This finding was confirmed in rat cortical neurons. RhoA is implicated in the assembly of stress fibers and focal adhesions at growing end which is a continuous process [5, 59, 130]. It is possible that we did not detect changes in the actin filament content if tread milling of actin is occurring at equal rates and the rate of actin polymerization equals the rate of actin depolymerization. Also, the actin content in the cytosol and cytoskeleton did not change by overexpressing wild-type of mutant RhoA. These data suggest that there were no change in WCL, cytosolic, or cytoskeleton, but we did not look into the membrane or nuclear fractions. To see how overexpressing these Rho GTPases affects the actin content in nuclear and membrane fractions will be a great future direction to shed light on the differential localization of these Rho GTPases. Overall, it can

be postulated based on the reported data that regulation of actin is not dependent on the prenylatable form of RhoA.

On the other hand, we did see an increase in the actin filament content in the cytoskeleton by overexpressing Rac1 in the neuroblastoma cells. This finding was observed (see Chapter III, Figure 3.3.1.) and also in the growth cones, filopodia and lamellipodia of the neuroblastoma cells and further confirmed in the rat cortical neurons (see Chapter III, Figures 3.3.2 and 2.3.3.). This indicates that the prenylatable form of Rac1 is involved in the regulation of actin content and goes along with the known facts about how Rac1 plays a pivotal role in the rearrangement of actin filament in the growing neurons [6, 17, 130]. The similar results from the rat cortical neurons strengthen the fact that these proteins are functioning in a similar way be it in neuroblastoma cells or the primary cell lines as in the case of rat cortical neurons. A limitation of using B35 neurons is that they are continuously dividing and are an abnormal cell type and thus can alter the pharmacological and neurological responses. Hence, the results obtained from primary culture provided a firm explanation of the process. Overall, this provides a better insight into the involvement of these Rho GTPases in regulating actin content in two different conditions.

Visualizing the cellular localization of actin filament in the growth cones, lamellipodia, and filopodia of the growing neurons by ICC helped to clarify the growth cones guidance. Further, immunoblotting for actin in cytosol and cytoskeleton provided the information on the differential localization of these Rho GTPases. This also supports

the previous work from our lab that an active pool of Rac1 is present is other compartments rather than just in the membrane.

The signal transduction pathways has been monitored concerning many diseases, including neurological disorders. The second set of experiments was designed to test the influence of prenylation on actin nucleator (ARP), MAPK effectors (JNK, ERK) and actin-depolymerizing factors (ADF, cofilin) pathways. All these effectors are downstream of the Rho GTPases and are involved in the actin de/polymerization at the end of growing neurons [7, 8, 15]. These pathways are also implicated in many neurological disorders like AD, ALS, PD, and spinal cord injury [6, 36, 54, 97, 129, 131]. The cytosolic ADF/cofilin has been reported in high levels in the AD brain [62], whereas phosphorylated JNK3 and activation of the canonical c-Jun apoptotic pathway modulate signaling that appear to increase motor neuron viability [132]. Interestingly, the cessation of motor neuron degeneration was achieved by initiating a cofilin pathway downstream of LIMK that induces motor neuron degeneration [132]. These reports indicate a role of map kinase member (MAP4K4), initiates a cascades allowing motor neuron degeneration. This can also serve as a druggable target for ALS therapeutics [132]. ERK1/2 subcellular localization is modulated by wild type α-syn in a phospholipase D (PLD1) dependent manner [133], and downregulation of PDL regulates the early onset of α-syn triggered neurodegeneration implicating the importance of subcellular localization and functionality of these effectors.

Our data suggest that geranylgeranylatable RhoA works by ERK and WAVE/ARP pathways. The total ERK was increased by overexpressing mutant RhoA (EmGFP-

RhoA^{C190A}) in the cytosol and ARP was found to be increased in the membrane fraction by overexpressing mutant RhoA (EmGFP-RhoA^{C190A}). The elevated ERK in the cytosol indicate that the location of ERK is important to manage the actin dynamics manages the functionality [133]. The activation of ERK in the cytosol by overexpressing RhoA also supports the dependency of ERK activation on prenylation. There is ongoing debate whether phosphorylation of ERK is beneficial or harmful. Whether elevated ERK phosphorylation is detrimental or beneficial is debatable, but the phosphorylation of ERK achieved by exogenous growth factors, estrogen favors neuroprotection [113]. Our data and that of others suggest that if we may be able to pharmacologically optimize the protective effect of ERK while blocking its detrimental effects for neurological disorders and neural damage from cerebral ischemia.

Similarly the increased Wiskott–Aldrich syndrome family verprolin-homologous protein complex (WAVE) and ARP 2/3 association in the membrane also showed that prenylation is required for this protein to be functional in the membrane. Although there has not been any evidence where RhoA directly regulates WAVE/ARP2/3, our data suggests that ARP 2/3 getting higher association in the membrane by overexpressing mutant RhoA, suggesting that RhoA might be channelizing WAVE/ARP2/3 via Rac1 as WAVE/ARP2/3 is directly downstream of the Rac1 [50, 134]. In support, knocking out RhoA in cells inhibited the ERK pathway is concurrent with our data.

Another important finding from these data is that the findings concur with previous reports of an active pool of Rho GTPases in cellular compartments other than membrane, which is the widely accepted dogma of small GTPases [7, 8]. It remains unclear how these

GTPases are active in the cytosol. Our data indicate that there might be active guanosine exchange factors (GEFs) present in the cytosol, similar to the membrane-associated activity that is activating these RhoA.

In the same way, Rac1 activity can be regulated by another signaling molecule or pathway. Rac1 forms a complex with WAVE to control actin polymerization. C-Jun N terminal kinase (JNK) is downstream of Rac1 and plays a critical role in regulating microtubule dynamics via MAP1B and DCX [135]. These reports suggest that Rac1 plays a pivotal role in regulating cytoskeletal dynamics. The data from our work suggest that this geranylgeranylatable Rac1 is proceeding via WAVE and JNK pathways. The overexpression of mutant Rac1 (EmGFP-Rac1^{C189A}) decreased the active JNK in the cytosol whereas the increased association of WAVE and ARP in the whole cell lysates. Supporting results were obtained by immunocytochemistry (ICC) as well. Previous reports from our lab have demonstrated an active pool of Rac1 in the cytosol [16], and this work suggests that these prevalent active Rac1 in the cytosol might be working via the JNK pathway under the influence of unknown GEFs present in the cytosol. The association of WAVE and ARP in the whole-cell lysates was exciting. The whole-cell lysates consist of four major fractions; cytosolic, membranous, nuclear and cytoskeleton fractions. The co-IP were performed only on the samples from the cytosolic and membrane fractions. While we did find an association of WAVE and ARP in whole cell lysate, the association in likely occurring in the cytoskeleton and/or the nucleus fractions, as we did not find any of the association in the cytosolic or membranous fractions.

The decreased WAVE/ARP association in the growth cones by overexpressing non-geranylgeranylatable Rac1 showed the importance of WAVE/ARP and JNK pathways in the formation of lamellipodia and filopodia, and hence, the axon guidance in the growth cone of the extending neurons. This also unveiled the importance of geranylgeranylation in the activation of these small GTPases, and hence, in turn, the activation of these downstream signaling. Also, by overexpressing geranylgeranylatable Rac1 or non-geranylgeranylatable Rac1, phosphorylation was decreased when compared to our control (EmGFP) suggesting that the significant change observed after overexpressing Rac1 is either due to prenylation of Rac1 or it is the trend of phosphorylation in cellular conditions that is creating this change.

The small Rho GTPases play a pivotal role in the regulation of neuronal signaling to initiates axon guidance. A relatively large number of downstream targets of Rho-family GTPases have been reported by *in vitro* studies as discussed above, but only a small number of signal pathways have been established by the *in vivo* studies [7, 14]. The manipulations of these Rho GTPases and its functionality is not done in animal model, which is a good field to look into to get the insight of Rho GTPases in animal model. There are so many effectors like ERK, JNK, and WAVE that are known to be involved in development mechanism of the neurons [34, 43, 51, 136]. But how these pathways are activated is still not clear. They are activated by so many other upstream regulators and hence are parts of so many networking important for the axon guidance during the normal neural development and any change in these pathways would likely lead to the onset of the neurological disorders [57, 127].

Hence, our third set of experiments was to determine how the change in these serine/threonine kinases lead to decreased actin nucleation. Pharmacologically inhibiting various signaling molecules within the pathways indicate that cross-talk occurs between the ARP and JNK pathways initiated by RhoA and Rac1. WAVE/ARP, JNK, ERK are parts of many networking pathways essential for normal axon guidance and growth, and hence have cross-talk between them.

Overall, this work provides a better understanding of the importance of prenylation and its requirement for the activation of Rho GTPases. Rho GTPases when prenylated can anchor to membrane or can stay in the cytosol and can activate different cascades depending on the differential location. As emerging studies emphasize the abnormal pathways downstream of Rho GTPases, only then will we have a better understanding of the pathways guided by the geranylgeranylatable form of Rho GTPases. Together, the field can guide the development of novel pharmacological inhibitors that target these pathways and eventually provide improved treatment options for preventing or treating neurological conditions.

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LIST OF ABBREVIATIONS

Abbreviations

RhoA …….. Ras homolog gene family member A

Rac1 …… Ras-related C3 botulinum toxin substrate 1

Cdc42 ……. Cell division control protein 42 homolog

GTPases ……..... Guanosine triphosphatases

GDP …… Guanosine diphosphate

GDIs …… GDP-dissociation inhibitors

GEFs …… Guanine nucleotide exchange factors

GAPs …… GTPase activating proteins

MAPK …… Mitogen-activated protein kinases

ERK …… Extracellular signal-regulated kinases

JNK ……..... c-Jun N-terminal kinases

WAVE ……..... WASP-family verprolin-homologous protein

WASP …… Wiskott-Aldrich syndrome protein

AD ……. Alzheimer's diseases

PD …….. Parkinson Disease

PAK …… Protein kinase

LIMKs ……. LIM kinases

ARP ……..... Actin related protein

HD ……..... Huntington diseases

PTMs …… Post-translational modification

CaaX ……... C—cysteine, A—aliphatic amino acid, X—any amino acid

ROCK …… Rho-associated kinase

NPFs ……. Nucleation promoting factors