EFFECTS OF RHOA AND RAC1 PRENYLATION ON ALZHEIMER'S DISEASE PROTEINS

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF BIOLOGY

COLLEGE OF ARTS AND SCIENCES

BY

RAWAND KHALED CHABAYTA, B.S.

DENTON, TX

AUGUST 2019

DEDICATION

To my parents, Khaled Chabayta, and Jamile Abdulrahim, thank you for everything.

ACKNOWLEDGMENTS

I would like to thank everyone in Hynds lab for their help and support. A big thanks to my tremendous mentor, Dr. DiAnna Hynds, for all you have done to help me throughout my PhD years at TWU. I really appreciate your help in my research, in helping me to grow as a scientist, and for your endless advice and support in my school and personal life. I can not thank you enough. I would also like to thank my committee members, Dr. Lynda Uphouse, Dr. Chris Brower, Dr. Jannon Fuchs, and Dr. Laura Hanson for serving on my committee, and for encouraging my research with great suggestions and feedbacks. Thank you for everything.

ABSTRACT

RAWAND KHALED CHABAYTA

EFFECTS OF RHOA AND RAC1 PRENYLATION ON ALZHEIMER'S DISEASE PROTEINS

DECEMBER 2019

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by brain extracellular amyloid plaques and intracellular neurofibrillary tangles. β amyloid (Aβ) plaques are produced from the cleavage of amyloid precursor protein (APP) by β-amyloid cleavage enzyme (BACE1) and γ-secretase. Intracellular neurofibrillary tangles are formed from the hyperphosphorylation of tau accomplished by increased kinase and/or decreased phosphatase activity. Clinically, the cholesterol-depleting drugs, statins, which also decrease the isoprenoid production, are associated with decreased incidence of AD. Geranylgeranyltransferase I activity adds a 20-carbon geranylgeranyl group (a type of prenylation) to proteins, and its activity decreases with age or AD. This enzyme prenylates Rho guanosine triphosphatases (GTPases), including RhoA and Rac1. Some evidence indicates that dysregulation of Rho GTPase or activation or prenylation is associated with altered AD pathological markers Aβ or tau. Here, we investigated how altered prenylation of Rac1 or RhoA affects AD pathological makers. We hypothesized that overexpressing Rac1 or RhoA that cannot be prenylated would increase APP and tau by increasing their processing secretases and kinases. To address

this, we overexpressed emerald green fluorescently protein (EmGFP)-tagged prenylatable (wild-type) Rac1 or RhoA or non-prenylatable Rac1 or RhoA and measured APP, Aβ, BACE1, γ-secretase, tau, phosphorylated tau and levels of one kinase that phohsphorylates tau, glycogen synthesis kinase 3β (GSK3β) and activation of BACE1. In whole cell lysates of B35 neuroblastoma cells, overexpressing prenylatable or non-prenylatable Rac1 did not alter APP, Aβ, BACE1, presenilin 2 (the active subunit of γ secretase), or GSK3β levels. However, APP levels in membranes were decreased by overexpressing either prenylatable or non-prenylatable Rac1, with the latter decreasing membrane-associated APP less than overexpressed wild-type Rac1. Cells overexpressing prenylatable or non-prenylatable RhoA increased total levels of tau, but only overexpression of wild-type RhoA increased levels of tau phosphorylation. The interpreted results showing increased Rac1 and decreased RhoA prenylation increases tau phosphorylation indicate a possible contribution to neurofibrillary tangle formation. However, since altering Rac1 or RhoA prenylation did not affect Aβ formation and evidence indicates that Rho GTPases function both upstream and downstream of AD marker production, RhoA GTPase prenylation, regardless of activation, may result from AD marker production instead of synthesis regulation.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

DISSERTATION INTRODUCTION

PROJECT RATIONALE AND AIMS

The project described within this dissertation is arranged in chapters that are designed and formatted for three manuscripts to be submitted to peer-reviewed scholarly journals. Because of this, the opportunity to provide a succinct description of the project and the organization of this dissertation is limited. Therefore, I provide an overview of the project rationale, major hypothesis and the specific aims to be accomplished. A short description of each of the chapters included within this work follows this section.

Alzheimer's disease (AD) is a fatal neurodegenerative disorder characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain, accompanied by oxidative stress, inflammation, and synaptic damage.[1] Cleavage of the transmembrane amyloid precursor protein (APP) through β secretase, rather than α secretase, creates a template for producing β-amyloid (Aβ), which aggregates to form disease-associated extracelllar plaques.[2] The second pathological hallmark of AD involves hyperphosphorylation of the microtubule associated protein, tau, which forms intracellular neurofibrillary tangles.[2]

Defects linked to AD, and other neurological diseases, include aberrant regulation of the Rho guanosine triphosphatases (GTPases).[3] Rho proteins, including RhoA and Rac1, are monomeric GTPases that act as switch poteins and are major regulators of the actin cytoskeleton, as well as other cellular functions.[4] Rho GTPases require prenylation for insertion into cell membranes to be properly localized to propagate appropriate signals.[4] Rho GTPases are prenylated by geranylgeranylation, which is the covalent attachment of a geranylgeranyl moiety from geranylgeranyl pyrophosphate (GGPP), a reaction catalyzed by geranylgeranyl transferase-I (GGTase-I).[5,6]

Prenylation of Rho GTPases is altered in many cases of AD. Levels of GGPP are significantly higher in animal models of ageing and AD, and in AD patients, but geranylgeranylation of Rho GTPases is decreased due to decreased activation of GGTase-I.[7,8] Some studies show that AD alters Rho GTPase levels or activity, while others suggest Rho GTPases contribute to AD pathology. For instance, Rac1 and RhoA activity is altered in human AD hippocampi,[9] and overall RhoA levels decrease in AD brains, where they co-localize with hyperphosphorylated tau in the neurofibrillary tangles.[10] Conversely, Rac1 inhibition decreases APP synthesis[11] and alters the selectivity of γ-secretase toward substrates other than APP.[12] Important for this study, prenylated, hence, membrane-associated RhoA and Rac1 decrease in ageing mice,[8] and prior results show that RhoA and Rac1 can be activated to allow

interaction with immediate downstream effectors without localizing to plasma membranes.[13]

Since Rho GTPases can be activated without being prenylated, it is possible that aberrantly-located, non-prenylated Rho GTPases may alter production and localization of APP and tau, leading to increased pathological markers of AD. It is hypothesized that compared to prenylatable Rac1 or RhoA, overexpressing Rac1 or RhoA GTPases that cannot be prenylated would increase the levels of APP and tau and increase the activity of secretases and kinases that lead to AD pathology. For the purpose of this study, prenylatable and non-prenylatable constructs for Rac1 and RhoA were used. Expressions of non-prenylatable RhoA or Rac1 prevents their insertion into membranes, allowing their activation in aberrant subcellular localizations, where they may increase AD pathological markers. Experiments were conducted in cell models of neurons, the main cell type affected in AD: 1) rat B35 neuroblastoma cells, and/or 2) cultured primary rat cortical neurons. B35 neuroblastoma cells, as a cell line, can provide a large number of cells to use in biochemical analyses. Furthermore, B35 cells are easy to transfect and differentiate to approximate neurons. Cortical neurons are directly affected in AD and embryonic neurons in culture have been extensively used to study mechanisms of AD, providing an appropriate second model for our experiments. In this study the hypothesis was tested using three Specific Aims:

1. To assess whether overexpressing non-prenylatable Rac1 or RhoA increases the production of tau, phosphorylated tau and the kinase GSK-3β, one kinase associated with tau hyperphosphorylation in AD, compared to overexpressing prenylatable Rac1 or RhoA. This was analyzed using western blotting and immunocytochemistry.
2. To determine whether overexpressing non-prenylatable Rac1 or RhoA increases the production of APP and the activity of the secretases, β-site APP cleaving enzyme 1 (BACE1) and presenilin compared to overexpressing prenylatable Rac1 or RhoA. This was analyzed using western blotting, cellular fractionation and enzyme activation assays.
3. To assess whether overexpressing non-prenylatable Rac1 or RhoA increases the production of β amyloid, compared to overexpressing prenylatable Rac1 or RhoA. This was analyzed using activation assays.

Performing these aims tested the hypothesis that the localization of RhoA and Rac1 affects the production and localization of AD-associated aggregation of APP and tau. If the hypothesis was correct, we should have observed increased production of AD-related proteins when we overexpress non-prenylatable form of RhoA or Rac1, compared to their overexpressed wild-type forms. By determining the role of RhoA and Rac1 in regulating the levels of AD pathological markers, we address the disagreement in the literature on whether Rho GTPase activity in

AD is upstream or downstream of the production of Aβ and hyperphosphorylated tau. This better understanding of the role of Rho GTPase prenylation in AD may facilitate the identification of novel therapeutic targets for treating the pathogenesis or symptoms associated with AD.

DISSERTATION ORGANIZATION

This work is arranged in five chapters (see Table 1). The first chapter provides an overview of the rational, aims and organization for the experimental project described herein; a project that is focused on performing sets of experiments to address the hypothesis that overexpressing non-prenylatable forms of the small GTPases, RhoA and Rac1, affects AD pathology. Both of these GTPases are ubiquitous regulators of the actin cytoskeleton.[9] In neurons, activation of RhoA by binding to guanosine triphosphate (GTP) leads to retraction of neuronal processes and consolidation of process shafts behind the extending growth cones, while GTP loading of Rac1 leads to increased actin nucleation and polymerization, promoting the extension of neuronal processes.[9]

Table 1.1. Organization of this dissertation

Chapter	*Topic*	*Submission*[a]	*Format*[b]
I	Organization of the dissertation	No	AMA[c]
II	Review of Rho GTPases in Alzheimer's Disease	*Molecular Neurobiology*	Journal's
III	Non-prenylatable Rac1 effect on Aβ and tau	*Cellular Signalling*	Journal's
IV	Non-prenylatable RhoA effect on Aβ and tau	*Cellular Signalling*	Journal's
V	Dissertation summary and conclusions	No	AMA[c]

[a]No indicates that the chapter is not planned to be submitted to a peer-reviewed journal for publication consideration. If the chapter will be submitted the intended journal is listed.
[b]Indicates the referencing format used in each chapter.
[c]American Medical Association.

Chapters II through IV are manuscripts that will be submitted for publications to peer-reviewed scholarly journals. Chapter II is a review of the literature focused on RhoA and Rac1 in AD, which will be submitted to *Molecular Neurobiology*, a journal that focuses on publishing neuroscience-based review articles written primarily by graduate students and postdoctoral scholars. Chapter III is a research article that reports results from all three Specific Aims concerning overexpression of non-prenylatable Rac1, and Chapter IV reports the results obtained for all three aims from overexpressing non-prenylatable RhoA. We plan to submit each of these manuscripts to *Cellular Signalling*, a journal that publishes reports on all types of cellular signal transduction and published work leading to the current research.[13]

CHAPTER II

PRENYLATION OF THE SMALL GTPASES RHOA AND RAC1 IN ALZHEIMER'S DISEASE

A Paper to be Submitted for Publication in *Molecular Neurobiology*

Rawand K. Chabayta and DiAnna L. Hynds
Department of Biology, Texas Woman's University, Denton, TX, 76204

Corresponding Author: DiAnna L. Hynds, Ph.D.
PO Box 425799
Denton, T 76204-5799
940-898-3235
Fax: 940-898-2382
dhynds@twu.edu
ORCID: 0000-0002-5500-5795

Acknowledgments: This study was funded by the Texas Woman's University (TWU) Department of Biology and grants from the National Science Foundation (DUE 0806963, 1154394), the TWU Research Emhancement Program, and the TWU Grant Proposal Development Fund.

Abstract:

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that is most often found in the elderly. AD leads to a gradual loss of cognitive skills and memory as well as the capability to perform daily activities. It is histopathologically characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain. Aberrant cleavage of the transmembrane amyloid precursor protein (APP) produces β amyloid (Aβ) plaques. Hyperphosphorylation of tau, a microtubule associated protein, forms intracellular neurofibrillary tangles. The molecular mechanisms that influence the development of these pathological hallmarks are not completely elucidated but a body of evidence implicates involvement of guanine triphosphatases (GTPases) of the Rho family. Rho GTPase activation has long been thought to require prenylation for proper insertion into the plasma membranes where it can be activated. However, Rho GTPases can be activated without being prenylated. So, it is possible that non-membrane-localized, non-prenylated Rho GTPases can alter production or processing of APP and tau, leading to increased AD pathological markers. This review covers AD hallmarks, the potential involvement of RhoA and Rac1 in AD pathology, and the effect of inhibiting RhoA or Rac1 activity on production of AD proteins, kinases and secretases.

Keywords: β-amyloid, Geranylgeranylation, Neurodegeneration, Post-translational modification, Small G proteins, Tau.

Introduction:

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that is histopathologically characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain, accompanied by signs of oxidative stress, inflammation, and synaptic damage [1-4]. Defects in Rho GTPase signaling pathways have been recently linked to AD and other neurological diseases [5]. Prenylation has long been thought to be required for Rho GTPase activation [6]. Rho GTPases are prenylated by geranylgeranylation, the covalent attachment of geranlgeranylpyrophosphate (GGPP) catalyzed by geranylgeranyl transferase-I (GGTase-I) [6]. In many cases, it is the prenylation of Rho GTPases that is altered in AD [7]. Levels of geranylgeranylpyrophosphate (GGPP) are significantly higher in animal models of ageing and AD, and in AD patients [7,8]. Some studies suggest that AD alters Rho GTPase levels or activity, while others suggest Rho GTPases contribute to AD pathology [reviewed in 9]. As AD pathological hallmarks are often seen in post-mortem tissue from patients not diagnosed with the disease, some aspects of AD may occur in normal ageing. Membrane-associated RhoA and Rac1 decrease in ageing mice [8] and our prior results show that RhoA and Rac1 can be activated without localizing to plasma membranes [10]. Since Rho GTPases may be activated (have the ability to bind GTP and interact with effectors) without being prenylated, it is possible that aberrantly located non-prenylated Rho GTPases can alter production and of tau and APP leading to increased hallmarks of AD.

Alzheimer's Disease:

AD is the major category of dementia in the older population. It is also "the sixth leading cause of death in the United States and the fifth leading cause of death in Americans aged 65 and older" [11]. Worldwide, there are about 47.5 million people suffering from AD [2]. In 2017, it was estimated that there were 5.5 million sufferers in the U.S. alone, with an estimated healthcare cost of 232 billion dollars [12]. According to statistical analyses, the incidence of AD would decrease by 2 million after 50 years if the onset of the disease could be delayed by 2 years [13]. Therefore, decreasing or being able to treat AD could significantly decrease the tangible and intanglible burdens on patients, their caretakers and family members.

AD was named after the German neuropathologist Dr. Alois Alzheimer in 1906 [14]. It is a neurodegenerative fatal disorder that is clinically characterized by progressive decline in memory, movement and cognitive skills, and a change in personality. Pathological markers for AD include the formation of neuritic extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain, along with oxidative stress, inflammation, and synaptic damage [14]. Despite the progress that has been made to understand AD progression, current treatments are limited to N-methyl-D-aspartate (NMDA) receptor antagonists and acetylcholinesterase inhibitors that may relieve symptoms but do not alter AD pathogenesis, as measured by postmortem analysis of extracellular β amyloid plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau [15].

Amyloid Plaques

Amyloid plaques are mainly comprised of the accumulated β-amyloid (Aβ) peptide in the hippocampus and neocortex. Many types of brain cells (e.g. astrocytes) produce Aβ throughout life and neurons produce the highest levels [16]. Amyloid plaques are small, round deposits consisting of 39 to 42 amino acid long Aβ peptides [17]. Aβ (approximately 4 kD in size) disrupts neurotransmission and it is formed in plaques from the proteolytic cleavage of amyloid precursor protein (APP), a transmembrane protein that is ubiquitously expressed. Aβ is found in small amounts in the cerebrospinal fluid and in the brain of normal individuals [18].

APP cleavage occurs by two pathways (Figure 2.1): the amyloidogenic pathway and the non-amyloidogenic pathway [18]. In the amyloidogenic pathway, the sequential cleavage of APP by β secretase (β-amyloid cleavage enzyme or BACE1) and the γ secretase complex containing presenilin, produces soluble APPβ (sAPPβ), APP intracellular domains (AICDs), and Aβ of two different lengths (40 and 42 amino acids, called Aβ40 and Aβ42, respectively) [19]. Under normal conditions, Aβ is released outside the neurons and is rapidly cleared by glial cells and/or degraded [20]. Aβ40 and Aβ42 are the two main forms of Aβ in the brain. Aβ40 is the major type in soluble Aβ. It makes up about 90% of total Aβ, and it is less pathogenic in AD development. However, Aβ42, which makes up about 10% of total Aβ, has been identified as the key feature in AD pathogenesis, as it has been found to be more toxic and fibrillate faster than

Aβ40 [21]. High levels of Aβ in the brain activate microglia, which leads to the production of reactive oxygen species and cytochemokines and, consequently, leads to neuronal damage [22]. In addition, Aβ can trigger the formation of neurofibrillary tangles, impair memory, alter synaptic plasticity, and cause dendritic abnormalities that will, in turn, cause neuronal death and synaptic failure [23].

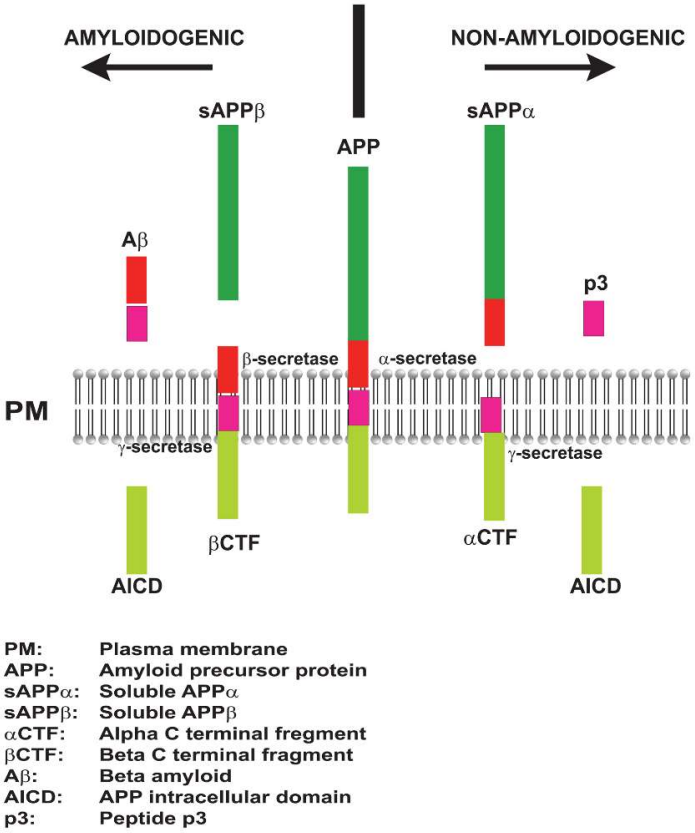

Fig. 2.1 Amyloid plaque formation. The amyloid precursor protein (APP) is cleaved by the amyloidogenic or the non-amyloidogenic pathways to form β amyloid (Aβ), which aggregates into plaques, or non-toxic peptides, respectively. In the amyloidogenic pathway, cleavage by β-secretase produces soluble APPβ and a 99 amino acid C terminal fragment (βCTF). Further cleavage by γ-secretase leads to the production of Aβ and the APP intracellular domain (AICD). APP cleavage by α-secretase (α-secretase) produces extracellular sAPPα and membrane-bound αCTF. Additional cleavage by γ-secretase produces an extracellular p3 peptide.

APP, β-secretase, and γ-secretase are all membrane proteins. APP is a type-I membrane protein with its carboxyl terminus located in the cytosol and its amino terminus located within the extracellular space. BACE1 is an aspartyl protease and γ-secretase is a multicomponent protease containing the active site presenilin [19]. During normal cell activities, β-amyloid peptide is usually produced in monomeric, soluble form, which is non-toxic to cells and can help modulate synaptic activity, or act as an antioxidant agent [24].

In the constitutive non-amyloidogenic pathway (Figure 2.1), APP is cleaved by α-secretase and results in the production of C31, or the α-C terminal fragment (CTF), which is then transformed into the APP intracellular domain (AICD) and p3 peptides by γ-secretase [19]. This pathway has been found to be neuroprotective as well as neurotrophic [25].

According to the "amyloid hypothesis," an increase in Aβ42 production or a decrease in Aβ42 degradation is the primary factor in the neuropathological cascade of AD [19]. From biochemical and genetic evidence, it is suggested that an increase in Aβ42 production represents the key mechanism for early-onset AD [26]. Dysregulation in β-amyloid's clearance, by passage across the blood brain barrier or by cellular influx, represents the key mechanisms for late-onset form of AD [27,28]. However, the presence of amyloid plaques in Aβ42-overexpressing BRI2-Aβ mice does not lead to loss of neurons or impairment of cognitive functions, indicating that Aβ42 may not be toxic to cells or lead to development of AD [20]. Similarly, plaques are often seen in patients with no

cognitive impairment at the time of death. In addition, immunotherapies targeting Aβ in AD models are effective in decreasing Aβ accumulation but do not improve AD symptoms or tau accumulation [20]. Because of the different time scales investigated by these experiments, it is important to perform additional studies to address the kinetics of Aβ production and clearance.

Neurofibrillary Tangles:

The second pathological characteristic of AD is the neurofibrillary tangles. Neurofibrillary tangles are primarily composed of the microtubule associated protein (MAP) tau, in hyperphosphorylated form [29]. Tau, one of the key components of the cytoskeleton, is a phosphoprotein that usually contains 2-3 moles of phosphate per mole of protein [29]. It interacts with tubulin and promotes tubulin assembly into microtubules, stabilizing their structure and providing structural support to cells, including neurons (Figure 2.2). These functions of tau are regulated by its degree of phosphorylation [29]. In the AD brain, tau is 3- to 4-fold more phosphorylated than in normal adult brain [30]. In this hyperphosphorylated state, tau is polymerized into paired helical filaments (PHFs) mixed with straight filaments (SFs) to form the neurofibrillary tangles. Hyperphosphorylated tau sequesters normal tau with microtubule-associated protein (MAP) 1 and MAP2 and promotes self-aggregation of microtubules into PHF/SF [30]. In neurons, tau is an axonal protein and is also found in lower levels in dendritic regions such as spines [31]. In the tau knockout model of AD mice, absence of tau is associated with reduced stability of microtubules,

reduced neurite extension and deterioration of neurons [32, 33]. In addition, tau has also been recently reported to be part of the pathway causing synaptotoxicity that is induced by β amyloid [34]. Adding β amyloid oligomers to primary neurons mislocalizes tau proteins to somatodendritic regions, leading to loss of spines, microtubules and synaptic activity [35].

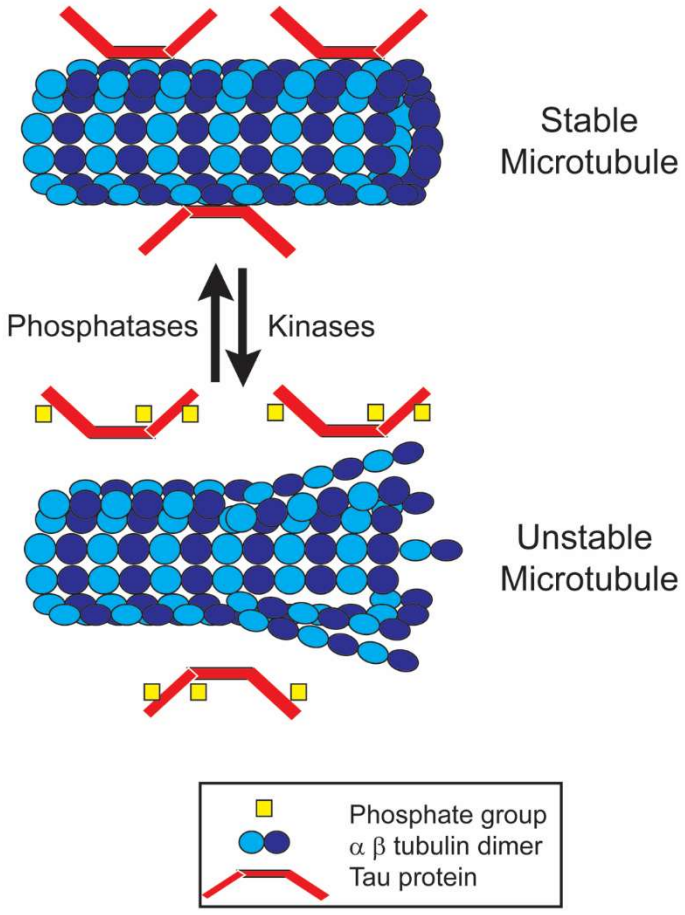

Fig. 2.2 Production of neurofibrillary tangles (NFTs). The second pathological hallmark of AD is the formation of intracellular NFTs, which are polymerized forms of hyperphosporylated tau and other microtubule binding proteins. Several kinases can phosphorylate over 70 sites on the six isoforms of tau, disrupting its normal microtubule-stabilizing function. Hypoerphyosphorylation of tau cause it to dissociate from microtubules, creating first paired helical filament (PHFs) and the NFTS along with destabilization and breakdowm of the microtubules.

Tau can be phosphorylated at over 38 serine or threonine residues, in addition to tyrosines that may also be phosphorylated, and is more highly phosphorylated on serine and threonine in AD brain than in age-matched normal controls [36]. Among the kinases that have been identified to play an important role in abnormal tau hyperphosphorylation are glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinases (CDKs), casein kinase 1 (CK1), protein kinase A (PKA), calcium and calmodulin-dependent protein kinase-II (CaMK II), and the MAP kinases ERK1/2 and stress-activated kinases are among the kinases that have been identified to play an important role in abnormal tau hyperphosphorylation [36]. For example, an increase in GSK3β activity has been linked to neurodegeneration, memory deficits, tau hyperphosphorylation, and β amyloid production [35]. In AD brains, GSK3β increases and co-localizes with neurofibrillary tangles [35].

Hyperphosphorylation of tau could result from increased kinase activity or decreased phosphatase activity [36, 37]. There are many phosphatases whose inactivation is implicated in regulating tau phosphorylation and in inhibiting tau hyperphosphorylation in AD [37]. These protein phosphatases (PPs) include PP-1, PP-2A, PP-2B and PP-5. Protein phosphatase 2-A (PP-2A) is responsible for over 70% of tau phosphatase activity in the human brain. Inhibition of PP-2A activity by okadaic acid, not only decreased dephosphorylation, but also promoted the activity of several tau kinases causing abnormal

hyperphosphorylation of tau in cultured cells [37]. In cultured neurons, inhibition of PP-2A was reported to be involved in apoptosis induced by APP [37].

Multifactorial Disease:

AD is a multifactorial complex disease influenced by the interaction of many factors including both genetic and epigenetic factors [38]. Aging is considered as the primary risk factor in AD and other aging-related problems, such as hypertension and diabetes, further increase AD risk [38]. In the brains of both aged and AD patients, excessive mitochondrial oxidative damage has been found. The exact mechanisms through which aging contributes to AD progression are not fully understood [39].

Genetic Epidemiology:

Late-onset and early-onset AD are accompanied by different factors of genetic epidemiology. Early-onset AD cases (1-5%) are caused by mutations in the amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PS1) gene on chromosome 14, or the presenilin 2 (PS2) gene on chromosome 1 [40]. The majority of AD cases, over 95%, display the late-onset form or the sporadic form of the illness. Expression of the E4 allele of the gene coding for apolipoprotein E (APOE) is the gene variation most correlated with late onset AD. The presence of one or two alleles of APOE4 raises the risk for AD by 3-fold and 15-fold, respectively [40].

Mutations in genes outside the APP or tau pathways have also been implicated in AD. For instance, Rac1 and RhoA activity is altered in human AD

hippocampus [41] and in other disorders of intellect (e.g. intellectual disability, autism spectrum disorders, and neurodevelopmental disorders). Mutations in Rac1 or in the genes that modulate Rac1 activity have been reported in central nervous system diseases [42]. Further studies are needed to elucidate the effects of mutated Rho GTPases on AD hallmarks in both natural and manipulated conditions. For instance, expressing Rho GTPases that cannot be prenylated will help elucidate the role of GTPase membrane localization on production of AD pathological markers.

Rho GTPases in Alzheimer's Disease:

Small GTPases have been implicated in production of Aβ and dysfunction of synapses in AD pathology [43]. Small GTPases are monomeric-guanine-nucleotide-binding molecules that play a role in many cellular functions, including growth, differentiation, endocytosis, cell adhesion, actin cytoskeleton organization and vesicle trafficking [44].

Small GTPases are among many proteins that undergo prenylation. Prenylation is accomplished by adding a 15-carbon farnesyl isoprenoid or a 20-carbon geranylgeranyl group to the C-terminus of a protein [45]. The isoprenoid synthesis starts with synthesis of geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP) by isoprenyl pyrophosphate synthetase [46]. The CAAX terminus of the protein (C is cysteine, A is an aliphatic amino acid and X is any amino acid) must undergo post-translational modification by geranylgeranyl transferase-I (GGTase-I) or geranylgeranyl transferase-II (GGTase-II), followed

by the cleavage of the -AAX by a peptidase, to add isoprenoids to the C-terminus of the protein. Geranylgeranylation by GGTase-I is restricted to proteins whose X-residue of CAAX contain isoleucine or leucine (includes Rho GTPases) [47]. This modification will cause targeting of the prenylated proteins to the plasma membrane for their activation and propagating signals [48]. It is commonly thought that prenylation is required for attaching Rho GTPases to plasma membrane for their activation [49]. Otherwise, proteins would not interact with their downstream effectors to propagate signaling [49]. Rho GTPases are not farnesylated or geranylgeranylated by GGTase-II; therefore, these mechasnisms are not discussed further.

Rac1 and RhoA are the most studied members of the Rho family GTPases. They are important key regulators of the actin cytoskeleton and have been implicated in playing effective roles in neurological morphogenesis [50]. RhoA activates the formation of focal adhesions and stress fibers and inhibits the growth and stability of dendritic spines, whereas Rac1 promotes neurite outgrowth and dendritic spine formation and is required for lamellipodia formation [50]. These rho proteins act as molecular switches cycling between an inactive GDP-bound and an active GTP-bound state [51, 52]. This cycling between the GTP- and GDP-bound forms is regulated by the positive regulators guanine-nucleotide-exchange factors (GEFs) and negative regulators GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Figure 2.3) [51, 52]. The interaction with GEFs promote the exchange of GDP for GTP.

GAPs increase the intrinsic GTPase activity that hydrolyses the GTP to GDP. GDIs sequester the GDP-bound GTPase in the cytosol [51, 52]. In their active state, Rho GTPases can interact with their downstream effector molecules to activate specific signal transduction pathways and exert their effects on various biological activities (Figure 2.3) [51, 52].

Defects in Rho GTPase signalling pathways have been recently linked to many neurodegenerative and other human diseases, such as Alzheimer's Disease, Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), cancers, pulmonary disorders, and cardiovascular diseases [53]. The Rho family of GTPases is the key regulator of actin cytoskeleton rearrangements [54]. Importantly, Rho GTPase dysfunction in these neurodegenerative disorders is linked to common abnormalities in the actin cytoskeleton. Therefore, it is important to understand if Rho signaling pathways contribute to those disorders. If so, some therapeutic interventions might be beneficial for multiple diseases.

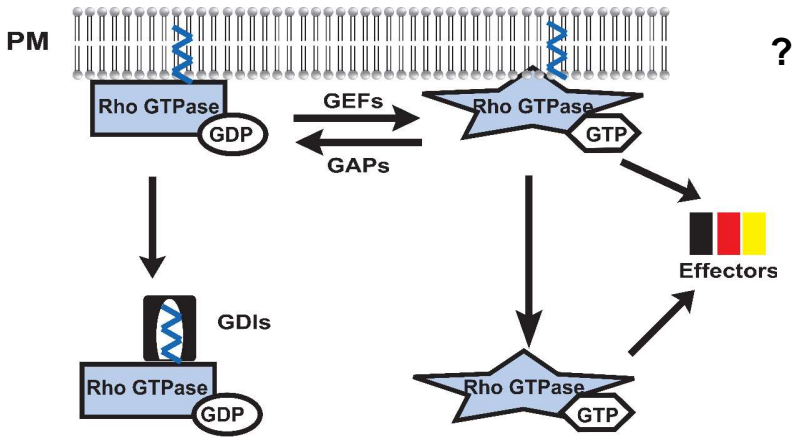

Fig. 2.3 Rho GTPase activation cycle. Isoprenoids (blue zigzag lines) are involved in the post-translational prenylation of Rho guanosine triphosphatases (GTPases) for their translocation of the cytosolic GTPase to the plasma membrane (PM). Rho GTPases are active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP). Guanosine exchange factors (GEFs) are responsible for exchanging GDP to GTP. GTPase activating peoteins (GAPs) increase the intrinsic phosphatase activity that hydrolyses GTP to GDP. Guanosine dissociation inhibitors (GDIs) require prenylation to sequester and solubilize the GDP-bound inactive GTPase in the cytosol. In their active state, Rho GTPases can interact with their downstream effectors to propagate signal transduction.

There are several ways to manipulate protein prenylation to determine whether prenylation of Rho GTPases is important in AD pathology. Statin treatment is one method used to inhibit all protein prenylation including that of RhoA and Rac1. Statins inhibit the mevalonate pathway by competitively inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase [55]. Levels of GGPP are significantly higher in aged mice and in AD patients [9,10]. The decrease in the activity of geranylgeranyl transferase-I (GGT-I) increases levels of geranylgeranyl pyrophosphate (GGPP) but decreases levels of geranylgeranylated Rho GTPases in AD [9]. In aged mice, membrane-

associated RhoA and Rac1 are decreased, while cytosolic RhoA and Rac1 are increased [9]. Our results suggest that Rho GTPases might be active (bind GTP and interact with effectors) without their localization to plasma membranes [7]. Therefore, altered subcellular localization of RhoA and Rac1 might affect the production of amyloid plaques and neurofibrillary tangles formation in AD or may result from AD pathology.

The role of Rho GTPases in animal models of AD has also been investigated and has led to conflicting results. Intracerebroventricular administration of a RhoGTPase activator in four-month old transgenic mice with double mutant form of APP 695 (TgCRND8), corrects behavioral disturbances, suggesting that Rho GTPase modulation might be beneficial in AD treatment [56]. Petratos et al. (2008) found that β amyloid decreases neurite outgrowth in SH-SY5Y cells by increasing RhoA activation (GTP binding), which then increases phosphorylation of collapsing response mediator protein-2 (CRMP-2) to prevent tubulin assembly [57]. In addition, increasing RhoA expression was found to increase dystrophic neurites, reduce synapses, and induce neurofibrillary tangles in 18-month-old AβPP transgenic mice [58]. No major changes were found in Rac1 (or a related GTPase, Cdc42), suggesting that altered subcellular targeting of RhoA is more likely to be implicated in AD [58]. However, Mendoza-Naranjo et al. (2007), have found that, in hippocampal cells, fibrillar $A\beta_{1–42}$ causes alterations in the neuronal actin cytoskeleton through activating Rac1 and Cdc42 [59]. The transcription of the APP gene in primary

hippocampal neurons is regulated by Rac1 [60]. Blocking Rac1 signaling also prevents the production of β amyloid *in vivo* as well as *in vitro*, by modulating APP processing by γ-secretase, suggesting that Rac1 signaling might be a potential therapeutic target for AD [61]. More studies are needed to understand the role of RhoGTPases in AD pathology.

Defects in Rho GTPase activity are also associated with production of β amyloid and reduced synapse plasticity in AD [42]. Aberrant activation of Rho GTPases may cause neurite retraction and increase toxic β amyloid formation [62]. The relationship between Rac1, RhoA, and the production of APP and tau hyperphosphorylation has been examined. For instance, in APP over-expressing transgenic mouse brains, RhoA expression is reduced within the synapses and upregulated in degenerating neurites [58]. Rho-associated, coiled-coil containing protein kinase 1 (ROCK 1) colocalizes with hyperphosphorylated tau [63]. ROCK1 protein levels are elevated in AD brains and the knockdown of ROCK1 reduces the levels of β-amyloid by stimulating the degradation of APP [64]. In AD brains, aberrant activation of p21-activated kinase (PAK) co-localized with activated Rac1, suggesting that Rac1 might initiate synaptic dysfunction and loss [65]. Moreover, γ-secretase enhances dendritic spine formation at the synapse by activating the Rac signaling pathway [66]. In one Alzheimer's mouse model (3xTg-AD), the knockdown of cyclin-dependent kinase 5 (CDK 5) decreased tau phosphorylation and increased Rac activity and p35 protein levels, leading to improvements in cognitive functions [67]. In addition, expression of dominant

negative Rac1 decreased the activity of γ-secretase in COS-7 fibroblast-like cells and led to CTF accumulation and reduction of APP intracellular domain production [68]. Taken together, some studies suggest the involvement of RhoA and Rac1 in AD pathology, in tau hyperphosphorylation and APP processing.

In addition to Rho GTPases affecting progression to AD pathology, there is evidence that pathological changes in AD also affect Rho GTPase function. β-amyloid production has been reported to affect RhoA and Rac1 activation [69, 70]. For instance, treatment with the toxic $A\beta_{1-42}$ activated RhoA and reduces neuronal survival through protein tyrosine phosphatase 1B (PTP1B) inhibition in PC12 cells [69]. Studies performed *in vitro* and *in vivo* have shown that Aβ may induce neurofibrillary tangles by interacting with Rac1 [70]. $A\beta_{1-42}$ was found to induce neuronal death and formation of reactive oxygen species through activating phosphatidylinositol 3-kinase (PI3K)/phosphoinositol-dependent kinase 1 (PDK1)/protein kinase C (PKC)/Rac 1 signaling and Rac-cytosolic phospholipase A_2-($cPLA_2$) -5- lipoxygenase (5-LO) pathways, suggesting Rac1 as a therapeutic target in AD pathology [71, 72]. In addition, $A\beta_{1-42}$ was reported to increase tau phosphorylation, induce synaptic loss and cause neuronal death [73]. The activation of the Wnt-PCP-RhoA/ROCK pathway is required for Aβ-driven synaptic loss [73]. While collectively the studies detailed above do not clearly indicate whether AD pathology affects Rho GTPases, or manipulating Rho GTPase prenylation affects AD pathological markers, we are not aware of any group currently assessing how altered Rho GTPase location by affecting its

prenylation can alter Rho GTPase signaling to AD-like pathology. This underscores the need to determine the importance of Rho GTPase localization in AD pathology.

Conclusion:

Defects in Rho GTPase signaling pathways have recently been linked to AD and other neurological diseases [51]. Prenylation of small GTPases has been associated with amyloid precursor protein (APP) processing, failure of synaptic plasticity and generation of reactive oxygen species causing oxidative stress [8]. Despite these findings and the use of statins to inhibit prenylation, little research has focused on how non-prenylated Rac1 and RhoA affect production of tau and APP and the activity of APP secretases and tau kinases. Since RhoA and Rac1 are altered in AD and they may be activated without being prenylated, it is possible that aberrantly- located non-prenylated Rho GTPases can alter production of APP and tau in ways associated with AD pathology.

Funding:

This study was funded by the Texas Woman's University (TWU) Department of Biology and grants from the National Science Foundation (DUE 0806963, 1154394) and the TWU Research Emhancement Program, and the TWU Grant Proposal Development Fund.

Conflicts of Interest:

The authors declare that they have no conflicts of interest.

Ethical Approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Carlo MD, Giacomazza D, Biagio PL (2012) Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools. J Physics: Condensed Matter 24: 244102.
2. Serranzo-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. CSH Persp Med 1:a006189.
3. Bukhari H, Glotzbach A, Kolbe K, Leonhardt G, Loosse C, Müller T (2017) Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease. Prog Neurobiol 156:189-213.
4. Iba M, Guo JL, Mcbride JD, Zhang B, Trojanowski JQ, Lee VM (2013) Synthetic tau fibrils mediate transmission of neurofibrillary tangles in a transgenic mouse model of Alzheimers-like tauopathy. J Neuroscience 33:1024-1037.
5. Nadif Kasri N, Van Aels L (2008) Rho-linked genes and neurological disorders. Pflugers Archiv: Eur J Physiol, 455:787-797.

6. Calero M, Chen CZ, Zhu W, Winand N, Havas KA, Gilbert PM, et al. (2003) Dual Prenylation Is Required for Rab Protein Localization and Function. Molecular Biology of the Cell 14:1852-1867.
7. Eckert G, Hooff G, Strandjord D, Igbavboa U, Volmer D, Müller W, Wood W (2009) Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients. Neurol Dis 35(2):251-257.
8. Afshordel S, Wood WG, Igbavboa U, Muller WE, Eckert GP (2014) Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain. J Neurochem 129:732-742.
9. Hottman D, Cheng S, Gram A, Leblanc K, Yuan L, Li L (2018) Systemic or Forebrain Neuron-Specific Deficiency of Geranylgeranyltransferase-1 Impairs Synaptic Plasticity and Reduces Dendritic Spine Density. Neuroscience 373:207-217.
10. Reddy JM, Samuel FG, Mcconnell JA, Reddy CP, Beck BW, Hynds DL (2015) Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated. Cell Signal 27 630-637.
11. Thies W, Bleiler L, Alzheimer's Association (2013) 2013 Alzheimer's disease facts and figures. Alzheimer's & Dementia: J Alzheimer's Dis 9:208-45.
12. Alzheimer's Association (2017) 2017 Alzheimer 's disease Facts and Figures. Alzheimer's & Dementia 2017 13:325-373.

13. Brookmeyer R, Gray S, Kawas C (1998) Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. Am J Pub Health 88:1337-1342.
14. Kazee AM, Johnson EM (1998) Alzheimers disease pathology in non-demented elderly. J Alzheimers Dis 1:81-89.
15. Weller J, Budson A (2018) Current understanding of Alzheimer's disease diagnosis and treatment. F1000Research 7: 1161.
16. Tanzi RE, Bertram L (2005) Twenty years of the Alzheimer's disease amyloid hypothesis: A genetic perspective. Cell 120:545-555.
17. Wesson DW, Nixon RA, Levy E, Wilson DA (2011) Mechanisms of neural and behavioral dysfunction in Alzheimer's disease. Mol Neurobiol 43:163-179.
18. Mura E, Zappettini S, Preda S, Biundo F, Lanni C, Grilli M, Cavallero A, Olivero G, Salamon A, Govoni S, Marchi M (2012) Dual effect of β-amyloid on α7 and α4β2 nicotinic receptors controlling the release of glutamate, aspartate and GABA in rat hippocampus. PLoS ONE, 7: e29661.
19. Zhang Y, Thompson R, Zhang H, Xu H (2011) APP processing in Alzheimer's disease. Mol Brian 4:3-16.
20. Kametani F, Hasegawa M (2018) Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimers disease. Front Neurosci 12:25.
21. Wesson DW, Nixon RA, Levy E, Wilson DA (2011) Mechanisms of neural and behavioral dysfunction in Alzheimer's disease. Mol Neurobiol 43:163-179.

22. Ray B, Chauhan NB, Lahin DK (2011) Oxidative insults to neurons and synapse are prevented by aged garlic extract (age) and s-ally-l-cysteine (sac) treatment in the neuronal culture and app-tg mouse model. J Neurochem 117:388-402.
23. Fu HJ, Liu B, Frost JL, Lemere CA (2011) Amyloid-B immunotherapy for Alzheimer's disease. CNS Neurol Dis-DR 9:197-206.
24. Grilli M, Lagomarsino F, Zappettini S, Preda S, Mura E, Govoni S, Marchi M (2010) Specific inhibitory effect of amyloid-β on presynaptic muscarinic receptor subtypes modulating neurotransmitter release in the rat nucleus accumbens. Neurosci 167:482-489.
25. Chow VW, Mattson MP, Wong PC, Gleichmann M (2009) An overview of APP processing enzymes and products. Neuromol Med 12:1-12.
26. Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegenration: lessons from the Alzheimer's amyloid β-peptide. Nature Rev Mol Cell Biol 8:101-112.
27. Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 57:178-201.
28. Selkoe DJ (2001) Clearing the brain's amyloid cobwebs. Neuron 32:177-180.
29. Iqbal K, Liu F, Gong CX, Alonso AC, Grundke-Iqbal I (2009) Mechanisms of tau-induced neurodegenration. Acta Neuropathol 118:53-69.
30. Iqbal K, Liu F, Gong CX, Grundke-Iqbal I (2010) Tau in Alzheimer disease and related tauopathies. Cur Alzheimer Res 7:656-664.

31. Papasozomenos SC, Binder LI (1987) Phosphorylation determines two distinct species of tau in the central nervous system. Cell Motil Cytoskel 8:210-226.
32. Harada A, Oguchi K, Okabe S, Kuno J, Terada S, Ohshima T, Lanté F, Buisson A (1994) Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature 369: 488-491.
33. Dawson H, Cantillana V, Jansen M, Wang H, Vitek M, Wilcock D, et al. (2010) Loss of tau elicits axonal degeneration in a mouse model of Alzheimers disease. Neuroscience 169: 516-531.
34. Frandemiche ML, Seranno SD, Rush T, Borel E, Elie A, Arnal I, et al. (2014) Activity-dependent tau protein translocation to excitatory synapse is disrupted by exposure to amyloid-β oligomers. J Neurosci 34:6084-6097.
35. Zempel H, Mandelkow E (2015) Tau missorting and spastin-induced microtubule disruption in neurodegeneration: Alzheimer disease and hereditary spastic paraplegia. Mol Neurodegen 10:68-80.
36. Planel E, Richter KE, Nolan CE, Finley JE, Liu L, Wen Y, et al. (2007) Anesthesia Leads to Tau Hyperphosphorylation through Inhibition of Phosphatase Activity by Hypothermia. Journal of Neuroscience 27:3090-3097.
37. Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin M, Terro F (2013) Tau protein phosphatases in Alzheimers disease: The leading role of PP2A. Ageing Res Rev 12:39-49.

38. Mao P, Reddy PH (2011) Aging and amyloid β-induced oxidative DNA damage and mitochondrial dysfunction in Alzheimer's disease: Implications for early intervention and therapeutics. Biochim Biophys Acta 1812:1359-1370.
39. Dubal DB, Broestl L, Worden K (2012) Sex and gonadal hormones in mouse models of Alzheimer's disease: What is relevant to the human condition? Biol Sex Diff 3(1):24-40.
40. Reitz C, Brayne C, Mayeux R (2011) Epidemiology of Alzheimer disease. Nature Rev Neurol 7:137-52.
41. Zamboni V, Jones R., Umbach A, Ammoni A, Passafaro M, Hirsch E., Merlo, G (2018) Rho GTPases in intellectual disability: From genetics to therapeutic opportunities. Int J Mol Sci 19:1821.
42. Huang G, Sun Z, Li H, Feng D (2017) Rho GTPase-activating proteins: Regulators of Rho GTPase activity in neuronal development and CNS diseases. Mol Cell Neurosci 80:18-31.
43. Nadif Kasri N, Van Aelst L (2008) Rho-linked genes and neurological disorders. Pflugers Archiv: Eur J of Phys 455:787-797.
44. Hooff GP, Wood WG, Muller WE, Eckert GP (2010) Isoprenoids, small GTPases and alzheimer's disease. Biochim Biophys Acta, 1801:896-905.
45. Shafer W (1992) Protein prenylation: genes, enzymes, targets, and functions. Annual Rev Genet 26:209-237.

46. Liang P, Ko T, Wang AH (2002) Structure, mechanism and function of prenyltransferases. Eur J Biochem 269:3339-3354.
47. Roskoski R (2003) Protein prenylation: A pivotal posttranslational process. Biochem Biophys Res Comm 303:1-7.
48. Benetka W, Koranda M, Eisenhaber F (2006) Protein prenylation: an (almost) comprehensive overview on discovery history, enzymology, and significance in physiology and disease. Monatshefte Für Chemie - Chemical Monthly 137(10):1241-1281.
49. Molnár G, Dagher M, Geiszt M, Settleman J, Ligeti E (2001) Role of prenylation in the interaction of Rho-family small GTPases with GTPase activating proteins. Biochemistry 40:10542-10549.
50. Auer M, Hausott B, Klimaschewski L (2011) Rho GTPases as regulators of morphological neuroplasticity. Annals Anat 193:259-266.
51. Spiering D, Hodgson L (2011) Dynamics of the Rho-family small GTPases in actin regulation and motility. Cell Adhes Migr 5:170-180.
52. Ridley AJ (2001) Rho family proteins: Coordinating cell responses. Trends Cell Biol 11:471-477.
53. Degeer J, Lamarche-Vane N (2013) Rho GTPases in neurodegeneration diseases. Exp Cell Res 319:2384-2394.
54. Govek EE, Hatten ME, Van Aelst L (2011) The role of rho GTPase proteins in CNS neuronal migration. Dev Neurobiol 71:528-553.

55. Samuel F, Hynds DL (2010) RHO GTPase signaling for axon extension: Is prenylation pmportant? Mol Neurobiol 42:133-142.
56. Musilli M, Nicolia V, Borrelli S, Scarpa S, Diana G (2013) Behavioral effects of rho GTPase modulation in a model of alzheimer's disease. Behav Brain Res 237:223-229.
57. Petratos S, Li QX, George AJ, Hou X, Kerr ML, Unabia SE, Francés S, Santpere G, Ferrer I, Galea E. (2008) The β-amyloid protein of Alzheimer's disease increases neuronal CRMP-2 phosphorylation by a rho-GTP mechanism. Brain 131:90-108.
58. Huesa G, Baltrons MA, Gomez-Ramos P, Moran A, Garcia A, Hidalgo J Francés S, Santpere G, Ferrer I, Galea E (2010) Altered distribution of RhoA in Alzheimer's disease and AβPP overexpressing mice. J Alzheimer's Dis: 19:37-56.
59. Mendoza-Naranjo A, Gonzalez-Billault C, Maccioni RB (2007) Aβ1-42 stimulates actin polymerization in hippocampal neurons through Rac1 and Cdc42 Rho GTPases. J Cell Sci 120:279-288.
60. Wang PL, Niidome T, Akaike A, Kihara T, Sugimoto H (2009) Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene. J Neurosci Res 87:2105-2114.
61. Desire L, Bourdin J, Loiseau N, Peillon H, Picard V, De Oliveira C, Bachelot F, Leblond B, Taverne T, Beausoleil E, Lacombe S, Drouin D, Schweighoffer F (2005) RAC1 inhibition targets amyloid precursor protein processing by γ-

secretase and decreases aβ production *in vitro* and *in vivo*. J Biol Chem 28045):37516-37525.

62. Stankiewicz TR, Linseman DA (2014) Rho family GTPases: Key players in neuronal development, neuronal survival, and neurodegeneration. Front Cell Neurosci 8:314.
63. Wang G, Huang Y, Wang L, Zhang Y, Xu J, Zhou Y, Lourenco GF2, Zhang B, Wang Y, Ren RJ, Halliday GM, Chen SD (2016) MicroRNA-146a suppresses ROCK1 allowing hyperphosphorylation of tau in Alzheimer's disease. Sci Rep 6:26697.
64. Henderson BW, Gentry EG, Rush T, Troncoso JC, Thambisetty M, Montine TJ, Herskowitz JH (2016) Rho-associated protein kinase 1 (ROCK1) is increased in Alzheimers disease and ROCK1 depletion reduces amyloid-β levels in brain. J Neurochem 138:525-531.
65. Ma Q, Yang F, Calon F, Ubeda OJ, Hansen JE, Weisbart RH, Beech W, Frautschy SA, Cole GM (2008) P21-activated kinase-aberrant activation and translocation in Alzheimer disease pathogenesis. J Biol Chem 283:14132-14143.
66. Inoue E, Deguchi-Tawarada M, Togawa A, Matsui C, Arita K, Katahira-Tayama S, Sato T, Yamauchi E, Oda Y, Takai Y (2009) Synaptic activity prompts γ-secretase–mediated cleavage of EphA4 and dendritic spine formation. J Cell Biol 185:551-564.

67. Posada-Duque RA, López-Tobón A, Piedrahita D, González-Billault C, Cardona-Gomez GP (2015) P35 and Rac1 underlie the neuroprotection and cognitive improvement induced by CDK5 silencing. J Neurochem 134:354-370.
68. Boo JH, Sohn JH, Kim JE., Song H, Mook-Jung I (2008) Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch1. Biochem Biophys Res Comm 372:913-917.
69. Aguilar BJ, Zhu Y, Lu Q (2017) Rho GTPases as therapeutic targets in Alzheimer's disease. Alzheimers ResTher 9(1):97.
70. Perez SE, Getova DB, He B, Counts SE, Geula C, Desire L, Coutadeur S, Peillon H, Ginsberg SD, Mufson EJ. (2012) Rac1b increases with progressive tau pathology within cholinergic nucleus basalis neurons in Alzheimers disease. Am J Pathol 180(2):526-540.
71. Manterola L, Hernando-Rodríguez M, Ruiz A, Apraiz A, Arrizabalaga O, Vellón L, Alberdi E, Cavaliere F, Lacerda HM, Jimenez S, Parada LA, Matute C, Zugaza JL (2013) 1–42 β-amyloid peptide requires PDK1/nPKC/Rac 1 pathway to induce neuronal death. Transl Psychiatry 3:e219.
72. Lee M, You H, Cho S, Woo C, Yoo M, Joe E, Kim J (2002) Implication of the small GTPase Rac1 in the generation of reactive oxygen species in response to β-amyloid in C6 astroglioma cells. Biochem J 366:937-943.
73. Sellers KJ, Elliott C, Jackson J, Ghosh A, Ribe E, Rojo AI, Jarosz-Griffiths HH, Watson IA, Xia W, Semenov M, Morin P, Hooper NM, Porter R, Preston

J, Al-Shawi R, Baillie G, Lovestone S, Cuadrado A, Harte M, Simons P, Srivastava DP, Killick R. (2018) Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil. Alzheimers Dement 14:306-317.

CHAPTER III

NON-PRENYLATED RAC1 IN ALZHEIMER'S DISEASE PATHOLOGICAL MARKERS

A Paper to be Submitted for Publication in *Cellular Signalling*

Rawand K. Chabayta and DiAnna L. Hynds
Texas Woman's University Department of Biology. Denton, TX 76204

Corresponding Author: DiAnna L. Hynds, Ph.D.
Professor, Department of Biology and
Affiliated Professor, Woodcock Institute
PO Box 425799
Denton, T 76204-5799
940-898-2359
Fax: 940-898-2382
dhynds@twu.edu

Highlights:

- Overexpressing non-prenylatable Rac1 does not alter the levels of the amyloid precursor protein, β amyloid, tau or tau hyperphosphorylation in B35 neuroblastoma whole cell lysates.

- Overexpressing non-prenylatable Rac1 does not increase or decrease the activity of β-secretase, presenilin 2 (the active subunit of γ-secretase) or the tau phsphorylating kinase glycogen synthase kinase 3 β.

- Overexpressing either wild-type or non-prenylatable Rac1 decreases levels of amyloid precursor protein decrease in isolated cell membranes; however; overexpressing non-prenylatable Rac1 does not decrease expression of amyloid precursor protein as much as overexpressing wild-type Rac1.

- We interpret these data to suggest that Rac1 likely has a minor role in producing β amyloid. However, the lack of a large effect on the production of Alzheimer's disease pathological markers may indicate that the major role for Rac1 in Alzheimer's disease pathology is downstream of β amyloid and tau aggregations.

Abstract:

Alzheimer's disease (AD) is currently the most common cause of dememtia in the elderly. AD is pathologically characterized by the formation of cerebral amyloid plaques, formed from aberrant cleavage of the amyloid precursor protein (APP), and neurofibrillary tangles, formed by aggregation of hyperphosphorylation of microtubule-associated protein tau. Dysregulation of Rac1 has been implicated in AD pathology. However, it is unclear whether Rac1 activity contributes to AD pathology or AD alters Rac1 activity. As a member of the Rho family of guanosine triphosphatases (GTPases), activated Rac1 is a key regulator in many cellular functions. Rac1 is activated by binding guanosine triphosphate (GTP), a process thought to require prenylation of Rac1, a post-translational modification. The role of subcellularly altered Rac1 in AD pathology has not been established. To address this, we investigated how overexpressing an emerald green fluorescent protein tagged, wild-type (EmGFp-Rac1) or non-prenylatable Rac1 construct (EmGFP-Rac1^{C189A}), affected the production of AD-associated pathological proteins, β- and γ-secretase and the tau-phosphorylating kinase GSK3β in B35 neuroblastoma cells. In cells transfected with either wild-type EmGFP-Rac1 or EmGFP-Rac1^{C189A}, APP levels decreased at the membrane compared to cells expressing EmGFP alone, with the decrease due to overexpressing EmGFP-Rac1^{C189A} occurred to a lesser extent. Overexpressing neither construct affected the total levels of intracellular or extracellular β amyloid, tau, GSK3β, γ- or β-secretase. Our interpretation of

these results is that the altering the subcellular localization of wild-type Rac1 plays a minor role in the processing of Aβ in the plasma membrane and its cytosolic localization leads to increased tau phosphorylation, suggesting a potential role in the formation of neurofibrillary tangles. Since overexpressing non-prenylatable Rac1 had either a lesser or no effect on production of AD markers, these results may further suggest that activation (GTP loading) of cytosol-localizated wild-type Rac1 may be important in tau pathology, but it is also possible that Rac1 plays a role in responding to AD pathological markers, rather than their production.

Keywords: β amyloid, Cortical neurons, Geranylgeranylation, GSK3β, Neuroblastoma, Rho GTPases, Secretases, Tau.

1. Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that is mostly observed in the elderly [1]. Worldwide, there are 47.5 million people suffering from AD. In 2017, there were about 5.5 million sufferers in the U.S. alone, with an estimated health-care cost of 232 billion dollars [2]. AD is histopathologically characterized by the formation of neurofibrillary tangles and amyloid plaques in the brain [3]. Neurofibrillary tangles are formed from the hyperphosphorylation of a microtubule associated protein, tau [4]. β amyloid

plaques are produced from the aberrant cleavage of the amyloid precursor protein (APP) [4].

Ras-related C3 botulinum toxin substrate 1, or Rac1, is a member of the Rho family of guanosine triphosphatases (GTPases) and a key regulator in neurite outgrowth, dendrite extension [5], lamellipodia formation [6], plasticity [7] and actin cytoskeleton organization [8]. Rac1 has been implicated in many neurological diseases, including AD [9]. Most studies have assessed whether Rac1 activation (ability to interact with effectors) influences disease states or progression. For instance, inhibition of Rac1 negatively regulates the synthesis of APP [10] and decreases β amyloid [11]. The knockdown of cyclin-dependent kinase 5 (CDK 5) decreases tau phosphorylation and increases Rac activity and leads to improvements in cognitive functions [12].

Rac1 activation is generally thought to require prenylation for the proper insertion into cell membranes to propagate signals [13]. Prenylation is a post-translational modification where a geranylgeranyl moiety is attached to Rho GTPases at their C terminal CaaX motif (where C is cysteine, a's are aliphatic amino acids and X is isoleucine or leucine) by geranylgeranyltransferase-I (GGTase-I) [13]. Rac1 is active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP) [14]. Rac1 prenylation has been thought to be required for proper localization to plasma membrane and activation by GTP loading [14]. However, in our previous work, we have found activated (bound to GTP), non-prenylated Rac1 in the cytosol,

suggesting that Rac1 might be active without its localization to plasma membranes [15]. Therefore, it is possible that aberrantly-located non-prenylated Rac1 can alter production of APP and tau leading to AD pathology.

We have generated a mutant, non-prenylated Rac1 construct referred to as EmGFP-Rac1^{C189A}, where cysteine is mutated to alanine in the CaaX motif [15]. When expressed, this mutated construct of Rac1 decreases Rac1 association with membranes and its activation by GTP loading, particularly at the plasma membrane [15]. Here, we assessed how EmGFP-Rac1^{C189A} expression in neuroblastoma cells affects APP and tau production as well as the activity of secretases and kinases that are associated with AD pathology.

2. Materials and Methods

2.1. Cell culture:

2.1.1. B35 rat neuroblastoma cells:

Unless otherwise noted, chemicals were purchased from Sigma (St. Louis, MO). B35 rat neuroblastoma cells were obtained from American Type Culture Collection (ATCC) in Manassas, Virginia (ATCC® CRL-2754™). Cells were routinely maintained in an incubator with 5% CO_2 at 37 °C. Neuroblastoma cells were grown in a medium containing Dulbecco's Modified Eagle's Medium and Nutrient Mixture F12 (DMEM/F12; Invitrogen, Carlsbad, CA) in a 1:1 ratio supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and penicillin/streptomycin (Sigma, St. Louis, MO). Cells were passed when 90-95%

confluent. For western blot experiments, cells were seeded in six-well plates at a density of 20,000 cells/cm^2 and for immunocytochemistry experiments, cells were seeded at a density of 5,000 cells/cm^2 on 12 mm glass coverslips (1.5 glass, Fisher, Houston, TX) in 24-well plates (tissue cultureware was from Fisher, Houston, TX) that were coated overnight at 37 °C with poly-D-lysine (Sigma, St. Louis, MO). Cells were maintained in serum-containing medium (SCM) to grow to the proper density, then after four hours of transfection using Lipofectamine 2000 or 3000 (Invitrogen, Carlsbad, CA), cells were placed in serum free media (SFM) for 24 hours to upregulate growth and trophic factor receptors.

2.1.2. Rat cortical neurons:

Animal procedures were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the TWU Institutional Animal Care and Use Committee (IACUC). Rat pups of both sexes were used in all experiments involving animals and potential sex differences were not assessed. Rat cortical neurons were prepared from newborn rat brains. Newborn Sprague-Dawley pups (Charles River, Wilmington, MA) were sacrificed by quick decapitation within 24 hours of birth. The dorsal cranium was removed, and meninges and blood vessels were removed from extracted brains using fine forceps. Cortices were dissected away from the rest of the brain. The cortical tissue was cut into small pieces with a sterile knife and then digested for 30 minutes in papain (2 mg/ml in high glucose-serum free Dulbecco's Modified

Eagle's Medium, HG-SFDMEM, Invitrogen, Carlsbad, CA) at 37 °C. Cells were pelleted in HG-SFDMEM for 5 minutes at 500 x *g* and the supernatant was removed. Cells were then resuspended in HG-SFDMEM and plated (20,000 cells/cm^2) on poly-D-lysine- (PDL, Sigma, St. Louis, MO) coated six-well plates and kept in the incubator. The media was changed after four hours to neurobasal media (NBM) with 10% B27 supplement (Thermo Fisher Scientific, Houston, TX). Cells were kept in NBM for a week to stabilize before transfections were performed. Six-well plates were coated by filling them with 2 ml PDL and incubating them for one hour at 37 °C. Excess PDL was removed and the plates were washed twice with phosphate-buffered saline (PBS) and then kept in the incubator at 37 °C.

2.2. Generation of expression vectors:

We previously made mammalian expression vector containing wild-type Rac1 that is N-terminally tagged with emerald green fluorescence protein (referred to as EmGFP-Rac1) [15]. This construct can be prenylated and was created by incorporating the open reading frame of Rac1 (Open Biosystems, Houston, TX) into a mammalian expression vector that fused EmGFP to the N terminus of Rac1 (Invitrogen, Carlsbad, CA). Rac1 mutant constructs were created using site-directed mutagenesis (kit from Invitrogen, Carlsbad, CA) to change the cysteine at the CaaX box (C = cysteine, a = any two aliphatic amino acids, X = any amino acid) to an alanine (referred to as EmGFP-Rac1^{C189A}). This

mutant construct is non-prenylated and cannot be attached to plasma membrane [15].

2.3. Transfection:

For all experiments, B35 cells were grown to approximately 95% confluency and cultures of primary neurons were transfected with plasmids to express emerald green fluorescent protein (EmGFP), wild-type EmGFP-Rac1 or non-prenylatable EmGFP-Rac1^{C189A}, using lipofectamine 2000 or 3000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. For western blotting, 3 µg of plasmid were used per well of a 6-well plate and 0.8 µg of plasmid were used per well of a 24-well plate for immunocytochemistry experiments.

2.4. Subcellular fractionation:

Following four hours of transfection, B35 rat neuroblastoma cells were placed in serum-free media for 24 hours. Then transfected and untransfected cells were subjected to fractionation into cytosolic (fraction 1) and membrane (fraction 2) fractions by using the S-PEK proteolysis kit (Calbiochem, Burlington, MA) according to the manufacturer's instructions. To assess the efficiency of fractionation, fractionated samples were subjected to western blotting for the membrane associated protein, GABA-A$_{\beta 2}$ receptor (Abcam ab186875, Cambridge, MA) , and the cytosolic and membranous protein, extracellular regulated kinase 1/2 (ERK1/2, Abcam ab184699, antibodies used at 1:500 and were from Abcam, Cambridge, MA).

2.5. Immunocytochemistry:

Following transfection, B35 rat neuroblastoma cells were placed in serum-free media for 24 hours. Cells were then fixed in 4% paraformaldehyde for 30 minutes and washed twice with PBS. Fixed cells were permeabilized and blocked in blocking buffer (PBS containing 0.1% triton X-100, 0.1% bovine serum albumin, and 1.5% pre-immune secondary-specific serum) for 30 minutes at room temperature. The cells were incubated with the primary antibody, rabbit anti-tau (Abcam ab76128, Cambridge, MA), at a 1:200 dilution in blocking buffer and incubated overnight at 4 °C. Following primary antibody incubation, cells were washed twice in blocking buffer, then incubated in 1:200 goat anti-rabbit IgG secondary antibody which was conjugated with Alexafluor 647 (Jackson immunolabs, West Grove, PA). The 1:200 dilution of secondary antibody was chosen because it was the lowest concentration at which we could observe substantial staining without having a large amount of non-specific binding, as determined in the absence of primary antibody. After the secondary antibody incubation, cells were washed twice with blocking buffer and twice with PBS. Then coverslips were mounted on clean slides with a drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vectashield, Vector Labs, Burlingame, CA) to stain the double-stranded DNA and mark nuclei. Digital images were captured through a 40X objective lens using a Nikon A1R-A1 confocal system with a Nikon Eclipse Ti inverted microscope and NIS elements imaging software (Nikon Instruments, Melville, New York). To assess

autofluorescence and set background levels in individual experiments, untransfected fixed cells were used. We did not observe appreciable fluorescence in primary-omitted controls. All conditions for capturing images, including exposure time and lamp intensity, were kept fixed and constant throughout each experiment.

2.6. Western blotting:

After transfection, B35 rat neuroblastoma cells were placed in SFM for 24 hours. Then media was discarded, and cells were washed gently with ice-cold 1X PBS. Ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM EDTA, and 1.0% IGEPAL CA-630), supplemented with 1X protease inhibitor cocktail (PIC, Sigma-Aldrich, St. Louis, MO) to prevent protein degradation, was added to each well and cells were incubated on ice for 15 minutes. Cells were then scraped, and lysates were transferred to microfuge tubes and centrifuged to pellet nuclei at 13,000 x *g* for 5 minutes. Supernatants were transferred to new microfuge tubes and stored at -20 °C until use.

Protein concentrations were determined using the Bicinchoninic Acid Protein Assay (BCA, Pierce, Houston, TX). The absorbance of the samples was measured at 562 nm on a plate reader. The protein concentrations of samples were calculated by comparing their absorbance to the standard curve of the bovine serum albumin (BSA).

Samples (30 µg lysates) were prepared in microfuge tubes using equivalent amounts of protein by diluting cell lysates 1:1 with 2X sample buffer

(Sigma, St. Louis, MO). Samples were then boiled for five minutes and were electrophoresed through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 130 V constant voltage for 90 minutes. Proteins from SDS-PAGE gels were transferred to nitrocellulose membranes at 400 mA constant current for 90 minutes using a wet BioRad electrotransfer apparatus. Following transfer, the membrane was blocked in 5% non-fat milk in tris buffered saline containing 0.1% tween-20 (TBST) for one hour at room temperature, then incubated overnight (with rocking) at 4 °C with primary antibody at a dilution of 1:5000. Primary antibodies included mouse anti-actin (Abcam ab8226, Cambridge, MA), rabbit anti-amyloid precursor protein (APP, Abcam ab32136, Cambridge, MA), rabbit anti-tau (Abcam ab76128, Cambridge, MA), rabbit anti-presenilin 2 (Abcam ab51249, Cambridge, MA), and rabbit anti-β-amyloid cleavage enzyme (BACE1, Abcam ab2077, Cambridge, MA). Membranes were washed with TBST four times for 10 minutes each before incubation in IRDye 680RD (infrared fluorescence dye) conjugated goat anti-rabbit secondary antibodies at a dilution of 1:5000 for one hour (with rocking) at room temperature. Following secondary antibody incubation, membranes were washed with TBST four times for 10 minutes each, then immunoreactive bands were visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band intensities were calculated by subtracting background from the total intensity then multiplying the result by area of the band (to compensate for any variation in the size of regions of interest defined around

each quantified band). All experiments were run at least three times. Interexperimental variability was controlled by normalizing data to those collected for untransfected cultures, which were not used for statistical comparisons. In some experiments, irrelevant lanes were removed from blots for clarity, but all displayed lanes are from the same blot and no individual sections of images were treated differently for display purposes.

2.7. ELISA Analysis:

We analyzed the level of β-secretase activity, extracellular and intracellular Aβ production, and levels of phosphorylated tau using enzyme-linked immunosorbent assay kits. For all ELISA analyses, B35 neuroblastoma cells were transfected with EmGFP alone, wild-type EmGFP-Rac1 or non-prenylatable EmGFP-Rac1^{C189A} for 4 hours, washed twice with PBS and placed in SFM for 24 hours at 37 °C in 95% humidity. Samples were scraped and centrifuged at 500 x *g* to pellet cells. Aliquots were saved from the conditioned medium in the supernatant and from the cell pellet after lysing. For each type of ELISA, samples were done in duplicate using either the supernatant (for extracellular Aβ) or the lysed cell pellet (for intracellular Aβ, β-secretase activity and tau hyperphosphorylation). For analysis, background levels were substracted from duplicate averages and relative amounts were determined by fit to either a linear or a quadratic curve.

2.7.1. β secretase activity assay:

The levels of β-secretase activity in samples were detected using a β-secretase activity assay fluorometric kit (Abcam, Cambridge, MA). Secretase activity levels were measured from cell extracts. For this, the medium was discarded, and cells were washed gently with ice-cold 1X PBS. Cells were resuspended in 100 µl of β-secretase extraction buffer and incubated on ice for 30 minutes. Cells were scraped, and lysates were transferred to microfuge tubes and centrifuged to pellet nuclei at 13,000 x *g* for 5 minutes at 4 °C using a cold microcentrifuge. Supernatants were collected and transferred to new clean microfuge tubes and kept on ice. Fifty µl of each sample were transferred to the 96-well plate coated with a β-secretase-specific peptide conjugated to two reporter molecules (supplied with the kit), whose cleavage unquenches a fluorescent signal. Quantification was carried out according to the supplied instructions. The plate was incubated in the dark at 37 °C for one hour and the fluorescence was measured on a fluorescent microplate reader at excitation/emission = 335/495 nm.

2.7.2. Measurement of amyloid β peptide 1-42:

Cells were pelleted by centrifugation at 500 x *g* for 5 minutes. A sample of conditioned medium was taken from the supernatant and Aβ levels were measured using a rat amyloid β peptide 1-42 (Aβ 1-42) ELISA kit (MyBioSource, San Diego, CA) according to the manufacturer's instructions. Plate wells were coated with one Aβ antibody and detection occurred through a sandwich with a

second Aβ antibody directed to a different epitope and conjugated to horse radish peroxidase, allowing Aβ quantification by colorimetric determination.

2.7.3. Measurement of phosphorylated microtubule-associated protein Tau:

Phosphorylated tau protein levels were measured using a rat phosphorylated microtubule-associated protein tau (pMAPT/pTAU) ELISA kit (MyBioSource, San Diego, CA) according to the manufacturer's instructions. This kit was also a sandwich ELISA with the plate wells coated with one anti-phospho-tau antibody and detection occurred with a second anti-phospho tau antibody conjugated to horse radish peroxidase, allowing phosphorylated tau quantification by colorimetric determination. Specific phosphorylation sites for either antibody were not identified by the kit.

2.8. Statistical analysis:

The sampling unit for all experiments was an individual culture. Each experiment was repeated at least three times. Each condition had three replicates in each immunocytochemical experiment, making a total N = 9. Each condition had a single culture in each western blotting experiment, making N = 3/condition in these experiments. All data were normalized to untransfected control and measurements for transfected cells with wild-type or mutant Rac1 were compared to cells transfected with EmGFP alone. Data were analyzed for differences across groups by using one-way univariant analyses of variance (ANOVA), with treatment as a fixed factor, and average fluorescence intensity

measurement as the dependent variable for all types of experiment. The level of significance was set at $\alpha = 0.05$. When equal variance could be assumed, differences from transfected EmGFP (control) cultures were determined using the Least Significant Difference (LSD). Tamhane's T2 post-hoc test was used when Levene's test for homogeneiety was significant and equal variances could not be assumed.

3. Results

3.1. Altering Rac1 prenylation did not affect tau levels in B35 cells

Rac1 dysregulation has been implicated in AD pathology [16]. The decrease in geranylgeranyltransferase-I (GGT-I) activity seen in ageing and AD leads to increased levels of geranylgeranyl pyrophosphate (GGPP), but decreased levels of prenylated proteins [17, 18]. Therefore, it is possible that altered prenylation of Rac1 might contribute to AD pathology in forming the amyloid plaques and the neurofibrillary tangles. To assess the effects of Rac1 prenylation on production of tau, we transfected cells with EmGFP alone, wild-type EmGFP-Rac1, and nonprenylatable EmGFP-Rac1^{C189A}.

Here, we assessed the amount of total tau and cytosolic tau in each condition using western blotting of whole cell lysates. We routinely assessed whether equivalent amount of protein was loaded in western blotting experiments by western blotting for actin. We did not observe changes in actin protein content across conditions in western blots from total cell lysates and analyses

conducted where each immunoreactive band was normalized to actin expression did not differ from analyses that did not include this normalization. Compared to cells transfected with EmGFP only, overexpressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change the levels of total tau obtained from whole cell lysates (Figure 3.1A and B). In AD, the majority of tau sequesters in aggregates in the cytosol, rather than being associated with microtubules [19]. Therefore, we assessed the levels of cytosolic tau in B35 cells expressing wild-type or nonprenylatable Rac1. Compared to cells transfected with EmGFP only, expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change the levels of cytosolic tau (Figure 3.1C and D).

Efficient separation of cytosolic and membrane fractions was consistently assessed by western blotting for proteins localized to specific subcellular fractions [15]. Here, western blots for the membrane-localized γ amino butyric acid A receptor β2 subunit (GABA-A$_{\beta 2}$) showed that this protein was found nearly exclusively in the membrane fraction, and western blots for the cytosolic extracellular-regulated kinase 1/2 (ERK 1/2) showed immunoreactive bands in both the cytosol and the membrane fractions since ERK 1/2 is translocated to the plasma membrane to interact in signalling complexes (Figure 3.1E, blots). Together, these data demonstrate efficient separation of cellular fractions. Descriptions of vector construction and morphological phenotypes elicited by overexpressing Rac1 have been previously described [15]. In B35 cells, quantification of Rac1 western blots showing both endogenous and expressed

proteins indicated that expressed proteins averaged 33.2-fold over the expression of endogenous proteins, with EmGFP-Rac1 or EmGFP-Rac1^{C189A} overexpression being between about 20 to 50 fold higher than the endogenous expression of Rac1 (Figure 3.1 E, graph). Transfection efficiencies were routinely monitored in immunocytochemical experiments using direct read of EmGFP expression in each condition. Transfection efficiencies ran between 90% and 98% in B35 cells (Figure 1F).

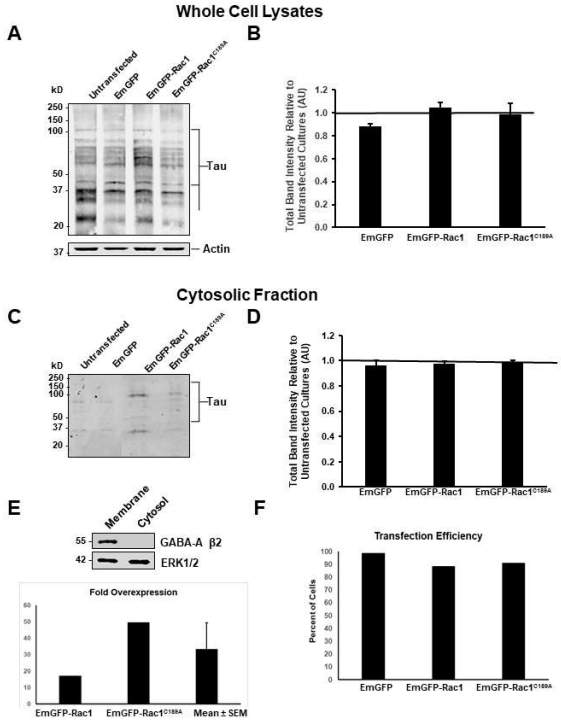

Fig. 3.1 Expression of EmGFP-Rac1^{C189A} did not affect total levels of tau or cytosolic tau. (A) Representative western blot of whole cell lysates from B35 cells either not transfected (untransfected) or transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} and blotted for all total tau (upper blot). Actin loading controls indicate equal protein loading in all conditions. (B) Immunoreactive tau bands were quantified by adding together the background-subtracted average fluorescence intensities for all tau immunoreactivity bands higher than 37 kD using the LiCor Odyssey CLx system for image capture and determination of average fluorescence intensities using a formula that corrects for variation

in band area. (C) Representative western blot for tau from the cytosolic fraction of B35 cells either untransfected or transfected with EmGFP, EmGFP-Rac1 and EmGFP-Rac1^{C189A}. (D) Quantification of protein immunoreactivity normalized to untransfected cultures and calculated as above indicates that altering the localization of Rac1 did not affect the total levels of Tau. (E) Representative western blots for the γ amino butyric acid receptor β2 subunit (GAGA-A$_{\beta 2}$, a membrane-localized protein) and for extracellular regulated knase 1/2 (ERK 1/2, a cytosolic protein that can associated with plasma membranes) demonstrates minimal GABA-A$_{\beta 2}$ in the cytosolic fraction and near equal amounts of ERK 1/2 in both the membrane and cytosol fractions, indicating efficient separation of cell fractions. The graph indicates the increase in expression comparing expressed EmGFP-Rac1 or EmGFP-Rac1C189A to the endogenous protein. The last bar indicates average fold increase in transgene expression compared to endogenous Rac1 expression across conditions (mean ± SEM). (F) Percent of cells transfected in each condition. Data in B and D are means ± SEM for 3 separate experiments. Horizontal line in B and D indicates the untransfected control level. N=3 cultures per condition and ANOVA analysis indicated that there was not a difference between groups.

We used immunocytochemistry for tau to determine if there were spatial accumulations of tau that were not revealed by western blotting analyses. We either did not transfect cells or transfected B35 cells with EmGFP, EmGFP-Rac1, or EmGFP-Rac1^{C189A} and assessed the mean fluorescence intensity of tau in regions of interest that encompassed an entire cell in each condition (15 cells/well were quantified with each condition performed in triplicate in each of 3 independent experiments, N = 9). From the number of cells in each photomicrograph (identified by staining with DAPI), EmGFP expression was evident only in transfected cells. Transfection efficiency was between 85% and 90% and only transfected cells were used for quantification. From observing the *Z* stacks and maximum projection images in each photomicrograph, tau was fairly evenly distributed through the cytoplasm and there were no apparent differences in the amount of tau expressed in each condition (Figure 3.2). Quantification of mean fluorescence intensity from transfected cells from a total

of nine cultures/condition confirmed that there were no significant differences between the total amounts of tau across experimental conditions, indicating that changing the localization of Rac1 does not affect tau levels (Figure 3.3).

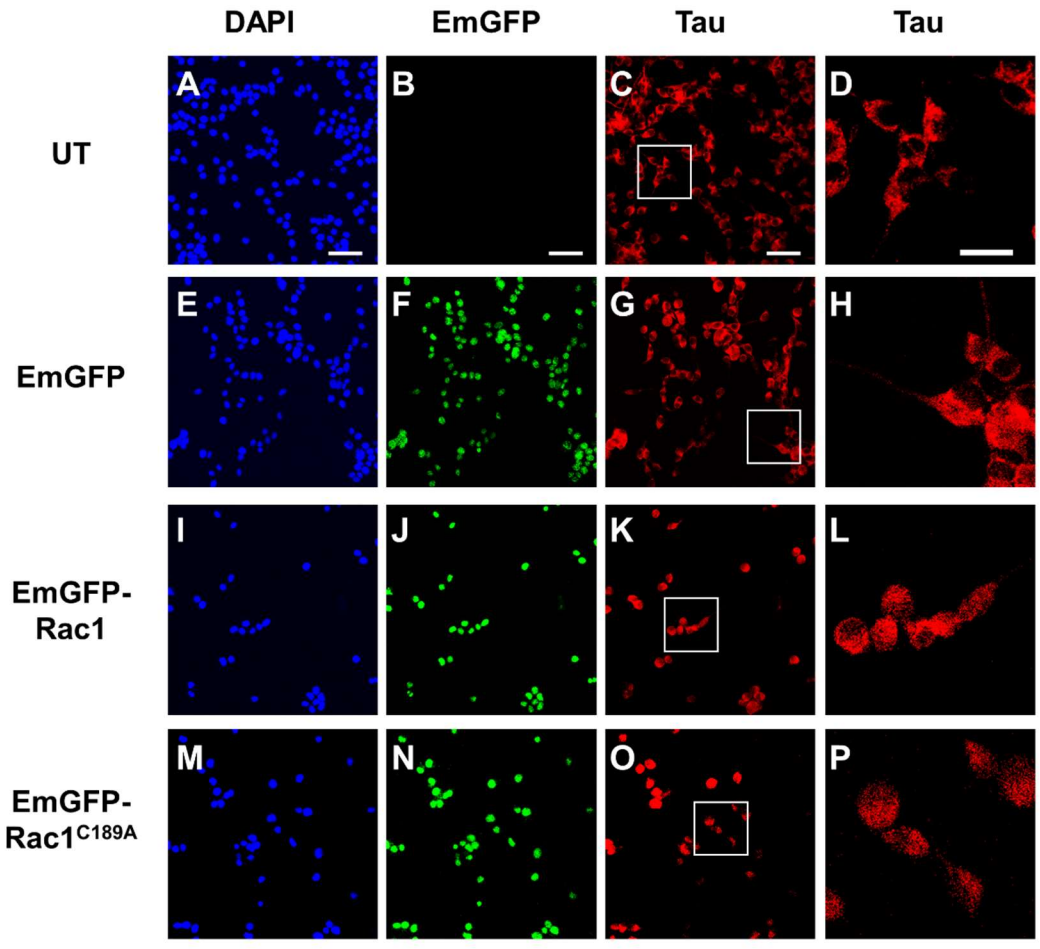

Fig. 3.2 Overexpressing non-prenylatable Rac1 did not alter tau expression in B35 cells as assessed using immunocytochemistry. Representative maximum projection images from Z stacks of B35 cells either untransfected (UT; A-D) or transfected with EmGFP only (E-H), EmGFP-Rac1 (I-L) or EmGFP-Rac1^{C189A} (M-P). Absence of EmGFP in

untransfected cells indicates successful transfection in the other conditions (compare B to F, J and N). Transfection occurred in 85% to 90% of transfected cells (determined by dividing the total number of transfected cells, indicated by EmGFP expression, by the total number of cells, indicated by DAPI staining). Tau immunoreactivity was observed in all cells labeled with rabbit anti-tau primary antibodies, followed by Alexafluor 647-conjugated secondary antibodies (C, G, K and O). Only cellular debris was observed when the primary antibody was omitted. Boxes in C, G, K and O labeling tau are areas shown at higher magnification in panels D, H, L and P, where tau immunoreactivity is evident in the cytoplasm and processes, but not nuclei. Images are representative of those captures from 3 independent experiments done in triplicate (N = 9). Scale bars in A, B and C = 50 μm and are valid for all images in the first 3 columns. Scale bar in D = 10 μm and is valid for panels D, H, L and P.

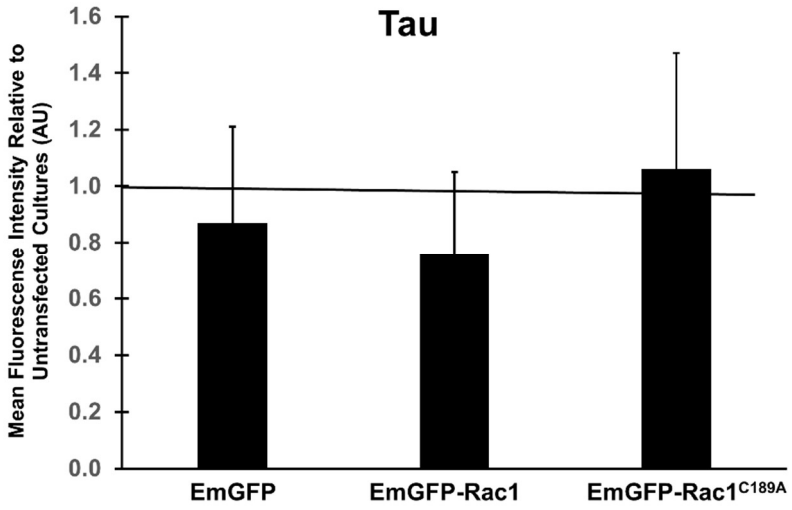

Fig. 3.3 Quantification from immunocytochemical experiments shows overexpressing non-prenylatable Rac1 does not alter tau levels. Quantification of the mean fluorescence intensity indicated that expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect tau levels, compared to cells transfected with EmGFP only. Horizontal line in the graph indicates the value for the untransfected control condition, which was used to normalize across experiments. Data are means ± SEM for each condition, which was performed in triplicate in each of 3 separate experiments (N = 9 cultures in each condition) with at least 5 cells being quantified for each experimental replicate.

3.2. Overexpressing EmGFP-Rac1 increases tau phosphorylation in B35 cells

Formation of neurofibrillary tangles is one of AD's hallmarks. These neurofibrillary tangles are primarily composed of the neuronal microtubule associated protein (MAP) tau, in a hyperphosphorylated form [20]. Even though altering the subcellular localization of Rac1 by overexpressing its non-prenylatable form (EmGFP-Rac1^{C189A}) did not affect tau levels, the amount of tau phosphorylation could be altered. To assess how Rac1 prenylation affects the phosphorylation of tau, we either did not transfect B35 cells or transfected them with EmGFP, EmGFP-Rac1, or EmGFP-Rac1^{C189A}. We then assessed the levels of phosphorylated tau in whole cell lysates using a phosphorylated tau ELISA kit. Compared to cells transfected with EmGFP only, expressing EmGFP-Rac1 significantly increased the level of phosphorylated tau, while transfection with EmGFP-Rac1^{C189A} did not (Figure 3.4). This suggests that Rac1 might be involved in neurofibrillary tangles formation.

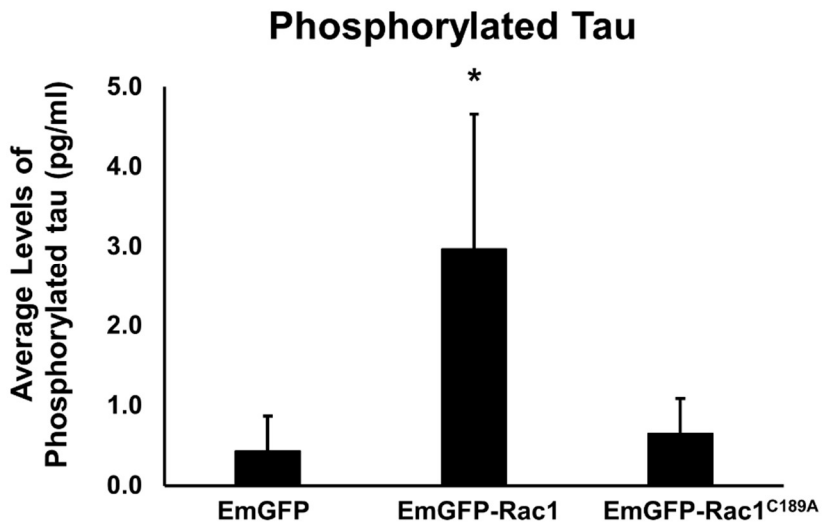

Fig. 3.4 Overexpression of EmGFP-Rac1, but not EmGFP-Rac1^{C189A}, increased levels of phosphorylated tau. B35 cells were transfected with EmGFP, EmGFP-Rac1 and EmGFP-Rac1^{C189A}. The levels of phosphorylated tau were detected using a phosphorylated microtubule-associated protein tau (PMAPT/PTAU) ELISA kit (MyBioSource) that recognizes phosphorylated tau from rat sources. Three separate experiments were run (N=3 per condition). Data are expressed as means ± SEM and the asterisk (*) indicates significant difference from cells expressing only EmGFP at $p \leq 0.05$ (ANOVA followed by a LSD posthoc test).

3.3. Overexpression of EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change GSK-3β levels in B35 cells

Many kinases can phosphorylate tau, but GSK-3β has been identified as one of the more important kinases that can induce tau hyperphosphorylation in AD [21]. Because overexpressing EmGFP-Rac1 increase tau phosphorylation, we next assessed whether these effects might be due to an increase in the steady-state levels of GSK-3β. After either not transfecting or transfecting B35

cells with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}, whole-cell lysates were subjected to western blotting against GSK-3β. Compared to cells transfected with EmGFP only, overexpressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change GSK-3β levels (Figure 3.5). Therefore, we interpret these data to suggest that GSK-3β is likely not involved in any Rac1-mediated phosphorylation of tau or that Rac1 influences the activation, rather than the levels, of this enzyme.

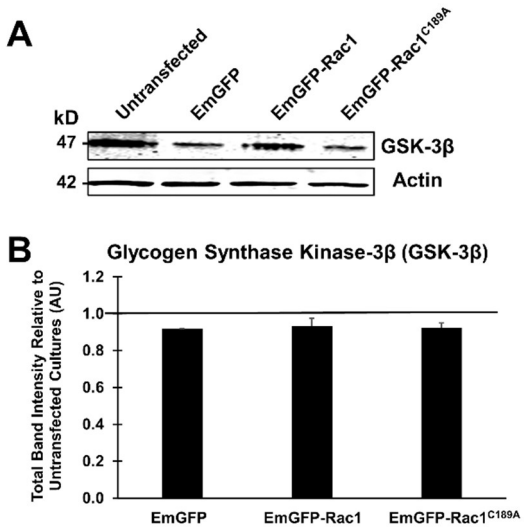

Fig. 3.5 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of GSK-3β. (A) Representative western blot for glycogen synthase kinase-3β (GSK-3β) of whole cell lysates from B35 cells either not transfected (Untransfected) or transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} (top blot). Equivalent level of protein loading was demonstrated by similar levels of actin being detected by anti-actin antibodies on the same blot. (B) Quantification of bands immunoreactive to anti-GSK-3β antibodies (Abcam) and visualized using goat anti-rabbit secondary antibodies conjugated to IRDye 680RD (LiCor) was determined from spot densitometry of immunoreactive bands using an Odyssey CLx infrared imaging system and correcting for an difference in area by applying the formula: (band intensity-background) * area. Conditions were normalized to the level of GSK-3β from the untransfected condition (horizontal line in graph in B) and data are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA indicated there were no significant differences between conditions.

3.4. Overexpressing either EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect total APP levels in B35 cells, but decreased membrane APP

Formation of amyloid plaques in the brain is the second AD hallmark. Amyloid plaques are primarily composed of the accumulated Aβ peptide. The aberrant cleavage of the amyloid precursor protein (APP) produces β amyloid plaques [4], which may be a precipitating event for AD according to the amyloid hypothesis [22]. To assess the effects of Rac1 prenylation on production of APP, we either did not transfect cells or transfected B35 cells with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. We assessed total levels of APP in each condition by western blotting whole cell lysates for APP levels. Compared to cells transfected with EmGFP only, expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect levels of APP (Figure 3.6).

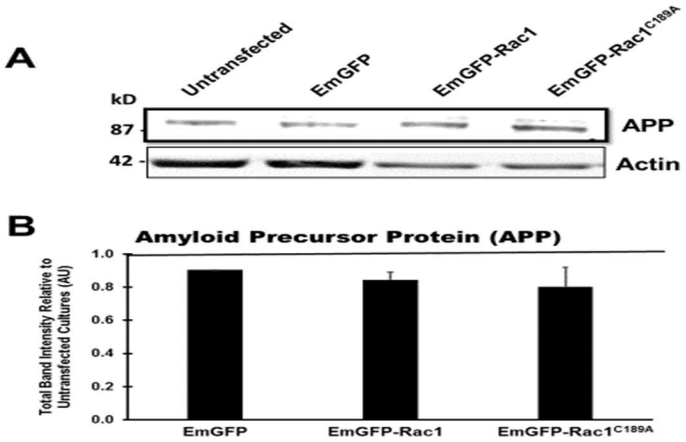

Fig. 3.6 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of APP. (A) Representative western blot of whole cell lysates of APP from B35 cells either not transfected (Untransfected) or transfected with EmGFP, EmGFP-Rac1 and EmGFP-Rac1^{C189A}. Vertical line in bottom blot indicates where irrelevant lanes for this analysis located on the same blot were removed to improve readability. (B) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in B is expressed as means ± SEM for 3 separate experiments. Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated that there were no significant differences across groups for levels of APP in whole cell lysates from B35 cells.

APP is a type I transmembrane protein [4]. Since prenylation of Rac1 directs it to the plasma membrane [15], its membrane association may allow a greater chance for Rac1 to influence the activity of APP processing. Therefore, we next assessed the effects of Rac1 prenylation on APP levels in cellular membranes. We predicted that overexpressing non-prenylatable Rac1 would decrease membrane APP levels. We either did not transfect cells or transfected B35 cells with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Then we separated

transfected cells into membrane and cytosolic fractions and assessed the total amount of APP in the membrane fraction by western blotting. Efficient separation of membrane fractions was routinely assessed by western blotting for ERK1/2 and for the β2 subunit of the GABA-A receptor (see Figure 3.1E). Western blots for membrane-associated APP suggested that overexpression of either EmGFP-Rac1 or EmGFP-Rac1^{C189A} decreased membrane APP levels, with EmGFP-Rac1 being more effective than EmGFP-Rac1^{C189A} (representative western blot in Figure 3.7A). Quantification showed that overexpression of either EmGFP-Rac1 or EmGFP-Rac1^{C189A} significantly decreased the amount of APP associated with membranes, compared to cells expressing EmGFP only (Figure 3.7B). Furthermore, overexpression of nonprenylatable EmGFP-Rac1^{C189A} increased membranous APP, but not back to levels observed in cells expressing only EmGFP (Figure 3.7B). The decrease in membrane APP but not total APP may indicate that Rac1 is involved in its processing and that it might influence the production of β amyloid.

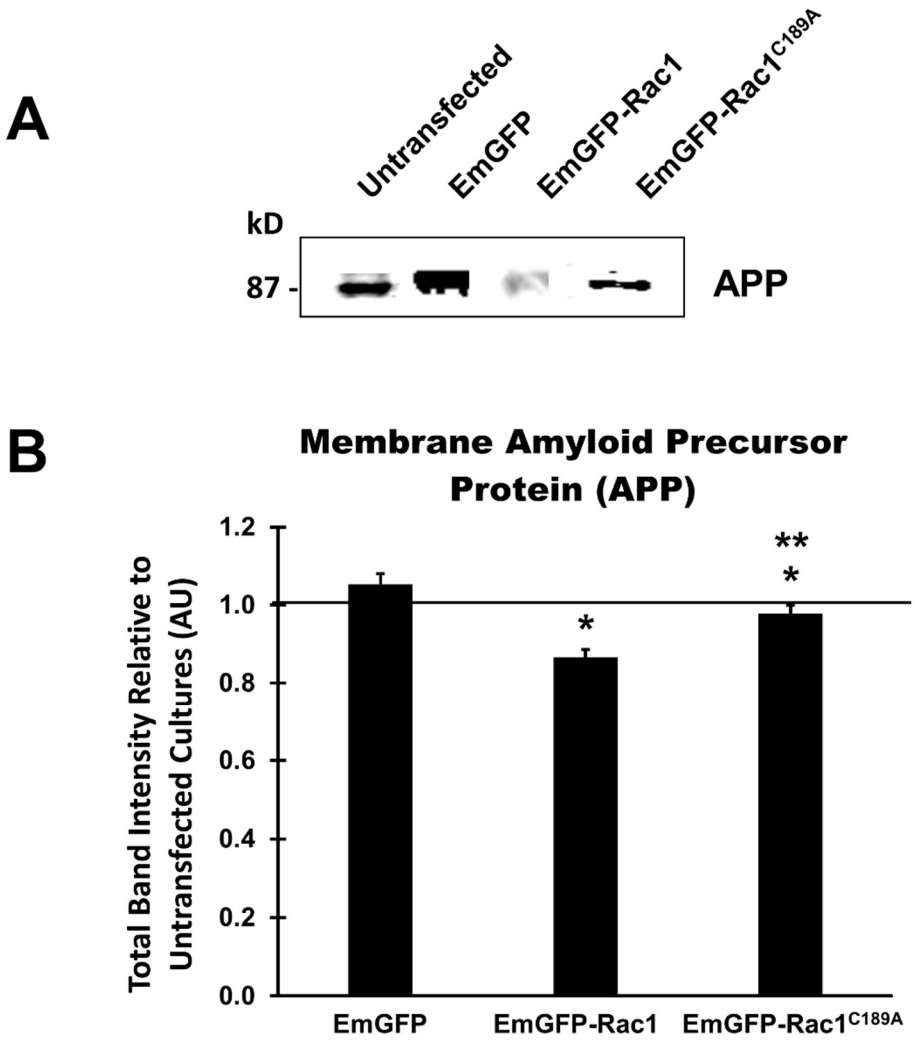

Fig. 3.7 Overexpression of EmGFP-Rac1 or EmGFP-Rac1^{C189A} decreased membrane amyloid precursor protein (APP) levels. (A) Representative western blot for APP from the membrane fractions of B35 cells either not transfected (untransfected) or transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. (B) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. * indicates significant difference compared to cells expressing EmGFP and ** indicate significant difference compared to cells expressing EmGFP-Rac1 at $p \leq 0.05$ (ANOVA and LSD posthoc). Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments.

3.5. Overexpressing either EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect intracellular or extracellular Aβ42 levels in B35 cells

Since overexpressing either wild-type or nonprenylatable Rac1 decreased the level of membrane-associated APP, it is logical to predict that Rac1 is involved in regulating the processing of APP to its metabolites, including Aβ peptides. To determine whether expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} affects production of β amyloid, we transfected cells with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Then we assessed the extracellular and intracellular β amyloid levels using a β amyloid ELISA kit. Compared to cells transfected with EmGFP only, expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect either the intracellular or extracellular levels of β amyloid (Figure 3.8). This suggests that, while Rac1 decreases membrane APP, it is not involved in further processing to Aβ.

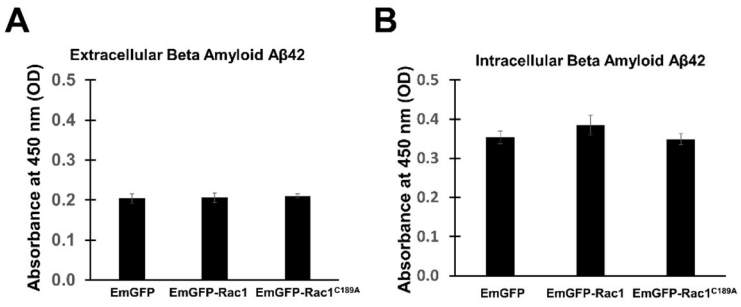

Fig. 3.8 Overexpression of EmGFP-Rac1^{C189A} did not affect extracellular or intracellular β amyloid Aβ42 levels. B35 cells were transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. The levels of extracellular (A) and intracellular (B) β amyloid Aβ42 were detected using an amyloid β peptide 1-42 (Aβ 1-42) ELISA Kit (MyBioSource) that was specific for rat Aβ42. N=3 cultures per condition. Analysis by ANOVA indicated that there were no significant differences between conditions.

3.6. Altering Rac1 prenylation did not affect total BACE1 levels or activity in B35 cells or primary cortical neurons

In AD, β amyloid is formed in the amyloidogenic pathway from the sequential cleavage of APP by β and γ-secretases [23]. β-secretase or the β-site APP cleaving enzyme 1 (BACE1) is an aspartyl protease, while γ-secretase is an intramembranous protease complex, with the active site being presenilin (PS2) [23]. To assess the effects of Rac1 prenylation on levels of BACE1, we either did not transfect or transfected B35 cells and rat neonatal primary cortical neurons with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Because we noted that overexpressing both prenylatable and non-prenylatable Rac1 decreased membrane-associated APP, we wanted to assess whether similar mechanisms occurred in primary neurons, as well as in neuroblastoma cells. We assessed the levels of BACE1 in each condition using western blotting of whole cell lysates. Compared to cells transfected with EmGFP only, expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change levels of BACE1 in B35 cells (Figure 3.9) or primary cortical neurons (Figure 3.10), which is consistent with the lack of change observed in β amyloid levels.

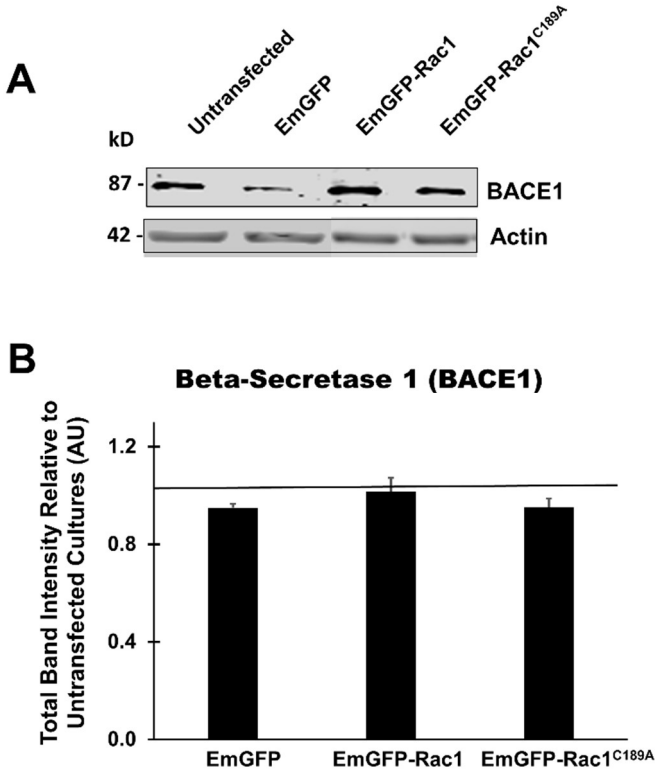

Fig. 3.9 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of BACE1 in B35 cells. (A) Representative western blots from B35 whole cell lysates for β-secretase 1 (BACE1) from cells transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} (top blot). Equivalent protein loading was demonstrating by similar levels of Actin immunoreactive bands across conditions (bottom blot). (B) Quantification of protein immunoreactivity was normalized to untransfected cultures and imaged using the Odyssey CLx infrared imaging system. Average band intensities were determined by applying this formula: (band intensity-background) * area of band, to correct for any variation in spot area. Data in B is expressed as means ± SEM for 3 separate experiments. Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated that there were no significant differences across conditions.

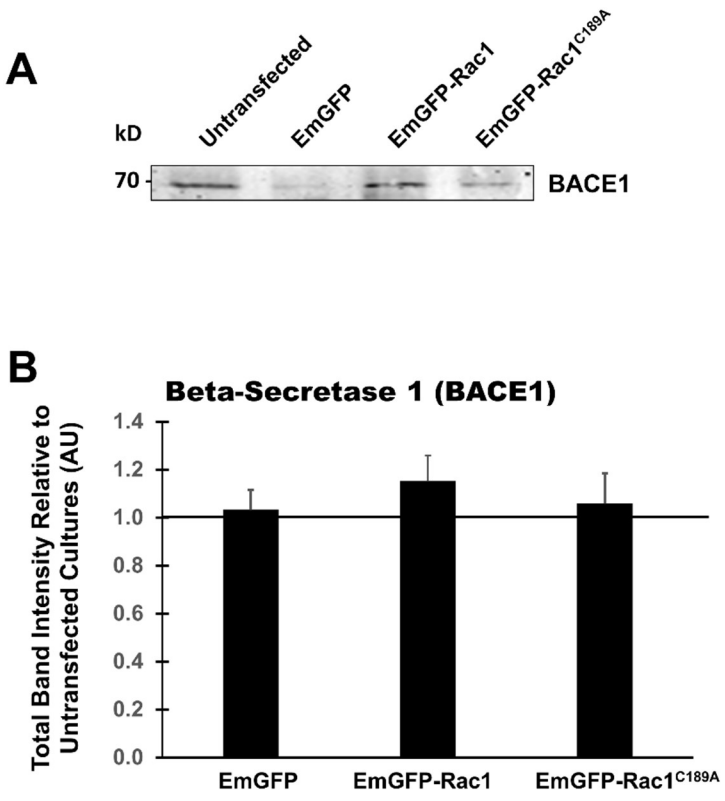

Fig. 3.10 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of BACE1 in primary cortical neurons. (A) Representative western blot of whole cell lysates for β-secretase (BACE1) from primary cortical neurons transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. (B) Quantification of immunoreactive bands was calculated from images captured using an Odyssey CLx infrared imaging system. Conditions were normalized to untransfected cultures and corrections for background and deviation in spot area size were calculated by applying the formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated that there were no significant differences across conditions.

Although altering the subcellular localization of Rac1 did not affect BACE1 levels, the enzyme activity is likely to be affected in many cases when the levels of enzyme do not change. To assess the β-secretase activity, we either did not transfect or transfected B35 cells with EmGFP, EmGFP-Rac1 or EmGFP-

Rac1^{C189A}. A β-secretase activity assay fluorometric ELISA kit was used to determine whether altering Rac1 prenylation affected β-secretase activity. Compared to cells transfected with EmGFP only, cells overexpressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not show evidence of altered β-secretase activity (Figure 3.11). Thus, it appears that the changes in APP membrane levels elicited by overexpressing either wild-type or non-prenylatable Rac1 do not involve activation of the amyloidogenic pathway.

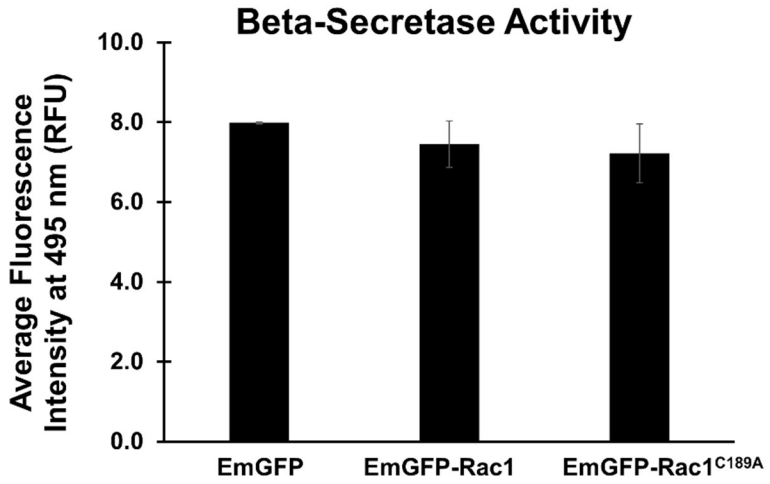

Fig. 3.11 Overexpression of EmGFP-Rac1^{C189A} did not affect β-secretase activity in B35 whole cell lysates. B35 cells were either not transfected or transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. The levels of β-secretase activity in samples were detected using a β-secretase activity assay fluorometric kit (Abcam). N=3 cultures per condition. Analysis by ANOVA indicated that there were no significant differences across experimental groups.

3.7 Altering Rac1 prenylation does not affect membrane or cytosolic BACE1 levels in B35 cells

Although we noted no change in BACE1 activity in whole cell lysates, BACE1 is a membrane protein [4], and it is prossible that Rac1 may only affect APP processing with membranes. Therefore, we next assessed the effects of Rac1 prenylation on localization of BACE1. We separated cells from each condition into membrane and cytosolic fractions and assessed the total amount of BACE1 in each fraction by western blotting. Observations of western blots for BACE1 levels in either the cytosol (Figure 3.12A) or membrane fractions (Figure 3.12B) did not shown any obvious differences across experimental conditions. When quantified, overexpression of either EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not alter the levels of the cytosolic (Figure 3.12C) or membranous (Figure 3.12D) BACE1, compared to cells transfected with EmGFP only. However, a BACE1 immunoreactive band was not consistently observed in cytosolic fractions, while a small amount of BACE1 was found in the cytosol of transfected cells. This may indicate that BACE1 and the amyloidogenic pathway are not responsible for the changes in membrane-associated APP.

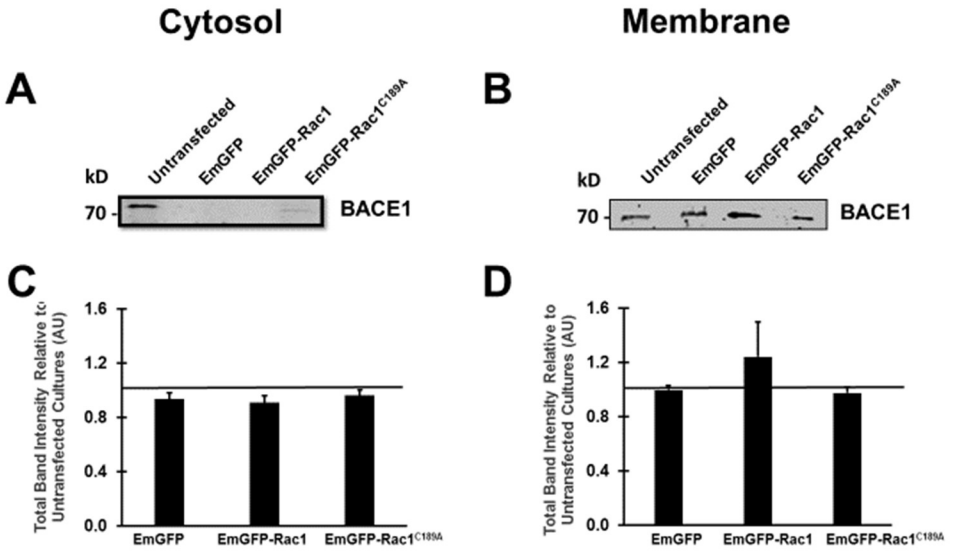

Fig. 3.12 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of cytosolic or membrane BACE1. Representative western blots for BACE1 from the cytosolic (A) and membrane (B) fractions of B35 cells transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not show observable differences in the amount of BACE1 in either fraction. Quantification of protein immunoreactivity normalized to untransfected condition (horizontal line in each graph) was determined from spot densitometry of bands from images captured using an Odyssey CLx (panel C for the cytosol fraction and panel D for the membrane fraction). Corrections for area differences and background substraction were calculated using this formula: (band intensity-background) * area of band. Data in C and D are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA indicated that there were not significant differences across the experiemntal conditions.

3.8. Overexpressing EmGFP-Rac1, but not EmGFP-Rac1^{C189A}, increased presenilin 2 levels in primary cortical neurons but not in B35 cells

The second enzyme involved in the production of Aβ is γ-secretase, which is active in both the amyloidogenic and the non-amyloidogenic processing of APP. To assess the effects of Rac1 prenylation on levels of presenilin 2 (PS2), the active component in cleaving APP, in B35 cells and primary cortical neurons,

we either did not tranfect or transfected cells with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. We assessed levels of PS2 in each condition using western blotting of whole cell lysates. Observation of PS2 western blots from B35 cells suggested little differences in PS2 levels across conditions (Figure 3.13A). Similar results were seen from PS2 western blots of cultured neonatal rat cortical neurons (Figure 3.13B). When quantified, expressing EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not change levels of PS2 in B35 cells, compared to cells transfected with EmGFP only (Figure 3.13C). However, in primary cortical neurons, overexpressing EmGFP-Rac1, but not EmGFP-Rac1^{C189A}, significantly increased PS2 levels, compared to cells transfected with EmGFP (Figure 3.13D). Since these experiments were conducted in neonatal neurons, rather than aged neurons, these results might be interpreted to indicate that PS2 might be important in neurodevelopment in this system.

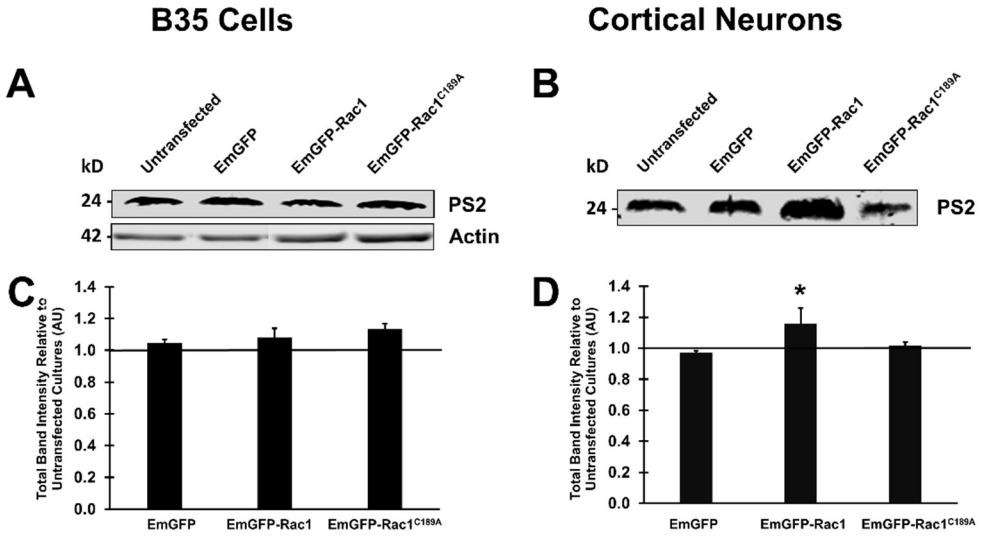

Fig. 3.13 Overexpression of EmGFP-Rac1 increased levels of Presenilin 2 (PS2) in primary cortical neurons, but not in B35 cells. Representative western blot of whole cell lysates for PS2 from B35 cells (A) or primary cultures of cortical neurons from neonatal rats (B) transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Quantification of protein immunoreactivity, normalized to untransfected condition (horizontal line in panels C and D) and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band indicated no differences in B35 whole cell lysates (C) but showed that overexpression of EmGFP-Rac1 significantly increased the amount of PS2 in lysates from cultured primary cortical neurons (D). Data in C and D were normalized to untransfected cultures and are expressed as means ± SEM for 3 separate experiments. * indicates significant difference compared to GFP at $p \leq 0.05$ (ANOVA and LSD posthoc).

3.9. Altering Rac1 prenylation does not affect membrane or cytosolic PS2 levels in B35 cells

Since PS2 is a membrane protein [4], we next assessed the effects of Rac1 prenylation on localization of PS2 in B35 cells. We separated transfected cells into membrane and cytosolic fractions and assessed the total amount of PS2 in each fraction by western blotting. Blots appeared to have similar amounts of PS2 across conditions within each fraction (Figure 3.14A and B for cytosol and

membrane fractions, respectively). Compared to cells transfected with EmGFP only, quantification of immunorective bands indicated that expressing either EmGFP-Rac1 or EmGFP-Rac1^{C189A} did not affect the levels of PS2 located in the cytosol (Figure 3.14C) or associated with cell membranes (Figure 3.14D). Together, these data could be intepreted to suggest that activation, rather than levels, of γ-secretase facilitate APP processing in the non-amyloidogenic pathway in B35 cells, as well as in primary cultured neurons.

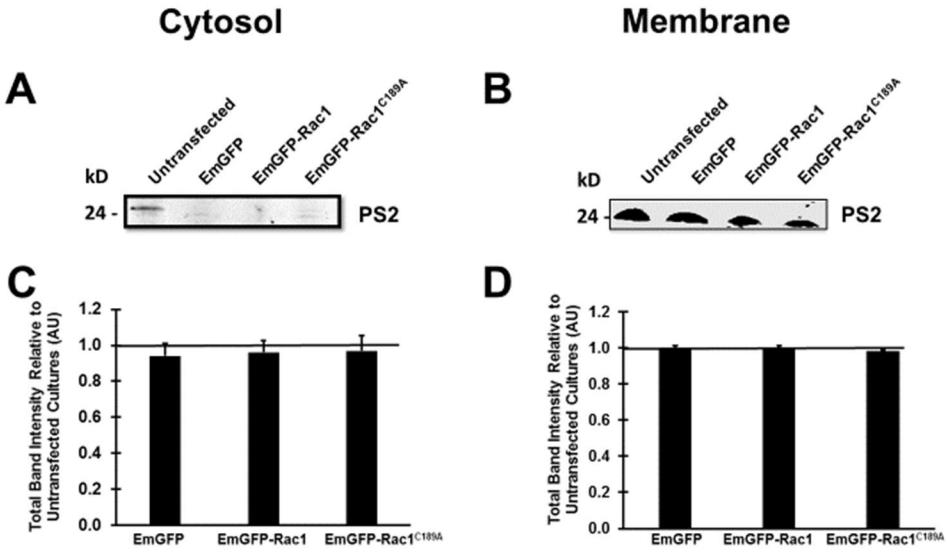

Fig. 3.14 Overexpression of EmGFP-Rac1^{C189A} did not affect levels of cytosolic or membrane presenilin 2 (PS2). Representative western blots for PS2 from the cytosolic (A) and membrane (B) fractions of B35 cells transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. Quantification of protein immunoreactivity from cytosol (C) and membrane (D) cellular fractions, normalized to the untransfected condition (horizontal line in each graph) from images captured using an Odyssey CLx infrared imaging system and calculated by applying this formula: (band intensity-background) * area of band to subtract background and correct for any variation in the area of regions of interest used for analysis. Data in C and D are expressed as means ± SEM for 3 separate experiments. Analysis using ANOVA indicated that there were no significant differences across conditions.

4. Discussion:

The primary purpose of this study was to determine the effects of Rac1 prenylation on AD hallmarks. We found that compared to cells expressing only the EmGFP protein tag, cells expressing either a wild-type Rac1 (EmGFP-Rac1) or a non-prenylatable Rac1 mutant (EmGFP-Rac1^{C189A}) did not alter the total levels of tau. However, overexpressing the wild-type form of Rac1 (EmGFP-Rac1), but not the non-prenylatable Rac1, did increase total tau phosphorylation, indicating that membrane-associated Rac1 may contribute to tau hyperphosphorylation and that the reduced prenylation reported in AD would be more likely to reduce rather than promote tangle formation. However, overexpressing the wild-type Rac1 or altering the subcellular localization of Rac1 did not increase levels of the kinase GSK-3β, a major phosphorylator of tau in AD. Although other tau kinases could be involved, this suggests that Rac1 is either not involved in progressing AD pathology through increasing hyperphosphorylation of tau to allow neurofibrillary tangle formation or that it plays a minor role, likely in its prenylated and membrane-bound state.

Interestingly, we found that expressing a non-prenylatable Rac1 mutant (EmGFP-Rac1^{C189A}) or wild-type Rac1 (EmGFP-Rac1) decreased the levels of amyloid precursor protein at the membrane compared to cells expressing EmGFP only. However, the non-prenylatable Rac1 was significantly less effective in decreasing membrane APP levels. The cellular compartmental changes in APP levels at the membrane were not reflected with changes in the

total levels of APP or the secretases associated with the amyloidogenic pathway, including β-secretase (BACE1) and the active subunit of γ-secretase (presenilin 2). Consequently, the extracellular or intracellular levels of β amyloid were not affected. This suggests that Rac1 may have an effect on the localization of APP, but that it is not likely to be directly involved in progressing AD pathology through increasing the production of β amyloid.

Several studies have implicated Rac1 in APP synthesis and γ-secretase activity. For instance, inhibition of Rac1 in mouse primary hippocampal neurons negatively regulated the synthesis of APP [24] and decreased the production of $A\beta_{42}$ by changing the selectivity of γ-secretase [25]. We found that overexpressing wild-type Rac1 in B35 neuroblastoma cells decreased APP at the membrane but did not significantly alter the total levels of APP or $A\beta_{42}$. It is also possible that altering prenylation of Rac1 affects the non-amyloidogenic pathway of APP processing, so we assessed the β secretase activity after expressing wild-type (EmGFP-Rac1) and mutant Rac1 (EmGFP-$Rac1^{C189A}$). β secretase levels should not be involved in the non-amyloidogenic pathway and our results confirmed that overexpressing non-prenylatable Rac1 mutant or wild-type Rac1 did not affect β secretase levels or activity. Together, our results show no effect of altering the subcellular localization of Rac1 on β amyloid formation. Since Rac1 has a role in AD pathology, the function of Rac1 might be downstream of the β amyloid or neurofibrillary tangles formation in AD. It should be noted that most previous studies assessed the role of Rac1 activation on aspects of AD.

Here, we were specifically investigating how Rac1 prenylation affected production of AD markers. While it is thought that prenylation is required for Rac1 to be activated by GTP loading, our prior work suggests that non-prenylated, cytosol-localized Rac1 can be activated and may affect pathways differently when it is localized to the cytosol [15]. Thus, it could be that the relevant pathways are located both cytosolically and associated with cellular membranes. Further studies are needed to investigate the transcriptional pathways activated by Rac1 and alternate pathways for secretase activation.

On the surface, altering Rac1 prenylation in our study did not affect APP or its secretases levels; however, previously-reported data have found a relationship between APP synthesis, γ-secretase and Rac1. For instance, γ-secretase enhances dendritic spine formation at the synapse by activating the Rac signaling pathway [26]. In addition, Rac1 inhibition decreased APP synthesis [24] and the selectivity of γ-secretase [25]. These prior results were obtained by measuring or manipulating the GTP-loading of Rac1. Our experiments did not manipluate Rac1 activation, but rather altered its localization. There are potentially some explanations for the disparate results. For example, it is possible that Rac1 is involved in APP processing that leads to non-amyloidogenic pathway through a mechanism that does not involve the secretases, BACE1 or presenilin. In the constitutive non-amyloidogenic pathway, APP is cleaved by α-secretase and results in the production of C83 or α-C terminal fragment (CTF), which is then transformed into APP intracellular domain

(AICD) and p3 peptides by γ secretase [27]. This pathway has been found to be neuroprotective as well as neurotrophic [28]. Through this pathway, APP induces its important neuronal functions such as synaptic plasticity, neurite formation or learning [29]. This might also explain our result showing a lack of β amyloid formation after expressing either wild-type or mutant Rac1. Interestingly, overexpressing wild-type Rac1, but not non-prenylatable Rac1, increased presenilin 2 levels in wole cell lysates of cultured neonatal rat cortical neurons, but not B35 neuroblastoma cells. This may suggest a role for presenilin in neurodevelopment, rather that AD, as at the time of experimentation, neonatal neurons in culture are still extending processes and forming synapses.

There are many lines of evidence that small GTPases prenylation is implicated in AD pathology. Statin treatment is one method used to inhibit all protein prenylation including that of Rac1. For instance, atorvastatin has implicated Rac1 prenylation and activation of geranylgeranyl transferase-I (GGT-I) to induce neuronal plasticity and protection [30]. Statin studies show a decrease in the levels of protein prenylation and a decrease in Aβ production by promoting the non-amyloidogenic pathway of APP [31]. Statins induce a decrease in isoprenoid levels and inhibit APP trafficking [32]. Our current results for Rac1 suggest that membrane-assoiated Rac1 is more likely assoiated with AD pathology that non-prenylatable Rac1. However, this is only one prenylated protein that would be affected by statins treatment and its effects could be overridden by other proteins affected by statins.

The vast majority of our experiments were performed in neuroblastoma cells, which, like all other cells, constitutively express endogenous Rac1. There is a possibility that competition occurred between the endogenous Rac1 and the expressed construct. However, the vector for all constructs used in this study drives expression using the human cytomegalovirus (CMV) immediate-early promoter/enhancer, which gives an average increase in expression of 33.2-fold over the level of expression of the endogenous Rac1. Still, additional studies are needed to be performed that remove potential confounds associated with expression of the endogenous protein. Using RNA silencing or conditional knockouts of Rac1 could be used to eliminate the possibility that the endogenous protein compensates for the expressed construct. This would allow for a better understanding and evidence for how preventing prenylation of Rac1 affects the localization and production of AD proteins or neurodegenerative disorders. More importantly, these experiments should be tested in a primary neuron system or animal models of AD.

5. Conclusions

The goal of this work was to assess how prenylation of the small GTPase, Rac1, affects the production of Alzheimer's disease markers including hyperphosphorylated tau and the production of Aβ. Since statins inhibit protein prenylation and their use is associated with decreasing the incidence of AD, we hypothesized that decreased prenylation of Rac1 would correlate with increased

AD markers. We found that overexpression of wild-type Rac1 can decrease membrane-associated APP and promote the phosphorylation of tau and that overexpressing a non-prenylatable form of Rac1 failed to do so. Furthermore, neither construct had an effect on the common tau kinase, GSK-3β, nor amyloidogenic processing proteins in B35 neuoblastoma cells, suggesting that the effects observed in this cell line affect AD markers through currently undefined means. We did observe that overexpression of wild-type Rac1 did increase the amount of presenilin 2 in primary cortical neurons, suggesting that additional studies should be performed in primary neurons. Overall, we interpret our results to suggest that prenylation of Rac1 plays a minor role in producing pathological markers of AD, perhaps indicating that their major roles in AD are downstream of hyperphosphorylated tau and Aβ.

Acknowledgments: The authors thank Prapti Mody for technical assistance and critical evaluation of this work and Dr. Jairus Reddy for constructing and providing the vectors for EmGFP, wild-type Rac1 and non-prenylatable Rac1. This work was funded by the TWU Department of Biology, grants for the National Science Foundation, the TWU Research Enhancement Program and the TWU Grant Proposal Preparation Program. Other than approval of the experiments that were initially approved by funding bodies, no funder was involved in the performance of experiments, interpretation of the results, or in the writing of this manuscript.

Declarations of Interest: None

Author Contributions: DiAnna Hynds and Rawand Chabayta jointly defined the study hypotheses, goals and methods to test hypotheses. Ms. Chabayta performed all the experiments under supervision of Dr. Hynds and wrote the initial draft of the manuscript. Both authors were involved in refining the manuscript for preparation of publication consideration. Both authors have approved submission of the final manuscript.

6. References:

1. Carlo, M. D., Giacomazza, D., & Biagio, P. L. Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools, Journal of Physics: Condensed Matter. 24 (2012) 244102.
2. Alzheimer's Association. 2017 Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia 2017. 13 (2017) 325-373.
3. Serranzo-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological alterations in Alzheimer disease, Cold Spring Harbor Perspectives in Medicine. 1 (2011) a006189.
4. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease, Progress in Neurobiology. 156 (2017) 189-213.

5. Curtis, I. D. Functions of Rac GTPases during Neuronal Development, Developmental Neuroscience. 30 (2007) 47-58.
6. Grabham, P. W. Microtubule and Rac 1-dependent F-actin in growth cones, Journal of Cell Science. 116 (2003) 3739-3748.
7. Martinez, L. A., & Tejada-Simon, M. V. Pharmacological inactivation of the small GTPase Rac1 impairs long-term plasticity in the mouse hippocampus, Neuropharmacology. 61 (2011) 305-312.
8. Govek, E. E., Hatten, M. E., & Van Aelst, L. The role of rho GTPase proteins in CNS neuronal migration, Developmental Neurobiology. 71 (2011) 528-553.
9. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders, Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.
10. Wang, P. L., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene, Journal of Neuroscience Research. 87 (2009) 2105-2114.
11. Boo, J. H., Sohn, J. H., Kim, J. E., Song, H., & Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch1, Biochemical and Biophysical Research Communications. 372 (2008) 913-917.
12. Posada-Duque, R. A., López-Tobón, A., Piedrahita, D., González-Billault, C., & Cardona-Gomez, G. P. P35 and Rac1 underlie the neuroprotection and cognitive improvement induced by CDK5 silencing, Journal of Neurochemistry. 134 (2015) 354-370.

13. Roberts, Patrick J., et al. Rho Family GTPase Modification and Dependence on CAAX Motif-Signaled Posttranslational Modification, Journal of Biological Chemistry. 283 (2008) 25150–25163.
14. Higgins, J. B., & Casey, P. J. The role of prenylation in G-protein assembly and function, Cellular Signalling. 8(1996), 433-437.
15. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated, Cellular Signalling. 27(2015), 630-637.
16. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease, Alzheimers Research & Therapy. 9(2017).
17. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients, Pharmacopsychiatry. 42(2009).
18. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain, Journal of Neurochemistry. 129(2014), 732-742.
19. Iqbal, Khalid, et al. Cytosolic Abnormally Hyperphosphorylated Tau But Not Paired Helical Filaments Sequester Normal MAPs and Inhibit Microtubule Assembly, Journal of Alzheimers Disease. 14(2008).

20. Iqbal, K., et al. Tau in Alzheimer Disease and Related Tauopathies, Current Alzheimer Research. 8 (2010) 656–664.
21. Papasozomenos, S. C., & Binder, L. I. Phosphorylation determines two distinct species of tau in the central nervous system, Cell Motility and the Cytoskeleton. 8(1987), 210-226.
22. Busciglio, J., Gabuzda, D. H., Matsudaira, P., & Yankner, B. A. Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells, Proceedings of the National Academy of Sciences. 90(1993) 2092-2096.
23. Zhang, Y., Thompson, R., Zhang, H., & Xu, H. APP processing in Alzheimer's disease, Molecular Brian. 4(2011).
24. Wang, P., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene, Journal of Neuroscience Research. 87(2009) 2105-2114.
25. Boo, J. H., Sohn, J. H., Kim, J. E., Song, H., & Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch, Biochemical and Biophysical Research Communications. 372(2008) 913-917.
26. Inoue, E., Deguchi-Tawarada, M., Togawa, A., Matsui, C., Arita, K., Katahira-Tayama, S., et al. Synaptic activity prompts γ-secretase–mediated cleavage of EphA4 and dendritic spine formation, The Journal of Cell Biology. 185(2009) 551-564.

27. Morgese, M. G., Schiavone, S., & Trabace, L. Emerging role of amyloid β in stress response: Implication for depression and diabetes, European Journal of Pharmacology. 817 (2017) 22-29.
28. Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. An Overview of APP Processing Enzymes and Products, NeuroMolecular Medicine. 12(2009), 1-12.
29. Moon, M., Kim, H., Li, Y., Kim, J., Jeon, Y., Won, H., Kim, J., Kwon, H., Choi, I., Ro, E., Joe, E, Choe, M., Kwon, H., Kim, H., Kim, Y., & Park, J. Involvement of small GTPase RhoA in the regulation of superoxide production in BV2 cells in response to fibrillar Aβ peptides, Cellular Signalling. 25(2013), 1861-1869.
30. Posada-Duque, Rafael Andrés, et al. Atorvastatin Requires Geranylgeranyl Transferase-I and Rac1 Activation to Exert Neuronal Protection and Induce Plasticity, Neurochemistry International. 62 (2013)433–445.
31. Ostrowski SM, Wilkinson BL, Golde TE, Landreth G. Statins reduce amyloid-β production through inhibition of protein isoprenylation, J Biol Chem. 282 (2007) 26832–26844.
32. Cole SL, Grudzien A, Manhart IO, Kelly BL, Oakley H, Vassar R. Statins cause intracellular accumulation of amyloid precursor protein, β-secretase-cleaved fragments, and amyloid β-peptide via an isoprenoid-dependent mechanism, J Biol Chem. 280(2005):18755–18770.

CHAPTER IV

NON-PRENYLATED RHOA IN ALZHEIMER'S DISEASE PATHOLOGICAL MARKERS

A Paper to be Submitted to *Cellular Signalling*

Rawand K. Chabayta and DiAnna L. Hynds
Department of Biology, Texas Woman's University, Denton, TX 76204

Corresponding Author: DiAnna L. Hynds, Ph.D.
Professor, Department of Biology and
Affiliated Professor, Woodcock Institute
PO Box 425799
Denton, T 76204-5799
940-898-2359
Fax: 940-898-2382
dhynds@twu.edu

Highlights:

- Overexpressing non-prenylatable RhoA does not alter the levels of the amyloid precursor protein, β amyloid, or secretases in the amyloidogenis pathways in B35 neuroblastoma whole cell lysates or cytosolic or membrane fractions.

- Overexpressing non-prenylatable RhoA does not alter the levels of β-secretase or presenilin 2 (the active subunit of γ-secretase) in primary cultures of neonatal cortical neurons.

- While most markers of AD were unaffected by overexpressing wild-type or non-prenylatable RhoA, overexpression of non-prenylatable, but not wild-type, RhoA increases tau phosphorylation in B35 neuroblastoma cells.

- Since the levels of the tau phosphorylating protein glycogen synthase kinase-3β (GSK-3β) did not increase in any condition, we interpret these data to indicate that the decrease in RhoA prenylation (as seen in Alzheimer's disease) may result in increase tau phosphorylation and tauopathies through altering kinases or phosphastases other than GSK-3β.

Abstract:

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that is characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain. Aberrant cleavage of the amyloid precursor protein (APP) produces β amyloid plaques. Hyperphosphorylation of tau, a microtubule associated protein, forms the neurofibrillary tangles. RhoA is a member of the Rho family of guanosine triphosphatases (GTPases) and a key regulator in many cellular functions. Dysregulation of RhoA has been implicated in AD pathology because decreased activity of prenyl transferases and increased in prenylation precursors happens with ageing or AD. Prenylation of RhoA has been thought to be required for its activation to ensure proper association with the plasma membrane. However, the role of subcellularly altered RhoA in AD pathology has not been established. To address this, we used a non-prenylatable RhoA construct (referred to as EmGFP-RhoA^{C190A}) and assessed how expressing this construct affects the levels of AD associated proteins, secretases and a tau kinase (GSK-3β), compared to overexpressing EmGFP alone or prenylatable, wild-type RhoA. Cells transfected with EmGFP-RhoA^{C190A} increased the total levels of tau and tau phosphorylation, but not the levels of GSK-3β, indicating that altering the subcellular localization of RhoA may promote the accumulation of hyperphosphoylated tau. We did not observe any conditions under which altering RhoA prenylation affected APP levels, β amyloid production or the levels or activity of presenilin 2 or β secretase. These results suggest that altering the

subcellular localization of RhoA in AD might be involved in the neurofibrillary tangles formation, but not APP processing.

Keywords: B35 neuroblastoma cells, β amyloid, primary cortical neuron culture, Rho prenylation, subcellular localization, tau.

1. Introduction

The fatal neurodegenerative condition Alzheimer's Disease (AD) is increasing in incidence and currently affects about 47.5 million older people worldwide [1,2]. In the next 20 years, the AD incidence is expected to reach about a million individuals/year, likely increasing the current health burden in the U.S., where about 5.5 million persons had AD in 2017, costing approximately 232 billion dollars [2, 3]. AD is characterized by formation of two pathological markers, the formation of extracellular amyloid plaques and intracellular neurofibrillary tangles generally beginning in cholinergic neurons. Non-pathological cleavage of the amyloid precursor protein (APP) is altered from α and γ secretase cleavage to aberrant cleavage by β and γ-secretases to eventually produce β amyloid plaques [4]. Increased kinase or decreased phosphatase activity can lead to hyperphosphorylation of the microtubule associated protein, tau, to form intracellular neurofibrillary tangles [4].

The Rho family of small guanosine triphosphatases (GTPases) are key regulators of the actin cytoskeleton and have roles in neurological morphogenesis [5]. In particular, activation of Ras homolog gene family, member

A (RhoA), a typical Rho GTPase member, promotes growth cone collapse and neuronal retraction *in vivo* [6]. More importantly, RhoA has been implicated in the pathogenesis of many neurological diseases, including AD [7]. For example, RhoA levels decrease in AD brains and they co-localize with hyperphosphorylated tau in the neurofibrillary tangles [8]. In addition, β-amyloid (Aβ42) treatment activates RhoA and reduces survival of neurons [9]. The exact mechanism through which RhoA may be involved in AD pathology is not known yet.

Post-translational modification of RhoA by prenylation allows its insertion into the plasma membrane where it is thought to be activated and able to interact with downstream effectors [10]. RhoA is geranylgeranylated by the addition of a 20-carbon isoprenoid, which is attached to the CaaX terminus to the cysteine residue [10]. This is followed by proteolysis of the last three C terminal amino acids (-aaX), which consist of two aliphatic amino acids followed by isoleucine or leucine. The prenylated cysteine is then methylated and RhoA associates with the plasma membrane, presumably leading to RhoA activation [10]. RhoA is considered active when it is bound to guanosine triphosphate (GTP), a switch from guanosine diphosphate (GDP) binding catalyzed by guanine nucleotide exchange factors (GEFs) and can activate downstream effectors to propagate signals [11]. RhoA is inactived through the action of either GTPase activating protein (GAPs) that facilitate GTP hydrolysis or guanosine dissociation inhibitors (GDIs) that sequester GDP-bound prenylatable RhoA in the cytosol [12].

However, in our previous studies, we have found activated non-prenylated RhoA that is bound to GTP and able to interact with effectors in the cytosol and other cell compartments, suggesting that RhoA might be active without localization to plasma membrane [13]. Since altered subcellular localization of RhoA might affect the production of amyloid plaques or neurofibrillary tangles, we have used a mutant non-prenylatable RhoA construct (referred to as EmGFP-RhoA^{C190A}), where the cysteine terminal of CAAX is mutated to an alanine AAAX. This mutated construct of RhoA decreases RhoA insertion into membranes [13]. Here, we assess how EmGFP-RhoA^{C190A} overexpression in neuroblastoma cells, with some work in primary cultures of cortical neurons, affects AD pathological hallmarks and the activity of secretases and kinases that aberrantly process APP and tau.

2. Materials and Methods

2.1. Cell Culture:

2.1.1. B35 Rat Neuroblastoma Cells: B35 rat neuroblastoma (American Type Culture Collection (ATCC® CRL-2754™, Manassas, VA) were routinely cultured at 5% CO_2 at 37 °C in 1:1 Dulbecco's Modified Eagle's Medium and Nutrient Mixture F12 (DMEM/F12; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Sigma, St. Louis, Missouri) and antibiotic/antimycotic (Sigma, St. Louis, MO) and passaged when 90-95% confluent. Cells were seeded in six-well plates at 20,000 cells/cm^2 for western blotting experiments and at 5,000 cells/cm^2 on 12

mm glass coverslips in 24 well plates for immunocytochemistry experiments. For immunocytochemical experiments, coverslips were coated overnight at 37 °C with poly-D-lysine (Sigma, St. Louis, MO). Once cells met the appropriate density in serum-containing medium (SCM) for each type of experiment, cultures were transfected for four hours using Lipofectamine 2000 or 3000 (Invitrogen, Carlsbad, CA). Cells were placed in serum-free medium (SFM) for 24 hours to upregulate growth and trophic factor receptors.

2.1.2. Rat Cortical Neurons: All procedures performed with animals were approved by the TWU Institutional Animal Care and Use Committee (IACUC). To prepare neonatal rat cortical neuron cultures, newborn (P1) Sprague-Dawley pups were sacrificed by quick decapitation following anesthetization on covered ice. The dorsal cranium was removed, and the brain was extracted. Meninges and blood vessels were removed using fine forceps. Cortices were separated from the rest of the brain and were cut into small pieces using sterile instruments. Tissue was digested for 30 minutes in 2 mg/ml papain in high glucose-serum free Dulbecco's Modified Eagle's Medium (HG-SFDMEM) at 37 °C. Cells were pelleted in HG-SFDMEM for 5 minutes at 500 x g and the supernatant was removed. Cells were then resuspended in HG-SFDMEM and plated (20,000 cells/ cm^2) on poly-D-lysine-(PDL) coated six-well plates and incubated at 37 °C. Plates were previously coated with 2 ml PDL for one hour at 37 °C. Excess PDL was removed and the plates were washed twice with PBS and then kept at 37 °C until use. Medium was changed after four hours to neurobasal media (NBM) with

10% B27 supplement (Thermo Fisher Scientific, Houston, TX). After a week in NBM transfections were performed.

2.2. Generation of Expression Vectors:

Mammalian expression vectors containing wild-type RhoA that is N-terminally tagged with emerald green fluorescence protein (referred to as EmGFP-RhoA) were made previously and all experiments were performed using these stocks [13]. This construct contains wild-type RhoA that can be prenylated (open reading frame from Open Biosystems) fused at its N-terminus to EmGFP under the human cytomegalovirus promotor (Invitrogen, Carlsbad, CA). Non-prenylatable RhoA (EmGFP-RhoA^{C190A}) constructs were made by using site-directed mutagenesis to change the cysteine at the CAAX box to an alanine. This mutant construct is non-prenylated and cannot be attached to plasma membrane, similar to that as previously reported for Rac1 [13] and has been also determined for RhoA (see results).

2.3. Transfection:

Treatments for all experiments were the same and consisted of transfecting cells with either 3 µg of plasmid per well on 12 mm glass coverslips (Fisher, Houston, TX) for immunocytochemical experiments or 0.8 µg of plasmid per 6 well plates for western blotting experiments. In each experiment, B35 cell or primary cultures of neonatal rat cortical neurons were transfected with constructs placed in Vivid Colors vector (Invitrogen, Carlsbad,CA) containing either EmGFP alone, EmGFP-RhoA or EmGFP-RhoA^{C190A} fused to the N-

terminus of each open reading frame using Lipofectamine 2000 or 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each plasmid placed EmGFP in frame with the gene of interest. Expression was under the human cytomegalo virus promotor and expression was routinely checked be western blotting for EmGFP and/or RhoA and direct fluorescence for expression of EmGFP in immunocytocemical experiments. In B35 cells, constructs were routinely expressed in about 90% of all cells at approximately 33.2-fold the amount of endogenously expressed protein. For primary cortical neurons, transfection occurred in approximately 10% of the cells, which were analyzed only in immunocytochemical experiments where the transfected cells could easily by identified for analysis (see [13] for similar data for Rac1, RhoA data for expression is described in this work and will be reported in a manuscript currently in preparation for consideration of publication in a peer-reviewed journal).

2.4. Subcellular Fractionation:

In some experiments, cell lysates were fractionated into cytosolic and membrane fractions to assess how relocating a majority of RhoA to the cytosol affected levels of AD pathological markers. Transfected and untransfected cells were fractionated into cytosolic (fraction 1) and membrane (fraction 2) fractions 24-hour post-transfection using the S-PEK proteolysis kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. To assess the efficiency of fractionation, fractionated samples were subjected to western blotting for the membrane associated protein, GABA-A (β2) receptor (Abcam

ab186875, Cambridge, MA), and the cytosolic and membranous protein, extracellular regulated kinase 1/2 (ERK1/2, Abcam ab184699, Cambridge, MA). The data are reported previously [13] and consistently showed much higher intensity for GABA-$A_{\beta 2}$ receptor immunoreactivity in the membrane compared to the cytosol fraction, whereas ERK1/2 was present in both membrane and cytosol fractions, as expected. Similar results are shown in the current work.

2.5. Immunocytochemistry:

B35 rat neuroblastoma cells were placed in SFM for 24 hours following four hours of transfection and fixed in 4% paraformaldehyde for 30 minutes at room temperature. Samples were washed twice with PBS and permeabilized with blocking buffer (0.1% Triton X-100, 0.1% bovine serum albumin, and 1.5% pre-immune secondary-specific serum in PBS containing for 30 minutes at room temperature) followed by exposure to appropriate primary (1:200 in blocking buffer overnight at 4 °C) and secondary antibodies (1:200 in blocking buffer two hours at room temperature) with washing by blocking buffer 2 X 10 minutes in between primary and secondary antibody exposure. Antibodies included rabbit anti-tau (Abcam ab76128, Cambredge, MA) primary antibody and goat anti-rabbit IgG secondary antibody conjugated with Alexafluor 647 (Jackson immunolabs, West Grove, PA). Negligible non-specific binding of secondary antibody was observed in samples where the primary antibody was omitted. Specificity of primary antibodies was reported by Abcam and is demonstrated in our own experiments. After washing twice with blocking buffer and twice with PBS,

coverslips were mounted on clean slides with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). Digital images were captured through a 40X objective lens by using a Nikon A1R-A1 confocal system with a Nikon Eclipse Ti inverted microscope and NIS elements imaging software (Nikon Instruments, Melville, NY). Untransfected cultures were used to determine levels of background fluorescence, which were negligible. We ran primary antibody-omitted controls in each immunocytochemical experiment to assess non-specific binding of secondary antibodies. We did not observe fluorescence in primary-omitted controls. All conditions for capturing images, including exposure time and lamp intensity, were kept fixed and constant throughout the experiment to allow comparison between different experimental conditions.

2.6. Western Blotting:

After transfection for four hours, B35 rat neuroblastoma cells were placed in SFM for 24 hours. Cells were washed gently with ice-cold 1X PBS and solubilized in ice-cold lysis buffer [25 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 1.5 mM EDTA, and 1.0% IGEPAL CA-630 supplemented with 1X PIC. All from Sigma-Aldrich, St. Louis, MO) on ice for 15 minutes. Cultures were scraped and lysates transferred to microfuge tubes and centrifuged to pellet nuclei at 13,000 x g for 5 minutes. Supernatants were transferred to new microfuge tubes and stored at -20 °C until use.

After determining protein concentrations (bicinchoninic acid protein assay, BCA, Sigma-Aldrich, St. Louis, MO), equivalent amounts of proteins (30 µg whole cell lysates or cell fractions) were diluted 1:1 with with 2X Laemmli buffer, boiled for five minutes and electrophoresed through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 130 V constant voltage for 90 minutes. Proteins were electrotransferred to nitrocellulose membranes at 400 mA constant current for 90 minutes. Following transfer, membranes were blocked in 5% non-fat milk for one hour at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBST). Blots were incubated overnight at 4 °C with 1:5000 primary antibodies, including mouse anti-actin (Abcam ab8226), rabbit anti-amyloid precursor protein (APP, Abcam ab32136), rabbit anti-tau (Abcam ab76128), rabbit anti-presenilin 2 (Abcam ab51249), or rabbit anti-β-amyloid cleavage enzyme (BACE1, Abcam ab2077; all from Abcam, Cambridge, MA). Membranes were washed with TBST four times for 10 minutes each before incubation in IRDye 680RD (infrared fluorescence dye) conjugated secondary antibodies at a dilution of 1:5000 for one hour (with rocking) at room temperature. After washing with TBST four times for 10 minutes each, bands were visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band intensities were calculated by subtracting background from the total fluorescence intensity of defined regions of interest and then multiplying by area of the band to correct for area variation. All experiments were run at least three times.

2.7. β Secretase Activity Assay:

β-secretase activity was detected using a β secretase (β-Secretase) activity assay fluorometric kit (Abcam, Cambridge, MA) according to the manufacturer's directions. In brief, following transfection, B35 rat neuroblastoma cells were placed in SFM for 24 hours. Cultures were washed gently with ice-cold 1X PBS and placed in 100 µl of β-secretase extraction buffer on ice for 30 minutes. Cells were scraped and lysates transferred to microfuge tubes and centrifuged to pellet nuclei at 13,000 x *g* for 5 minutes at 4 °C. Supernatants were transferred to new microfuge tubes on ice and 50 µl aliquots (in duplicate) were placed in a 96 well plate coated with anti-β-amyloid (supplied with the kit). Results were quantified according to the kit instructions and corrected for background and estimated the amount of β-secretase based on a standard curve. After one hour of incubation in the dark at 37 °C, fluorescence was measured on a fluorescent microplate reader at excitation/emission = 335/495 nm.

2.8. Measurement of Amyloid β Peptide 1-42:

After B35 cells had grown to near confluency, cultures were transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} for 4 hours and switched to SFM. 24 hours post transfection, cells were collected, and β amyloid levels were

measured using a rat amyloid β peptide 1-42 (Aβ 1-42) ELISA kit (MyBioSource) according to the manufacturer's instructions.

2.9. Measurement of Phosphorylated Microtubule-Associated Protein Tau:

Cells were grown to approximately 90% confluency and transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. After 4 hours, media was changed to SFM for 24 hours and cells were collected. The amount of phosphorylated tau protein levels in each sample (performed in duplicate) were measured using a rat phosphorylated microtubule-associated protein tau (PMAPT/PTAU) ELISA Kit (MyBioSource) according to the manufacturer's instructions.

2.10. Statistical Analysis:

Individual cultures were the sampling units for all experiments with each experiment being repeated at least three times. Immunocytochemical experiments were performed having three cultures for each experimental condition. After running three separate experiments for immunocytochemical analyses, the total N = 9/condition. In western blotting experiments, each experimental condition had a single culture in each of the three separate experiments, making a total N = 3/condition in western blotting analyses. In most experiments, data were normalized to the untransfected control condition and measurements for transfected cells with wild-type or mutant RhoA were compared to cells transfected with EmGFP alone. Data were initially analyzed

using a two-way analyses of variance (ANOVA) with treatment and experiment as independent variables. If there was little inter-experimental variance, the random independent factor for experimental day was removed from the analysis. Because this condition was met in nearly all experiments, data were generally analyzed by univariant ANOVA, unless otherwise indicated, for differences across groups with treatment as a fixed factor, and average fluorescence intensity measurement (from both western blotting and immunocytochemical experiments) as the dependent variable. The level of significance was set at $\alpha = 0.05$. If the ANOVA analysis was significant, pairwise comparisons were assessed across groups using either least significant difference (LSD) or Scheffé post-hoc tests when equal variances across groups were demonstrated by a non-significant Levene's test. In analyses where equal variances across groups could not be assumed (when Levene's test was significant), Tamhane's T2 post-hoc test was used to determine differences between treatment groups. Differences from cultures of transfected wild-type or non-prenylatable RhoA were compared to those transfected with EmGFP alone (control) in both experiments that were normalized to untransfected control cultures and for experiments where raw data is reported.

3. Results:

3.1. Expressing EmGFP-RhoA^{C190A} significantly increased tau levels in B35 cells

Dysregulation of RhoA has been implicated in the pathology of AD [14]. Important for the analyses taken in this work, ageing and AD are associated with an increase in geranylgeranyl pyrophosphate (GGPP) levels, likely due to their accumulation because of the activity of geranylgeranyltransferase-I (GGT-I), the enzyme responsible for transferring geranylgeranyl moieties to RhoA, is decreased [15, 16]. Therefore, it is a logical assumption that altered prenylation of RhoA might contribute to the formation of amyloid plaques and the neurofibrillary tangles in AD pathology. To assess the effects of RhoA prenylation on production of tau, we transfected cells with EmGFP, EmGFP-RhoA, and EmGFP-RhoA^{C190A} and assessed the amount of total tau using western blotting of whole cell lysates. Since tau is a cytosolic, microtubule-interacting protein, in some experiments we fractionated cells and assessed the amount of tau present in the cytosol in each condition. We routinely assessed whether equivalent amount of protein was loaded in western blotting experiments by blotting for actin after loading equivalent amounts of protein to each well of the gels. We did not observe changes in actin protein content across conditions in western blots from total cell lysates (see actin blot in Fig. 4.1A).

Western blot analysis demonstrated several bands immunoreactive with anti-tau antibodies (Fig. 4.1A). There are six isoforms of tau, ranging in size from

about 37 to 50 kD in their unmodified forms. However, tau proteins can be post-translationally modified in several ways, including phosphorylation, which affects both their migration on SDS-PAGE gels and decreases their ability to bind to and stabilize microtubules. Therefore, we assessed whether overexpressing non-prenylatable RhoA affected tau levels using western blotting. Compared to cells transfected with EmGFP only, expressing EmGFP-RhoA^{C190A} significantly increased the levels of total tau obtained from whole cell lysates while expressing EmGFP-RhoA did not (Fig. 4.1A, B). In AD, tau usually sequesters in aggregates in the cytosol [17]. So, we also assessed whether overexpressing non-prenylatable RhoA increased the levels of tau in the cytososl of B35 cells. Compared to cells transfected with EmGFP only, expressing neither EmGFP-RhoA nor EmGFP-RhoA^{C190A} significantly changed the levels of cytosolic tau (Fig. 4.1C, D). Efficient separation of cellular fractions was demonstrated by observed immunoreactive bands only for a nuclear-associated protein (histone 1.1), a membrane-associated protein (calnexin) and a protein found in nuclear, membrane and cytosolic cellular fractions (Fig. 4.1E). Transfection efficiencies determined through identifying transfected cells using EmGFP fluorescence and dividing by the total number of cells (identified by DAPI staining) averaged 70.5% (Fig. 4.1F).

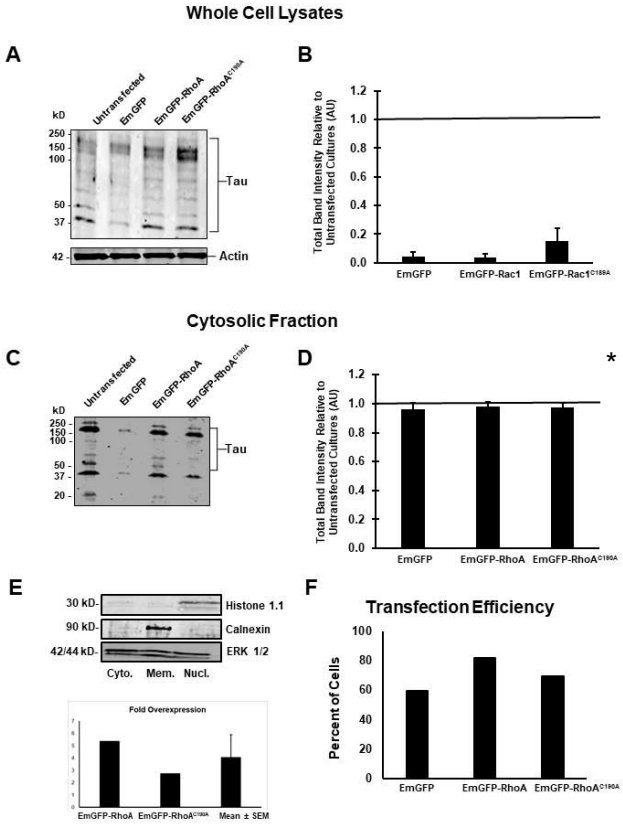

Fig. 4.1 Expression of EmGFP-RhoA^{C190A} increased total levels of tau. (A) Representative western blot of whole cell lysates from B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} showing bands immunoractive to anti-tau antibodies (A, top blot) in experiments with nearly equal loading of protein as assessed by blotting for actin (A, bottom blot). (B) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated by adding all the bands of tau protein btween approximately 37 and 250kD using the Odyssey CLx infrared imaging system by applying this formula: (total bands intensities-background) * area of bands to correct for both background level of staining and differences in the sizes of areas in assessed regions of interest. (C) Representative western blot for tau from the cytosolic fraction of B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (D) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated as described above for panel B. (E) Representative western blots showing efficient separation of cell fractions by blotting for proteins known to be localized to the nucleus (histone 1.1, top blot), membrane (calnexin, middle blot) or in the nucleus, cytosol and associated with membranes (Erk 1/2, bottom blot). Proteins expressed from plasmid averaged 4-fold higher expression than that of endogenous RhoA (F) Transfection efficiencies determined by the percent of transfected cells ranged between 60% and 82%. Data in B and D are expressed as means ± SEM for 3 separate experiments. Horizontal lines in B and D indicate the measure tau immunoreactivity in the untransfected control condition and was used to normalize across experiments. * indicates significant difference compared to cells transfected with EmGFP at $p \leq 0.05$ (ANOVA and LSD posthoc). N=3 cultures per condition.

We performed immunocytochemical experiments to assess the localization of tau when the localization of RhoA is altered. We did not transfect or transfected B35 cells with EmGFP, EmGFP-RhoA, or EmGFP-RhoA^{C190A} and assessed the mean fluorescence intensity of tau in each condition. From the number of cells in each photomicrograph (identified by staining with DAPI. Fig. 4.2A, E, I, M), EmGFP expression was evident only in transfected cells. Transfection efficiency was about 82% (Fig. 4.2F, J, N).

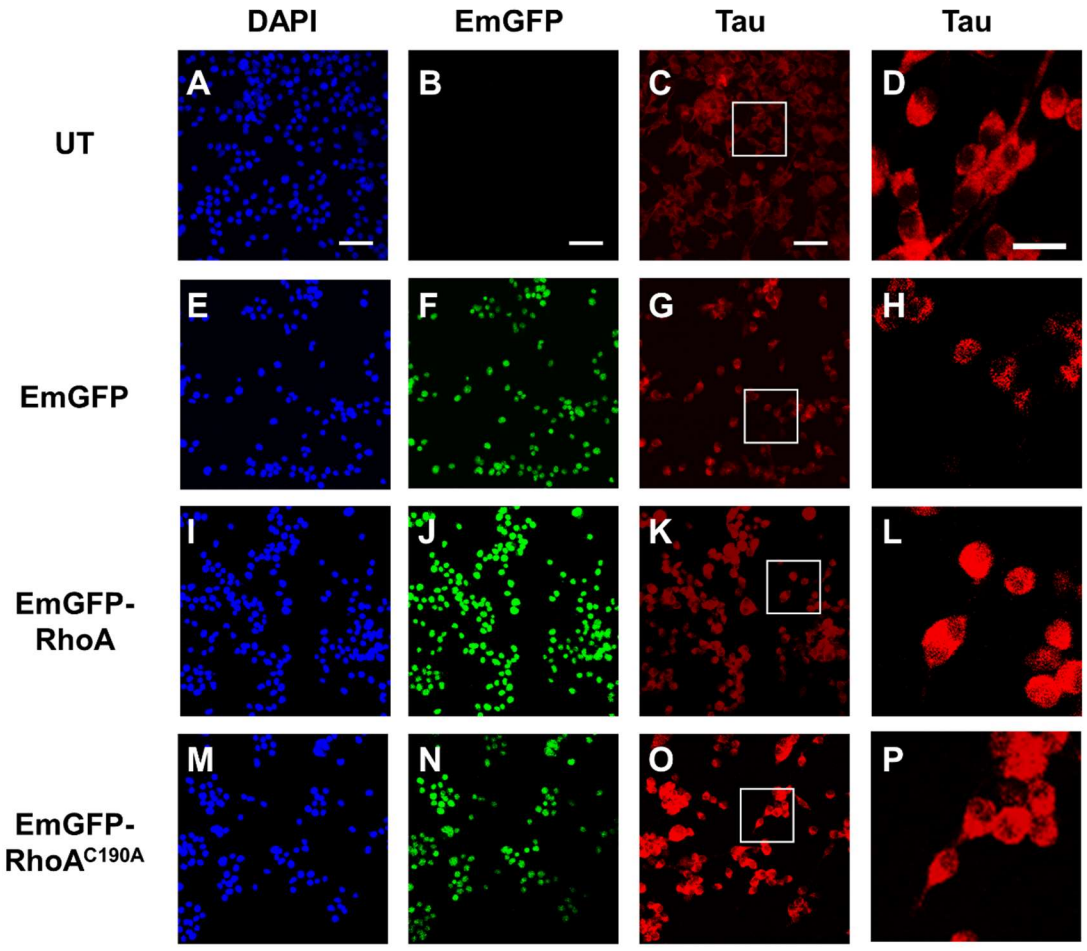

Fig. 4.2 Expressing EmGFP-RhoA^{C190A} did not change tau levels in B35 cells as assessed using immunocytochemistry. Representative images of B35 cells, either untransfected (UT; A-D), transfected with EmGFP only (E-H), or transfected with EmGFP-RhoA (I-L) or EmGFP-RhoA^{C190A} (M-P) indicated that transfection occurred in the majority of cells (total cells indicated by DAPI staining (A, E, I, M). transfected cells are indicated by EmGFP expression and subsequent fluorescence (B, F, J, N). Tau immunoreactivity is shown in the last two columns indicating fluorescence at lower (C, G, K, O) and higher (D, H, L, P) magnification. Boxes in images in the third column indicate that they are increased in images in the fourth column. Scale bars in A, B and C are 50 μm and is valid for all images in the first three columns, while the scale bar in D is 10 μm and is valid for images in the fourth column (D, H, L, P).

We quantified the mean fluorescence intensity for tau immunoreactivity using cell bodies as the analyzed regions of interest. Each of three separate

experiments had three cultures for each experimental condition and we analyzed at least five cells from each replicate culture (N = 9 cultures), with at least 45 cells analyzed in each experimental condition. Quantification of the mean fluorescence intensity indicated that expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not affect tau levels, compared to cells transfected with EmGFP only (Fig. 4.3).

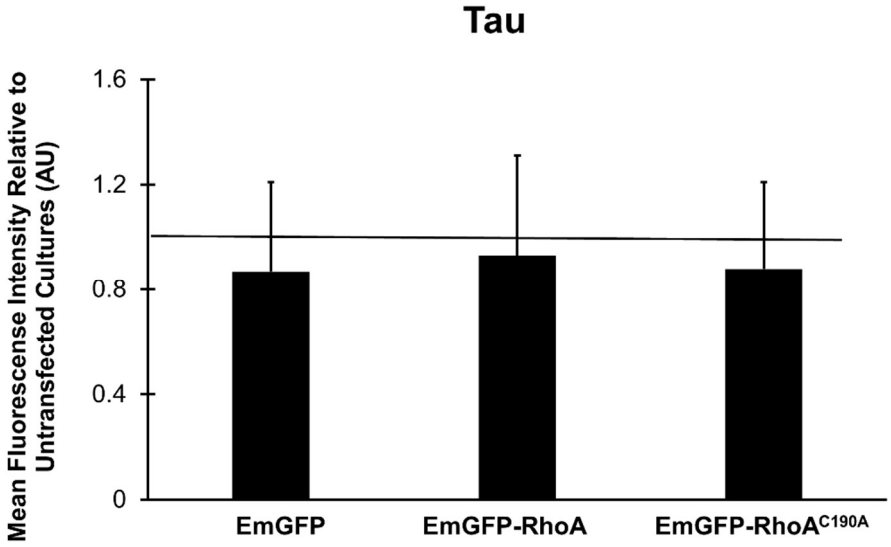

Fig. 4.3 Quantification from immunocytochemical experiments shows overexpressing non-prenylatable RhoA did not alter tau levels. Quantification of the mean fluorescence intensity indicated that expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not affect tau levels, compared to cells transfected with EmGFP only. Horizontal line in the graph indicates the value for the untransfected control condition, which was used to normalize across experiments. Data are means ± SEM for each condition, which was performed in triplicate in each of 3 separate experiments (N = 9 cultures in each condition) with at least 5 cells being quantified for each experimental replicate. Analysis by ANOVA indicated that there were no significant differences across groups.

3.2 Altering RhoA prenylation increases tau phosphorylation in B35 cells

In AD, the neurofibrillary tangles are mainly composed of the hyperphosphorylated protein tau [18]. To assess the effects of RhoA prenylation on production of phosphorylated tau, we transfected cells with EmGFP, EmGFP-RhoA, or EmGFP-RhoA^{C190A}. Then we assessed the levels of phosphorylated tau in whole cell lysates using a phosphorylated tau ELISA kit. Compared to cells transfected with EmGFP only, overexpressing EmGFP-RhoA^{C190A}, but not EmGFP-RhoA, significantly increased levels of phosphorylated tau (Fig. 4.4).

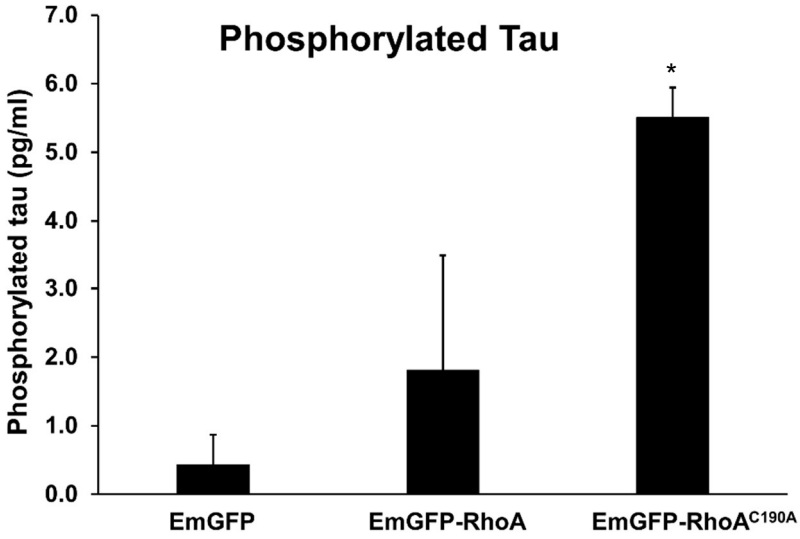

Fig. 4.4 Expression of EmGFP-RhoA^{C190A} increased levels of phosphorylated tau. B35 cells were transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. The levels of phosphorylated tau were detected using a rat phosphorylated microtubule-associated protein tau (PMAPT/PTAU) ELISA kit (MyBioSource). Total amounts of phosphorylated taus were estimated from a standard curve from samples supplied with the kit. Data are means ± SEM for N=3 cultures per condition. *indicates significant difference from cultures transfected with EmGFP alone using a univariant ANOVA followed by a least significant difference (LSD) post-hoc pairwise comparison at $p \leq 0.05$.

3.3 Altering RhoA prenylation did not change GSK-3β levels in B35 cells

We next assessed the effects of RhoA prenylation on steady state levels of GSK-3β, an important kinase that can induce hyperphosphorylation of tau [19]. After transfecting the cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}, B35 whole cell lysates were subjected to western blotting against GSK-3β. While some modest difference was seen in individual experiments (Fig 4.4A, top blot), equivalent protein loading was demonstrated by relatively little variation in the amount of actin across conditions (Fig. 4.5A, bottom blot). Quantification assessed by average band fluorescence intensity after correcting for background and area variations indicated that expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not change GSK-3β steady state levels, compared to cells transfected with EmGFP only, (Fig. 4.5).

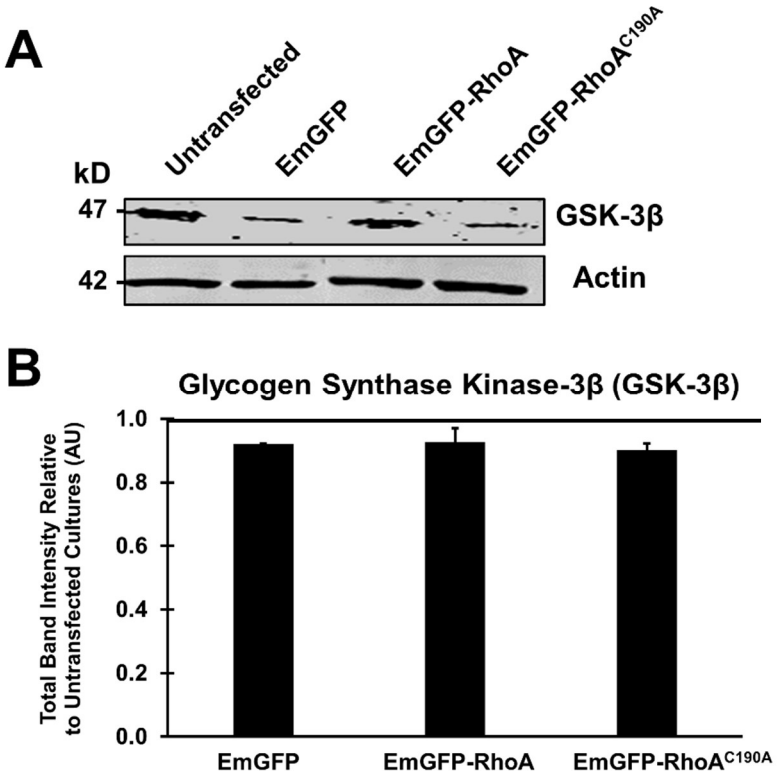

Fig. 4.5 Expression of EmGFP-RhoA^{C190A} did not affect levels of GSK-3β. (A) Representative western blot for GSK-3β from whole cell lysates of B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} (top blot) and actin loading controls (bottom blot). (B) Quantification of protein immunoreactivity for GSK-3β was normalized to the untransfected condition and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments. Analysis be ANOVA indicated that there were no significant differences between groups.

3.4 Altering RhoA prenylation did not change APP levels in B35 cells

In AD, the aberrant cleavage of the amyloid precursor protein (APP) produces β amyloid plaques [20]. β amyloid overproduction and its aggregation into plaques is considered the primary event in AD pathology, according to the amyloid hypothesis [21]. To assess how RhoA prenylation affects APP levels

and processing, we transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and first assessed the total levels of APP in each condition using western blotting. Western blot of whole cell lysates for APP showed little difference in levels across conditions (Fig. 4.6A, top blot), while equal loading of gels was confirmed by nearly equivalent amounts of actin across experimental conditions (Fig. 4.6A, bottom blot). Compared to cells transfected with EmGFP only, expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not significantly affect the levels of APP (Fig. 4.6B).

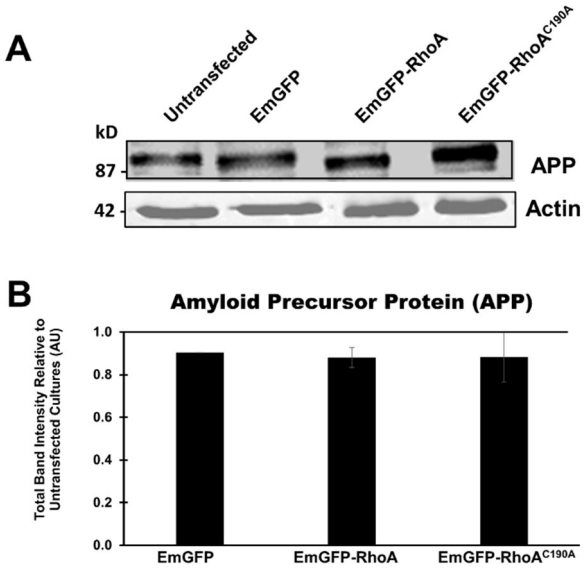

Fig. 4.6 Expression of EmGFP-RhoA^{C190A} did not affect levels of APP. (A) Representative western blot of whole cell lysates of APP from B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} showing little difference in the amount of APP (top blot) or loading control (actin, bottom blot). (B) Quantification of protein immunoreactivity using the Odyssey CLx infrared imaging system where conditions were normalized to the untransfected cultures and calculated by subtracting background from band intensity and adjusting for differences in area by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Horizontal line in B indicates the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated no significant differences across groups.

We next assessed the effects of RhoA prenylation on localization of APP. We transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and separated cells into membrane and cytosolic fractions to assess the total amount of APP in membranous fraction by western blotting. In western blots for APP associated with the membrane, no significant difference between EmGFP transfected cell and those transfected with EmGFP-RhoA or EmGFP-RhoA^{C190A} was seen (Fig. 4.7A). This was confirmed by quantification where expressing either EmGFP-RhoA or EmGFP-RhoA^{C190A} did not change membrane APP levels, compared to cells transfected with EmGFP only (Fig. 4.7B). The experiments support that prenylation of RhoA does not affect APP levels.

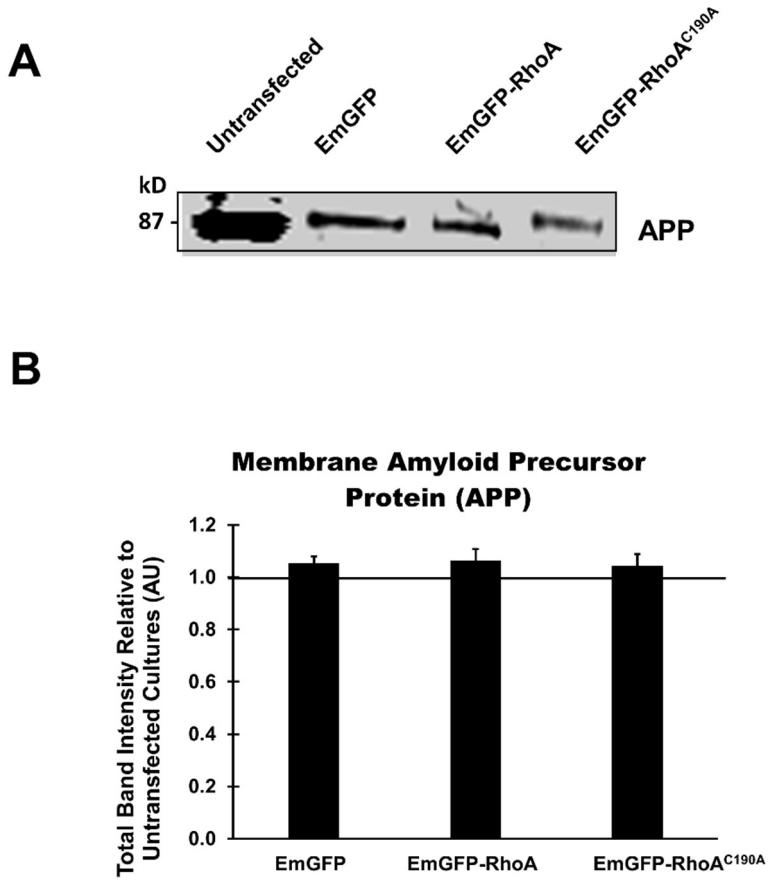

Fig. 4.7 Expression of EmGFP-RhoA^{C190A} did not affect levels of membrane-associated amyloid precursor protein (APP). (A) Representative western blot for APP from the membrane fractions of B35 cells either not transfected or transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (B) Quantification of protein immunoreactivity was normalized to untransfected condition (horizontal line in B) and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA indicated that there were no significant differences between groups.

3.5 Altering RhoA prenylation does not affect intracellular or extracellular Aβ42 levels in B35 cells

The above experiments indicate that prenylation of RhoA does not affect levels of APP. However, it is possible that decreasing RhoA prenylation could affect β amyloid levels while maintaining the levels of APP. To determine

whether expressing non-prenylatable RhoA increases production of β amyloid, we transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and assessed the extracellular and intracellular β amyloid levels using a β amyloid ELISA kit. Compared to cells transfected with EmGFP only, expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not affect either the intracellular or extracellular levels of β amyloid (Fig. 4.8)

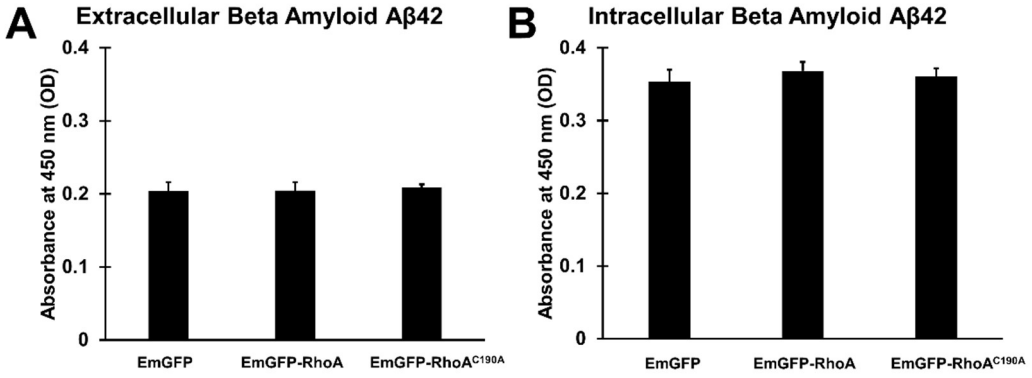

Fig. 4.8 Expression of EmGFP-RhoA^{C190A} did not affect extracellular or intracellular β amyloid Aβ42 levels. B35 cells were transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. The levels of extracellular and intracellular β amyloid Aβ42 were detected using a Rat Amyloid β Peptide 1-42 (Aβ 1-42) ELISA Kit (MyBioSource). N=3 cultures per condition. Analysis by ANOVA indicated that there were no significant differences between experimental groups.

3.6 Altering RhoA prenylation does not affect total BACE1 levels or activity in B35 cells or primary cortical neurons

All experiments assessing how RhoA prenylation affects APP and β amyloid levels suggest that its prenylation does not play a signficiant role. However, it is still possible that non-prenylatable RhoA could affect the enzymes

that process APP to β amyloid. In the amyloidogenic pathway, β amyloid is formed from the sequential cleavage of APP by β and γ-secretases [22]. To assess the effects of RhoA prenylation on production of BACE1 (β secretase), we transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and assessed levels of BACE1 in by western blotting of B35 whole cell lysates. Western blots for BACE1 (Fig. 4.9A, top blot; loading controls for actin, Fig. 4.9A, bottom blot) did not show any appreciable differences across conditions. Quantification of western analyses shows that there were no significant differences between experimental groups (Fig. 4.9B).

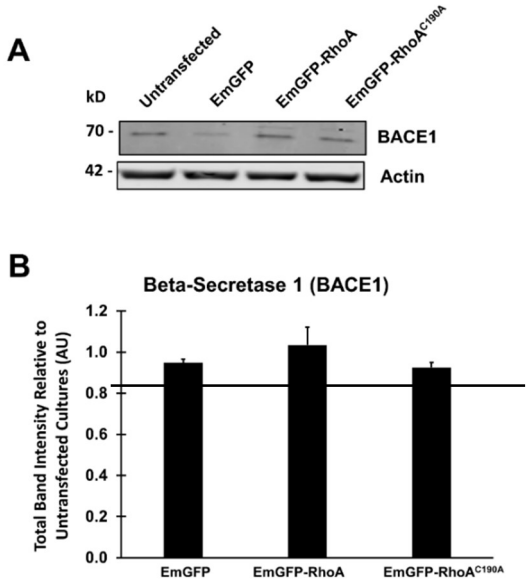

Fig. 4.9 Expression of EmGFP-RhoA^{C190A} did not affect levels of BACE1 in B35 cells. (A) Representative western blots of whole cell lysates for BACE1 from B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} (top blot) and actin loading controls showing equal protein loading across conditions (bottom blot). (B) Quantification of protein immunoreactivity was normalized to untransfected condition (horizontal line) and calculated using background and area corrected band intensities on an Odyssey CLx infrared imaging system. Data in B are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA indicated no significant differences across groups.

B35 cells are a good model for neurons. However, they are a transformed cell line that, unlike mature neurons, continue to divide. We minimize this in our experiments by performing them in SFM, which induces differentiation in B35 cells. However, it is likely that neurons affected by AD (e.g. cortical neurons) may respond differently to changes in RhoA prenylation. Therefore, we also assessed levels of BACE1 in primary cultured rat cortical neurons from postnatal day 1 neonates. Expression levels of transgenes are generally low in primary neurons (approximately 10%). It is, therefore, not surprising that we did not observe an obvious difference in the levels of BACE1 in primary neurons (Fig 4.10A). When we quantified western blots from primary neurons, there were no statistically significant difference between cells overexpressing non-prenylatable RhoA or wild-type RhoA and cells overexpressing EmGFP only (Fig 4.10B).

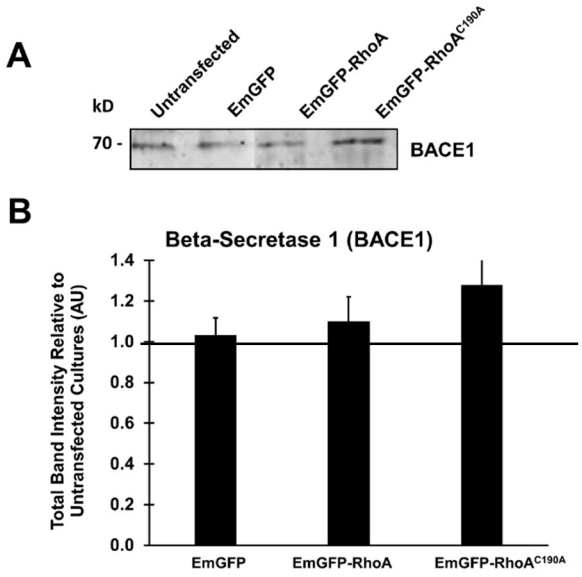

Fig. 4.10 Expression of EmGFP-RhoA^{C190A} did not affect levels of BACE1 in primary cortical neurons. (A) Representative western blot of whole cell lysates for BACE1 from primary cortical neurons transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (B) Quantification of protein immunoreactivity normalized to untransfected condition (horizontal line in B) and calculated using an Odyssey CLx infrared imaging system. Data in B are expressed as means ± SEM for 3 separate experiments. ANOVA analysis indicated no significant differences between conditions.

Together, each of the experiments analyzing APP, β amyloid or BACE1 levels do not support expressing non-prenylatable RhoA affects APP levels or processing. However, none of these assays assessed the activity of APP processing enzymes. It is possible that we could not observe subtle changes in APP or β amyloid level, but RhoA prenylation could still affect APP processing enzyme activity. Since β-secretase is the first enzyme in the amyloidogenic pathway, we next assessed whether expressing non-prenylatable RhoA affected BACE1 activity. To do this, we transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and used a β-secretase activity assay fluorometric kit to determine the β-secretase activity. Compared to cells transfected with EmGFP only, expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not affect the β-secretase activity (Fig. 4.11).

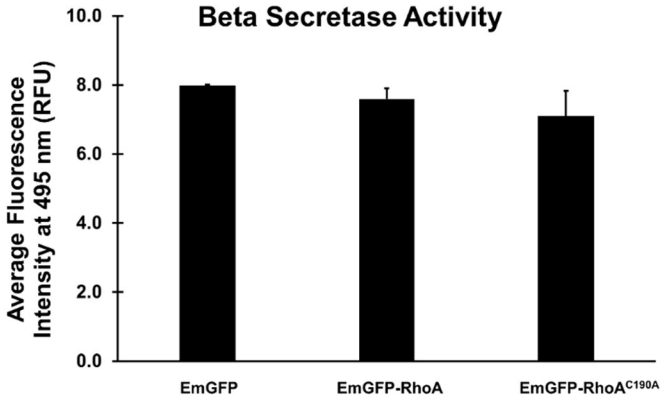

Fig. 4.11 Expression of EmGFP-RhoA^{C190A} did not affect β secretase activity. B35 cells were transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. The levels of β-secretase activity in samples were detected using a β-Secretase activity assay fluorometric kit (Abcam). N=3 cultures per condition. ANOVA analysis indicated that there were no significant differences between groups in this assay.

3.7 Altering RhoA prenylation did not affect membrane or cytosolic BACE1 levels in B35 cells

We next assessed the effects of RhoA prenylation on localization of BACE1, because this enzyme is membrane-associated. We separated transfected cells into membrane and cytosolic fractions and assessed the total amount of BACE1 in each fraction by western blotting. Western blots of the amount of BACE1 in the cytosol (Fig 4.12A, top blot) and associated with membranes (Fig. 4.12A, bottom blot) indicated that there was less BACE1 in the cytosol, as expected (Fig 4.12A). Quantification of each fraction, compared to cells transfected with EmGFP only, indicated that expressing either EmGFP-RhoA or EmGFP-RhoA^{C190A} did not affect the levels of the cytosol (Fig 4.12B) or membranous (Fig. 4.12C) BACE1. We interpret these experiments to suggest that altering the localization of RhoA does not affect the first proteolytic enzyme in the amyloidogenic pathway, BACE1.

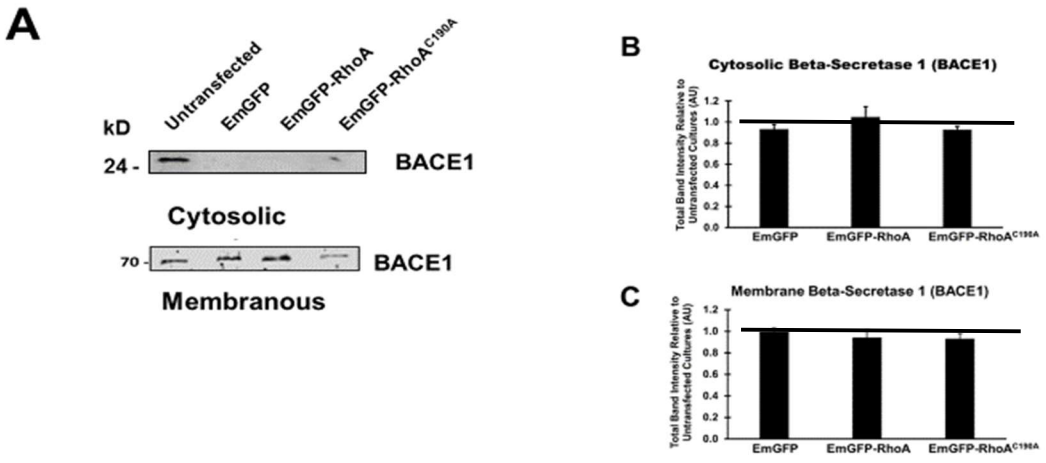

Fig. 4.12 Expression of EmGFP-RhoA^{C190A} did not affect levels of cytosolic or membrane BACE1. (A) Representative western blots for BACE1 from the cytosolic (top blot) and membrane (bottom blot) fractions of B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (B, C) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in B and C are expressed as means ± SEM for 3 separate experiments. Horizontal line in B and C indicates the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated that there were no significant differences between groups in either the cytosol (B) or membrane (C) fractions.

3.8. Expressing EmGFP-RhoA^{C190A} did not affect presenilin 2 levels in primary cortical neurons or in B35 cells

Each of the experimental sets we performed to assess how RhoA subcellular localization affects the amyloidogenic pathway indicated that neither starting material (APP) nor product (β amyloid) or the first proteolytic enzyme (BACE1) is altered by overexpressing EmGFP-RhoA^{C190A} in B35 cells or rat cortical neurons. However, RhoA could still affect the amyloidogenic pathway by

altering the second proteolytic enzyme, γ-secretase, which is active in both the amyloidogenic and non-amyloidogenic pathways. To assess whether RhoA prenylation affects production of the active subunit of γ-secretase, presenilin 2 (PS2), in B35 cells and primary cortical neurons, we transfected cells with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A} and assessed levels of PS2 in each condition using western blots of whole cell lysates. Western blots for PS2 indicated that expressing EmGFP-RhoA or EmGFP-RhoA^{C190A} did not change levels of PS2 in B35 cells (Fig. 4.13A) or in primary cortical neurons (Fig. 4.14A), compared to cells transfected with EmGFP only. Quantification of band intensities showed that expression of EmGFP-RhoA^{C190A} did not significantly alter the levels of PS2 compared to cells transfected with either EmGFP or EmGFP-RhoA in B35 neuroblastomas (Fig. 4.13B) or cultured primary neurons (Fig. 4.14B).

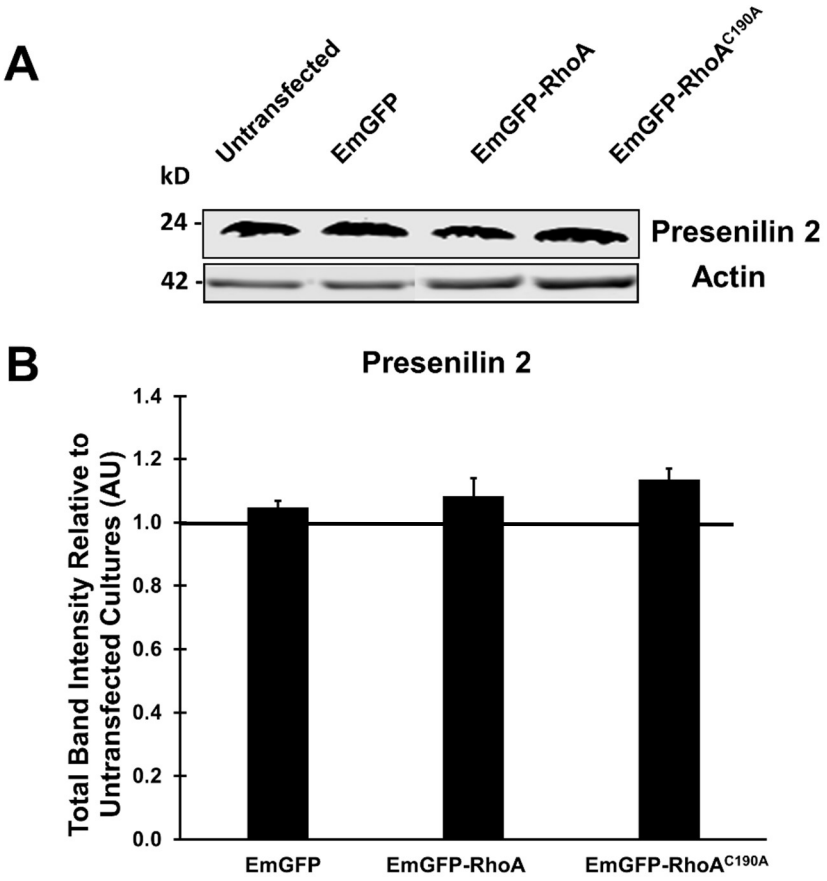

Fig. 4.13 Expression of EmGFP-RhoA^{C190A} did not affect levels of presenilin 2 (PS2) in B35 cells. (A) Representative western blot for PS2 of whole cell lysates from B35 cells transfected with EmGFP, EmGFP-RhoA and EmGFP-RhoA^{C190A}. (B) Quantification of protein immunoreactivity, normalized to the untransfected condition (horizontal line), imaged using the Odyssey CLx infrared imaging system and calculated by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA indicated that there were no significant differences across experimental groups.

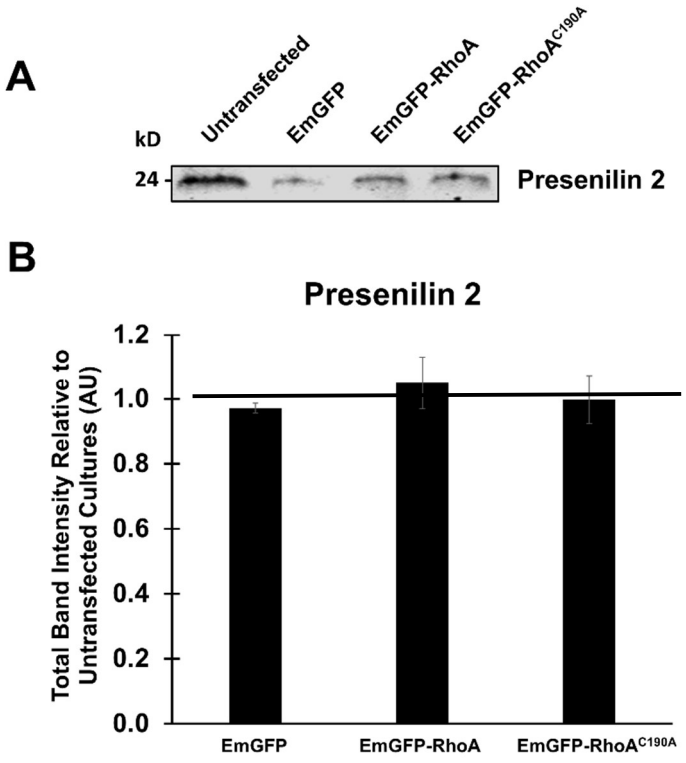

Fig. 4.14 Expression of EmGFP-RhoA^{C190A} did not affect levels of presenilin 2 (PS2) in primary cortical neurons. (A) Representative western blot for PS2 of whole cell lysates from primary cultures of P1 rat cortical neurons transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (B) Quantification of protein immunoreactivity was normalized to the untransfected condition (depicted by horizontal line in B), imaged using an Odyssey CLx infrared imaging system and calculated by applying this formula: (band intensity-background) * area of band. Data in B are expressed as means ± SEM for 3 separate experiments. Analysis by ANOVA did not indicate any significant differences between experimental groups.

3.9 Altering RhoA prenylation did not affect membrane or cytosolic PS2 levels in B35 cells

Altering RhoA prenylation translocates this protein to the cytosol. Therefore, it is possible that if RhoA interacted with γ-secretase, we may see differences in the level of the PS2 active subunit when cells are transfected with

non-prenylatable RhoA. Translocating γ-secretase to the cytosol would be expected to decrease its membrane levels and, in turn, affect APP processing. We separated transfected cells into membrane and cytosolic fractions and assessed the total amount of PS2 in each fraction by western blotting. Western blots for PS2 indicated that less PS2 was present in the cytosol as expected (Fig. 4.15A) than in the membrane (Fig. 4.15B) fraction and that these levels did not appear to change when B35 cells were transfected with EmGFP, EmGFP-RhoA, or EmGFP-RhoA^{C190A}. Quantification from images captured using a LiCor Odyssey CLx system and assessed for average band intensity confirmed no significant change in the levels of the membranous or the cytosolic PS2 (Fig. 4.15C and Fig. 4.15D).

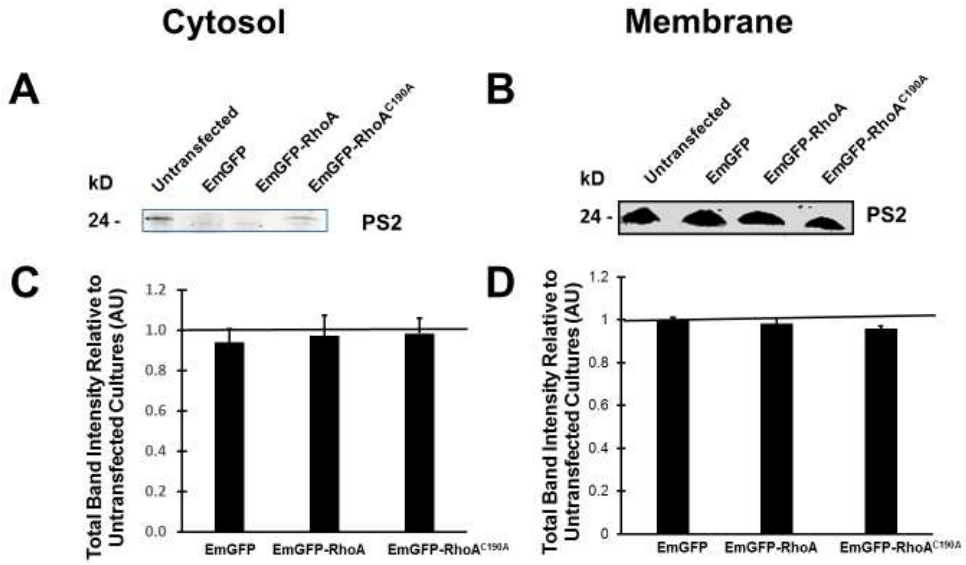

Fig. 4.15 Expression of EmGFP-RhoA^{C190A} did not affect levels of cytosolic or membrane presenilin 2 (PS2). (A) Representative western blot for PS2 from the cytosolic (A) and membrane (B) fractions of B35 cells transfected with EmGFP, EmGFP-RhoA or EmGFP-RhoA^{C190A}. (C, D) Quantification of protein immunoreactivity was normalized to untransfected condition and calculated by using the Odyssey CLx infrared imaging system by applying this formula: (band intensity-background) * area of band. Data in C and D are expressed as means ± SEM for 3 separate experiments. Horizontal lines in C and D indicate the untransfected control condition that was used to normalize across experiments. Analysis by ANOVA indicated that there were no significant differences between experimental conditions for PS2 located in the cytosol (C) or the membrane (D) fractions.

4. Discussion:

The main purpose of this study was to determine the importance of inhibiting RhoA prenylation and its effects on AD hallmarks. We found that, compared to cells expressing EmGFP only, cells expressing the non-prenylatable RhoA mutant (EmGFP-RhoA^{C190A}), but not wild-type RhoA (EmGFP-RhoA), significantly increased the total levels of tau, and the amount of tau phosphorylation. However, this did not involve an increase in the levels of GSK-

3β, a major phosphorylator of tau in AD. These data suggest that altering prenylation of RhoA could be involved in formation of neurofibrillary tangles in AD. To determine whether this is the case, a more detailed analysis of the potentially involved kinases and phosphatases is needed. Furthermore, our current results do not rule out any role that RhoA might play downstream of the formation of neurofibrillary tangles.

RhoA has been reported subcellularly altered in AD patients and in AD mouse models [8, 9]. In addition, in an AD mouse model, inhibiting RhoA activity significantly decreased the production of Aβ42, suggesting that RhoA signaling might be involved in APP cleavage [23, 24]. We tested the effects of overexpressing wild-type RhoA or non-prenylatable RhoA mutants on the levels of APP and its secretases, BACE1 and presenilin. We found that, compared to cells expressing EmGFP only, overexpressing either wild-type RhoA (EmGFP-RhoA) or a non-prenylatable RhoA mutant (EmGFP-RhoA^{C190A}) did not affect the total levels of APP, BACE1 and presenilin levels, or β-secretase activity. Whether at the membrane or in the cytosol, levels of APP, BACE1 and presenilin did not change when non-prenylatable RhoA mutant (EmGFP-RhoA^{C190A}) or wild-type RhoA (EmGFP-RhoA) was expressed. Consequently, the β-secretase activity was not affected. This suggests that RhoA does not affect APP processing and that the main function of RhoA might be controlled through activating GEFs, rather than location to affect production of β-amyloid. Given the lack of effect of overexpressing either wild-type or non-prenylatable RhoA on

APP processing, it is also possible that the main effects of RhoA are downstream of β-amyloid formation in AD. For instance, in PC12 cells, as well as in human neuroblastoma cells, RhoA activation was observed when cells were treated with Aβ42 [25].

We tested the effects of expressing wild-type RhoA or its non-prenylatable mutant on levels of extracellular and intracellular Aβ42, the pathological form of β-amyloid. We did not find any significant changes. This is in agreement with the rest of our results showing RhoA is not involved in APP processing through the amyloidogenic pathway by β- and γ-secretases.

The results we report are consistent with reports from some laboratories, but inconsistent with others. For instance, Rho prenylation inhibition by pitavastatin was found to reduce total levels of tau [26]. Treatment with the toxic $A\beta_{42}$ activated RhoA and reduced neuronal survival through protein tyrosine phosphatase 1B (PTP1B) inhibition [25]. Studies performed *in vitro* and *in vivo* have shown that Aβ induced neurofibrillary tangles by interacting with Rac1 [27]. Inhibiting the RhoA-ROCK pathway was reported to restore the disruption of blood-brain barrier induced by $A\beta_{42}$ [28]. Aβ was reported to increase tau phosphorylation, induce synaptic loss and cause neuronal death [29]. Together, these experiments demonstrate the complexity involved in how RhoA affects AD pathology and suggest that RhoA might function downstream of AD pathological markers.

There are many lines of evidence that small GTPases are implicated in AD pathology. Many results derive from treatment with cholesterol-lowering statins, which inihbit the rate-limiting enzyme for the mevalonate pathway, HMG-CoA reductase. Here, statins inhibit all protein prenylation, including that of RhoA. One study suggests that statins decrease the levels of protein prenylation and Aβ production by promoting the non-amyloidogenic pathway of APP [30]. In addition, statins induce a decrease in isoprenoid levels and inhibit APP trafficking [31]. Thus, decreased protein prenylation might be the likely source for promoting the non-amyloidogenic pathway and altering APP processing.

Overall, we interpret this study to suggest that the non-prenylatable RhoA mutant (EmGFP-RhoA^{C190A}) is not involved in one of AD's hallmarks, in production of β-amyloid through APP cleavage. It is possible that RhoA is involved in APP processing that leads to the non-amyloidogenic pathway as RhoA did not affect the β-secretase activity [30]. Also, as stated before, RhoA might not be upstream of production of AD's hallmarks, it might be downstream of β-amyloid plaque or neurofibrillary tangle formation.

Since all experiments in this study were conducted in neuroblastoma cells, with a few experimental results confirmed in primary cultures of cortical neurons, and each type of cell expresses endogenous RhoA, it is possible that competition has happened between the expressed construct and the endogenous RhoA, however unlikely this may be with such high levels of construct overexpression.

The previous studies demonstrating roles for RhoA in AD suggest that further studies need to be performed. Experiments that may shed some mechanistic insight into the role of RhoA in AD include reducing the influence of endogenously-expresed RhoA using RNA silencing or conditional knockouts techniques. This would allow for a better understanding of how inhibiting RhoA prenylation affects the localization and production of AD proteins. Most importantly, because cell culture results do not always correlate with animal models, these experiments should be tested in a primary neuron system or animal models of AD.

5. Conclusion

Prenylation of the small GTPase, RhoA, by the addition of a geranylgeranyl moiety targets the modified protein to the plasma membrane, where it can theoretically become active and affects the production of AD markers like hyperphosphorylated tau and the production of Aβ. Rationale leading to this idea includes studies performed with statins, which inhibit protein prenylation and cholesterol production and studies investigating prenylation and prenylation precursors in AD. Statin use is generally correlated with decreased incidence of AD, while the prenylation precursor GGPP increases in AD due to decreased geranylgeranyl transferase I activity and subsequent decreases in small GTPase activation. Based on this, we hypothesized that decreased prenylation of RhoA would increase AD markers like hyperphosphorylated tau and β-amyloid. Overexpression of non-prenylatable RhoA (EmGFP-RhoA^{C190A}),

increased tau and tau phosphorylation, but did not affect APP level, production of β-amyloid or levels or activities of the processing enzymes BACE1 and presenilin 2. Further, overexpressing non-prenylatable RhoA did not affect levels of the common tau kinase, GSK3, suggesting that cytosol-localized RhoA might activate a different kinase or inactivates a phosphatase to increase tau phosphorylation. We interpret our results to suggest that prenylation of RhoA might play a role in producing neurofibrillary tangles, but not β-amyoid plaques in AD pathology.

Acknowledgments: This work was funded by the TWU Department of Biology, and grants from the TWU Research Enhancement Program and the TWU Grant Proposal Preparation Program, as well as grants for the National Science Foundation. No funder was involved in the performance of experiments interpretation of the results, or in the writing of this manuscript beyond the approval of the experiments that were initially proposed. The authors thank Prapti Mody for technical assistance and critical evaluation of this work and Dr. Jairus Reddy for creating and providing us with the vectors for EmGFP, wild-type RhoA and non-prenylatable RhoA.

Declarations of Interest: None

Author Contributions: Rawand Chabayta proposed goals and she and DiAnna Hynds jointly defined the study hypotheses and methods to be used to test the hypotheses. Dr. Hynds supervised experiments, all of which were performed by Ms. Chabayta. Ms. Chabayta wrote the initial draft of the manuscript. Both

authors were involved in editing and refining the manuscript of submission to *Cellular Signalling*. Both authors have approved submission of the final manuscript.

6. References

1. Carlo, M. D., Giacomazza, D., & Biagio, P. L. Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools, Journal of Physics: Condensed Matter. 24 (2012) 244102.
2. Alzheimer's Association. 2017 Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia 2017. 13 (2017) 325-373.
3. Brookmeyer, R., Gray, S., & Kawas, C. Projections of alzheimer's disease in the united states and the public health impact of delaying disease onset, American Journal of Public Health. 88(1998) 1337-1342.
4. Serranzo-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological alterations in Alzheimer disease, Cold Spring Harbor Perspectives in Medicine. 1 (2011) a006189.
5. Curtis, I. D. Functions of Rac GTPases during Neuronal Development, Developmental Neuroscience. 30 (2007) 47-58.
6. Deng, Qiyue, et al. Small Nogo-66-Binding Peptide Promotes Neurite Outgrowth through RhoA Inhibition after Spinal Cord Injury, Brain Research Bulletin. 99 (2013) 140–144.

7. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders, Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.
8. Huesa, G., Baltrons, M. A., Gomez-Ramos, P., Moran, A., Garcia, A., Hidalgo, J., et., al. Altered distribution of RhoA in alzheimer's disease and AβPP overexpressing mice, Journal of Alzheimer's Disease: JAD. 19 (2010) 37-56.
9. Petratos, S., Li, Q. X., George, A. J., Hou, X., Kerr, M. L., Unabia, S. E., et., al. The β-amyloid protein of alzheimer's disease increases neuronal CRMP-2 phosphorylation by a rho-GTP mechanism, Brain: A Journal of Neurology. 131(2008) 90-108.
10. Roberts, Patrick J., et al. Rho Family GTPase Modification and Dependence on CAAXMotif-Signaled Posttranslational Modification, Journal of Biological Chemistry. 283 (2008) 25150–25163.
11. Higgins, J. B., & Casey, P. J. The role of prenylation in G-protein assembly and function, Cellular Signalling. 8(1996), 433-437.
12. Samuel, F., & Hynds, D. L. RHO GTPase Signaling for Axon Extension: Is Prenylation Important? Molecular Neurobiology. *42* (2010) 133-142.
13. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated, Cellular Signalling. 27(2015), 630-637.
14. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer’s disease, Alzheimers Research & Therapy. 9(2017).

15. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients, Pharmacopsychiatry. 42(2009).
16. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain, Journal of Neurochemistry. 129(2014), 732-742.
17. Iqbal, Khalid, et al. Cytosolic Abnormally Hyperphosphorylated Tau But Not Paired Helical Filaments Sequester Normal MAPs and Inhibit Microtubule Assembly, Journal of Alzheimers Disease. 14(2008).
18. Iqbal, K., et al. Tau in Alzheimer Disease and Related Tauopathies, Current Alzheimer Research. 8 (2010) 656–664.
19. Papasozomenos, S. C., & Binder, L. I. Phosphorylation determines two distinct species of tau in the central nervous system, Cell Motility and the Cytoskeleton. 8(1987), 210-226.
20. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease, Progress in Neurobiology. 156 (2017) 189-213.

21. Busciglio, J., Gabuzda, D. H., Matsudaira, P., & Yankner, B. A. Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells, Proceedings of the National Academy of Sciences. 90(1993) 2092-2096.
22. Zhang, Y., Thompson, R., Zhang, H., & Xu, H. APP processing in Alzheimer's disease, Molecular Brian. 4(2011).
23. Zhou, Y. Nonsteroidal Anti-Inflammatory Drugs Can Lower Amyloidogenic A 42 by Inhibiting Rho, Science. 302 (2003) 1215–1217.
24. Herskowitz, J. H., et al. Pharmacologic Inhibition of ROCK2 Suppresses Amyloid- Production in an Alzheimers Disease Mouse Model, Journal of Neuroscience. 33 (2013) 19086–19098.
25. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease, Alzheimers Research & Therapy. 9(2017).
26. Hamano, Tadanori, et al. Pitavastatin Decreases Tau Levels via the Inactivation of Rho/ROCK, Neurobiology of Aging. 33 (2012) 2306–2320.
27. Perez, S. E., Getova, D. P., He, B., Counts, S. E., Geula, C., Desire, L., et al. Rac1b Increases with Progressive Tau Pathology within Cholinergic Nucleus Basalis Neurons in Alzheimers Disease, The American Journal of Pathology. 180 (2012) 526-540.
28. Park, J., Baik, S. H., Han, S., Cho, H. J., Choi, H., Kim, H. J., et al. Annexin A1 restores Aβ1-42-induced blood-brain barrier disruption through the inhibition of RhoA-ROCK signaling pathway, Aging Cell. 16 (2016) 149-161.

29. Sellers, K. J., Elliott, C., Jackson, J., Ghosh, A., Ribe, E., Rojo, A. I., et al. Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil, Alzheimers & Dementia. 14 (2018) 306-317.

30. Ostrowski SM, Wilkinson BL, Golde TE, Landreth G. Statins reduce amyloid-β production through inhibition of protein isoprenylation, J Biol Chem. 282 (2007) 26832–26844.

31. Cole SL, Grudzien A, Manhart IO, Kelly BL, Oakley H, Vassar R. Statins cause intracellular accumulation of amyloid precursor protein, β-secretase-cleaved fragments, and amyloid β-peptide via an isoprenoid-dependent mechanism, J Biol Chem. 280(2005):18755–18770.

Chapter V

DISSERTATION SUMMARY AND CONCLUSIONS

The purpose of this work was to determine how inhibiting prenylation of Rac1 or RhoA affects the production and localization of Alzheimer's disease (AD) proteins: amyloid precursor protein, β amyloid, and tau, in B35 neuroblastoma cells with some confirmation in primary cortical neuron cultures. We focused on these Rho proteins, Rac1 and RhoA, since these monomeric-guanine-nucleotide binding molecules have been implicated in many neurological diseases, including AD. [1,2,3] Rho guanosine triphosphatases (GTPases) activity has been examined in AD, but how affecting prenylation to alter Rho GTPase localization might alter production of AD markers has not been established. In this study, we tested the following hypotheses:

1. ***Expressing non-prenylatable Rac1 or RhoA would increase the production of tau, phosphorylated tau and the kinase GSK-3β.***
2. ***Expressing non-prenylatable Rac1 or RhoA would increase the production of APP and the activity of the secretases, β-site APP cleaving enzyme 1 (BACE1) and presenilin at the membrane and in the cytosol.***
3. ***Expressing non-prenylatable Rac1 or RhoA would increase the production of extracellular and intracellular β amyloid.***

Neurofibrillary tangles formation is one of AD's neuropathological hallmarks.[4] These tangles are mainly composed of hyperphosphorylated tau.[5] In the first set of experiments, we assessed how expressing wild-type or non-

prenylated RhoA or Rac1 affects production of total tau and hyperphosphorylated tau. In B35 neuroblastoma cells, overexpressing wild-type RhoA or non-prenylatable Rac1 did not affect levels of tau, phosphorylated tau or GSK-3β levels, while overexpressing non-prenylatable RhoA increased tau levels and the amount of phosphorylated tau, but not GSK-3β levels. Overexpressing wild-type Rac1 also increased tau phorphorylation without affecting GSK3β levels. These data suggest that overexpressing non-prenylatable RhoA or wild-type Rac1 may be involved in the the formation of neurofibrillary tangles, but the mechanism through which non-prenylatable RhoA works does not involve the common tau phosphorylating enzyme, GSK-3β. These findings correlate with other research data. For instance, in AD brains, immunohistological experiments have shown that RhoA was altered in subcellular compartments whereas Rac1 was not. [6] RhoA levels increased in neurons and were found to be co-localized with hyperphosphorylated tau in the neurofibrillary tangles.[6] In APP overexpressing transgenic mouse brains, RhoA expression was reduced within synapses and increased in degenerating neurites.[6] In addition, tau aggregation in AD glial cells leads to neuronal dysfunction by activating the RhoA pathway through its activation of Rho kinase.[7]

In B35 neuroblastoma cells, overexpressing wild-type Rac1 increased phosphorylated tau but not levels of tau. These findings are not in agreeement with other research data. For instance, in AD patients, a negative correlation was found between phosphorylated tau and Rac1. [8] In a triple transgenic AD

mouse, the knockdown of cyclin-dependent kinase 5 (CDK 5) decreased tau phosphorylation and increased Rac activity and p35 protein levels, leading to improvements in cognitive functions.[9] Since B35 neuroblastoma cells were used instead of an AD model, this might be one reason why phosphosorylated tau levels did not decrease as in AD models when overexpressing wild-type Rac1. Additionally, altering the subcellular localization of Rac1 does not appear to play a role in AD as mentioned above.[10] In fact, the results suggest that membrane localization of Rac1, as seen when the wild-type is overexpressed, may play a role in increasing tau phosphorylation. As the neurofibrillary tangles are found in AD models, but not in B35 neuroblastoma cells, Rac1 might also function upstream and downstream of neurofibrillary tangles. However, it is also likely that the subcellular localization of Rac1, the modification manipulated by overexpressing non-prenylatable Rac1, does not contribute to the mechanisms used in previous reports showing that Rac1 protects against tau phosphorylation and neurofibrillary tangle formation. However, the subcellular distribution changes when overexpressing wild-type Rac1[10] in that more wild-type Rac1 associates with the plasma membrane, likely increasing the amount of GTP-loaded, activated Rac1 at this location and leading to the subsequent increase in tau phosphorylation.

This study focuses specifically on the kinase, GSK-3β, because an increase in GSK-3β activity has been linked to neurodegeneration, memory deficits, tau hyperphosphorylation, and β amyloid production [11] and GSK-3β is

increased and co-localized with neurofibrillary tangles in AD brains.[11] Tau is phosphorylated at over 30 serine/threonine residues in AD.[12] The results from this study did not find any changes in GSK-3β levels when expressing wild-type or non-prenylated RhoA or Rac1, despite the demonstration that overexpressing non-prenylatable RhoA increases tau levels and phosphorylation. For future studies, other mechanisms such as known signaling pathways that lead to tau transcription or phosphorylation need to be investigated. For example, the transcription factor peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) can be activated by AMP-activated protein kinase (AMPK), which in turn, stimulates mitochondrial biogenesis and causes mitochondrial malfunctions and production of reactive oxygen species.[13] This signaling pathway will lead to tau aggregation, one of the AD hallmarks.[13] In conclusion, in B35 neuroblastoma cells, overexpressing mutant RhoA increases tau production and phosphorylation, but not levels of GSK-3β. This suggests that RhoA may be upstream of neurofibrillary tangles formation in AD pathology but does not rule out any effects from RhoA downstream of AD pathological processes.

Formation of amyloid plaques is the other neuropathological AD hallmark. β-amyloid plaques are produced from the aberrant cleavage of the amyloid precursor protein (APP). In the amyloidogenic pathway, the sequential cleavage of APP by β and γ-secretases produces soluble APPβ (sAPPβ) and β amyloid.[14] In this study, it was also assessed how expressing prenylatable or non-prenylatable RhoA and Rac1 affected levels of total APP and its secretases,

BACE1 and presenilin. In B35 neuroblastoma cells, overexpressing either prenylatable RhoA or Rac1 or non-prenylatable RhoA or Rac1 did not affect levels of APP or levels of its secretases, BACE1 and presenilin. These findings contradict other research data. For instance, Rac1 inhibition was found to decrease APP synthesis and the selectivity of γ-secretase.[15] The reasons behind this discrepancy are not clear, but likely reflect effects of changing the subcellular localization of Rac1. However, when presenilin levels in rat cortical neurons were tested, overexpressing prenylatable Rac1 increased presenilin levels. The increase in presenilin levels only in cortical neurons, but not in B35 neuroblastoma cells, when overexpressing prenylatable Rac1 might indicate that Rac1 has an important function in neurodevelopment. This is a possibility because the experiments were not performed with aged neurons, but rather with neonatal neurons that were still developing networks. Future experiments should focus on how increasing cytosolic Rac1 affects the selectivity of γ-secretase, especially given the finding that γ-secretase enhances dendritic spine formation at the synapse by activating the Rac signaling pathway.[16]

One reason that explain why results in B35 cells did not support other research findings might be that Rac1 functions downstream of β amyloid formation in AD. Another explanation might be that the endogenous proteins expressed in B35 neuroblastoma cells compensate for the expressed protein. Although the latter is unlikely as proteins expressed from plasmids occur at much higher levels (an average of 33.2-fold higher) than endogenous Rac1. So, for

future studies, endogenous RhoA and Rac1 will be knocked out and cells will be transfected using the non-prenylatable constructs.

In this study, it was also assessed how expressing wild-type or non-prenylatable RhoA and Rac1 in B35 neuroblastoma cells affected production of membranous APP and levels of its secretases, BACE1 and presenilin, at the membrane and in the cytosol. Compared to cells expressing EmGFP only, expressing both wild-type and non-prenylatable Rac1 decreased membrane APP levels, while expressing wild-type or non-prenylatable RhoA did not affect membranous APP levels. However, compared to wild-type Rac1, overexpression of non-prenylated Rac1 increased membranous APP levels, but not back to the levels seen in cells overexpressing EmGFP alone. One reason for seeing differences in cellular fractions, but not in whole cell lysates, could be that the majority of each protein is located in one cell fraction. For instance, if the majority of APP is located in the membrane, any APP located in the cytosol might mask changes when assessed in whole cell lysates. On the other hand, expressing either wild-type or non-prenylatable RhoA or Rac1 did not affect BACE1 and presenilin secretase levels for proteins associated with membranes or located in the cytosol. Since these secretase levels do not change at the membrane or in the cytosol, it is logical that total levels of the secretases assessed from whole cell lysates do not change as well. The decrease in APP levels at the membrane when expressing wild-type or mutant Rac1 suggests that Rac1 is involved in APP processing through a mechanism that does not involve

the secretases, BACE1 or presenilin. It is also possible that Rac1 prenylation is involved in APP processing that leads to non-amyloidogenic pathway.

Since it is possible that Rac1 might be involved in the non-amyloidogenic pathway of APP processing, it was assessed the β secretase activity after overexpressing wild-type and mutant Rac1. β secretase activity should not be involved in the non-amyloidogenic pathway and this is what was observed after expressing either wild-type or mutant Rac1, with no changes occurring in β secretase activity. In the study it was also tested the β secretase activity after expressing wild-type and mutant RhoA and did not see any changes.

In the last set of experiments, the effect of expressing wild-type or non-prenylated RhoA and Rac1 on the production of β amyloid in B35 neuroblastoma cells was tested. β amyloid plaques are usually produced outside the cell. But, there is evidence that intracellular β amyloid gets formed first and then is released into the extracellular space to form the extracellular amyloid plaques.[17] First, it was tested how expressing wild-type or non-prenylated RhoA or Rac1 affect the levels of extracellular β amyloid Aβ42. Results showed no significant difference in Aβ42 levels between overexpressing EmGFP and wild-type Rac1 or non-prenylated Rac1 or between overexpressing EmGFP and wild-type RhoA or non-prenylated RhoA in B35 neuroblastoma cells. This might be explained due to the fact that there were also no significant changes in the APP levels or levels of β and γ-secretases, BACE1 and presenilin in the previous sets of experiments.

Another explanation might be due to formation of intracellular β amyloid in B35 cells after expressing the wild-type or non-prenylatable constructs of RhoA and Rac1 that might require more time to secrete β amyloid into the extracellular space. This hypothesis was tested in the next experiment by overexpressing wild-type or non-prenylatable RhoA or Rac1 and assessing the effects on the levels of intracellular Aβ42, which also showed no significant differences between experimental conditions. Other explanations might be due to the type of cell lines that are used in the experiment, as B35 neuroblastoma cells might not produce enough Aβ42. For future studies, different cell lines should be used, or in *vivo* AD models should be used to run such experiments.

Rho GTPases are key regulators of the actin cytoskeleton and play effective roles in neurological morphogenesis.[18,19] In this study, it was found that inhibiting Rac1 prenylation decreased membrane APP, a transmembrane protein that is important in regulating many cellular functions including synaptic plasticity and synaptogenesis. It also found that inhibiting RhoA prenylation increased tau levels and phosphorylation. While tau normally stabilizes microtubules, increased expression and phosphorylation of tau may indicate that decreasing RhoA prenylation may lead to the development of a hallmark of AD, the formation of neurofibrillary tangles. Results from all the experiments reported in this dissertation are summarized in Table 5.1.

Table 5.1. Similarities and differences of results between constructs

Expressed Constructs	***EmGFP-Rac1***	***EmGFP-Rac1^{C189A}***	***EmGFP-RhoA***	***EmGFP-RhoA^{C190A}***
Total tau Levels	No Change	No Change	No Change	Increased
GSK-3β Levels	No Change	No Change	No Change	No Change
Phosphorylated Tau Levels	Increased	No Change	No Change	Increased
Total APP Levels	No Change	No Change	No Change	No Change
Total BACE1 Levels	No Change	No Change	No Change	No Change
Total presenilin Levels	No Change	No Change	No Change	No Change
Cytosolic BACE1	No Change	No Change	No Change	No Change
Cytosolic Presenilin	No Change	No Change	No Change	No Change
Membranous APP	Decreased	Decreased	No Change	No Change
Membranous BACE1	No Change	No Change	No Change	No Change
Membranous Presenilin	No Changes	No Changes	No Changes	No Change
β-Secretase Activity	No Changes	No Changes	No Change	No Change
Extracellular β amyloid Levels	No Changes	No Changes	No Change	No Change
Intracellular β amyloid Levels	No Changes	No Changes	No Change	No Change

[a]Table shows the similarities and differences between the expressed wild-type and mutated constructs of Rac1 and RhoA. The significant differences in levels or activation of AD proteins, secretases and kinase in B35 neuroblastoma cells are shown in gray shaded cells.

Future studies need to be performed as an alternative route to help explore the role of Rho GTPase cellular localization in AD. For instance, significant knowledge could be gained by knocking down or knocking out endogenous Rac1 and RhoA and transfecting cells using non-prenylatable constructs. This would eliminate the possibility that the endogenous proteins expressed in B35 neuroblastoma cells compensate for the expressed protein. Using both non-prenylatable constructs to transfect cells would help provide an additional way to study the effects of inhibiting both Rac1 and RhoA on AD hallmarks or on signaling cascades.

More studies are also needed to elucidate the signaling pathways of differently localized and active RhoA and Rac1 that lead to tau transcription, secretases activation or β-amyloid processing. Moreover, AD models should be used in experiments, as well, to study the role of altered Rho GTPases localization on the different stages of the disease. These studies would help better understand the role of Rho GTPases in AD and other neurodegenerative disorders.

These studies raise more questions on how non-prenylation of other RhoGTPase proteins such as CDC42 or Rab affect the production and localization of AD's proteins, secretases and kinases. Studying those other GTPases proteins would give insights into the molecular mechanisms of Rho GTPases in AD and other neurodegenerative disorders and is important to understand how these proteins function dependently and independently.

CUMULATIVE REFERENCE SECTION

CHAPTER I

1. Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological Alterations in Alzheimer Disease. Cold Spring Harbor Perspectives in Medicine. 1 (2011).
2. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease. Progress in Neurobiology. 156 (2017) 189–213.
3. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders. Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.
4. Higgins, J. B., & Casey, P. J. The role of prenylation in G-protein assembly and function. Cellular Signalling. 8 (1996) 433–437.
5. Liang, P. H., Ko, T. P., & Wang, A. H. J. Structure, mechanism and function of prenyltransferases. European Journal of Biochemistry. 269 (2002) 3339–3354.
6. Roskoski, R. Protein prenylation: a pivotal posttranslational process. Biochemical and Biophysical Research Communications. 303 (2003) 1–7.
7. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-

pyrophosphate is altered in male Alzheimer patients. Pharmacopsychiatry. 42 (2009).

8. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain. Journal of Neurochemistry. 129 (2014) 732–742.

9. Huang, G. H., Sun, Z. L., Li, H. J., & Feng, D.F. Rho GTPase-activating proteins: Regulators of Rho GTPase activity in neuronal development and CNS diseases. Molecular and Cellular Neuroscience. 80 (2017) 18–31.

10. Huesa, G., Baltrons, M. A., Gómez-Ramos, P., Morán, A., García, A., Hidalgo, J., et al. Altered Distribution of RhoA in Alzheimers Disease and AβPP Overexpressing Mice. Journal of Alzheimers Disease. 19 (2010) 37–56.

11. Wang, P. L., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene. Journal of Neuroscience Research. 87 (2009) 2105–2114.

12. Boo, J. H., Sohn, J. H., Kim, J. E., Song, H., & Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch1. Biochemical and Biophysical Research Communications. 372 (2008) 913–917.

13. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated. Cellular Signalling. 27 (2015) 630-637.

CHAPTER II

1. Carlo, M. D., Giacomazza, D., & Biagio, P. L. Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools, Journal of Physics: Condensed Matter. 24 (2012) 244102.

2. Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological Alterations in Alzheimer Disease. Cold Spring Harbor Perspectives in Medicine. 1 (2011).

3. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease. Progress in Neurobiology. 156 (2017) 189–213.

4. Iba, M., Guo, J. L., Mcbride, J. D., Zhang, B., Trojanowski, J. Q., & Lee, V. M.-Y. Synthetic Tau Fibrils Mediate Transmission of Neurofibrillary Tangles in a Transgenic Mouse Model of Alzheimers-Like Tauopathy. Journal of Neuroscience. 33 (2013) 1024–1037.

5. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders. Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.

6. Calero, M., Chen, C. Z., Zhu, W., Winand, N., Havas, K. A., Gilbert, P. M., Collins, R. N., et al. Dual Prenylation Is Required for Rab Protein Localization and Function. Molecular Biology of the Cell. 14 (2003) 1852–1867.

7. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients. Pharmacopsychiatry. 42 (2009).

8. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain. Journal of Neurochemistry. 129 (2014) 732–742.

9. Hottman, D., Cheng, S., Gram, A., Leblanc, K., Yuan, L. L., & Li, L. Systemic or Forebrain Neuron-Specific Deficiency of Geranylgeranyltransferase-1 Impairs Synaptic Plasticity and Reduces Dendritic Spine Density. Neuroscience. 373 (2018) 207–217.

10. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated. Cellular Signalling. 27 (2015) 630-637.

11. Thies, W., Bleiler, L. 2013 Alzheimer's disease facts and figures. Alzheimer's & Dementia: J Alzheimer's Dis. 9 (2013) 208-45.

12. Alzheimer's Association. 2017 Alzheimer 's disease Facts and Figures. Alzheimer's & Dementia 2017. 13 (2017) 325-373.

13. Brookmeyer, R., Gray, S., & Kawas, C. Projections of Alzheimers disease in the United States and the public health impact of delaying disease onset. American Journal of Public Health. 88 (1998) 1337–1342.

14. Kazee, A. M., Johnson, E. M. Alzheimers disease pathology in non-demented elderly. J Alzheimers Dis. 1 (1998) 81-89.

15. Weller, J., Budson, A. Current understanding of Alzheimer's disease diagnosis and treatment. F1000Research. 7 (2018) 1161.

16.Tanzi, R, E., Bertram, L. Twenty years of the Alzheimer's disease amyloid hypothesis: A genetic perspective. Cell. 120 (2005) 545-555.

17. Wesson, D, W., Nixon, R, A., Levy, E., Wilson, D, A. Mechanisms of neural and behavioral dysfunction in Alzheimer's disease. Mol Neurobiol. 43 (2011) 163-179.

18. Mura, E., Zappettini, S., Preda, S., Biundo, F., Lanni, C., Grilli, M., Cavallero, A., Olivero, G., Salamon, A., Govoni, S., Marchi, M. Dual effect of B-amyloid on

α7 and α4β2 nicotinic receptors controlling the release of glutamate, aspartate and GABA in rat hippocampus. PLoS ONE. 7 (2012) e29661.

19. Zhang, Y., Thompson, R., Zhang, H., Xu, H. APP processing in Alzheimer's disease. Mol Brian. 4 (2011) 3-16.

20. Kametani, F., Hasegawa, M. Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimers disease. Front Neurosci. 12 (2018) 25.

21. Wesson, D, W., Nixon, R, A., Levy, E., Wilson, D, A. Mechanisms of neural and behavioral dysfunction in Alzheimer's disease. Mol Neurobiol. 43 (2011) 163-179.

22. Ray, B., Chauhan, N, B., Lahin, D, K. Oxidative insults to neurons and synapse are prevented by aged garlic extract (age) and s-ally-l-cysteine (sac) treatment in the neuronal culture and app-tg mouse model. J Neurochem. 117 (2011) 388-402.

23. Fu, H, J., Liu, B., Frost, J, L., Lemere, C, A. Amyloid-B immunotherapy for Alzheimer's disease. CNS Neurol Dis-DR. 9 (2011) 197-206.

24. Grilli, M., Lagomarsino, F., Zappettini, S., Preda, S., Mura, E., Govoni, S., Marchi, M. Specific inhibitory effect of amyloid-β on presynaptic muscarinic receptor subtypes modulating neurotransmitter release in the rat nucleus accumbens. Neurosci. 167 (2010) 482-489.

25. Chow, V, W., Mattson, M, P., Wong, P, C., Gleichmann, M. An overview of APP processing enzymes and products. Neuromol Med. 12 (2009) 1-12.

26. Haass, C., Selkoe, D, J. Soluble protein oligomers in neurodegenration: lessons from the Alzheimer's amyloid B-peptide. Nature Rev Mol Cell Biol. 8 (2007) 101-112.

27. Zlokovic, B, V. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 57 (2008) 178-201.

28. Selkoe, D, J. Clearing the brain's amyloid cobwebs. Neuron. 32 (2011) 177-180.

29. Iqbal, K., Liu, F., Gong, C, X., Alonso, A, C., Grundke-Iqbal, I. Mechanisms of tau-induced neurodegenration. Acta Neuropathol. 118 (2009) 53-69.

30. Iqbal, K., Liu, F., Gong, C, X., Grundke-Iqbal, I. Tau in Alzheimer disease and related tauopathies. Cur Alzheimer Res. 7 (2010) 656-664.

31. Papasozomenos, S, C., Binder, L, I. Phosphorylation determines two distinct species of tau in the central nervous system. Cell Motil Cytoskel. 8 (1987) 210-226.

32. Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Lanté, F., Buisson, A. Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature. 369 (1994) 488-491.

33. Dawson, H., Cantillana, V., Jansen, M., Wang, H., Vitek, M., Wilcock, D., et al. Loss of tau elicits axonal degeneration in a mouse model of Alzheimers disease. Neuroscience. 169 (2010) 516-531.

34. Frandemiche, M, L., Seranno, S, D., Rush, T., Borel, E., Elie, A., Arnal, I., et al. Activity-dependent tau protein translocation to excitatory synapse is disrupted by exposure to amyloid-B oligomers. J Neurosci. 34 (2014) 6084-6097.

35. Zempel, H., Mandelkow, E. Tau missorting and spastin-induced microtubule disruption in neurodegeneration: Alzheimer disease and hereditary spastic paraplegia. Mol Neurodegen. 10 (2015) 68-80.

36. Planel, E., Richter, K, E., Nolan, C, E., Finley, J, E., Liu, L., Wen, Y., et al. Anesthesia Leads to Tau Hyperphosphorylation through Inhibition of Phosphatase Activity by Hypothermia. Journal of Neuroscience. 27 (2007) 3090-3097.

37. Martin, L., Latypova, X., Wilson, C, M., Magnaudeix, A., Perrin, M., Terro, F. Tau protein phosphatases in Alzheimers disease: The leading role of PP2A. Ageing Res Rev. 12 (2013) 39-49.

38. Mao, P., Reddy, P, H. Aging and amyloid B-induced oxidative DNA damage and mitochondrial dysfunction in Alzheimer's disease: Implications for early intervention and therapeutics. Biochim Biophys Acta. 1812 (2011) 1359-1370.

39. Dubal, D, B., Broestl, L., Worden, K. Sex and gonadal hormones in mouse models of Alzheimer's disease: What is relevant to the human condition? Biol Sex Diff. 3 (2012) 24-40.

40. Reitz, C., Brayne, C., Mayeux, R. Epidemiology of Alzheimer disease. Nature Rev Neurol. 7 (2011) 137-52.

41. Zamboni, V., Jones, R., Umbach, A., Ammoni, A., Passafaro, M., Hirsch, E., Merlo, G. Rho GTPases in intellectual disability: From genetics to therapeutic opportunities. Int J Mol Sci. 19 (2018) 1821.

42. Huang, G., Sun, Z., Li, H., Feng, D. Rho GTPase-activating proteins: Regulators of Rho GTPase activity in neuronal development and CNS diseases. Mol Cell Neurosci. 80 (2017) 18-31.

43. Nadif Kasri, N., Van Aelst, L. Rho-linked genes and neurological disorders. Pflugers Archiv: Eur J of Phys. 455 (2008) 787-797.

44. Hooff, G, P., Wood, W, G., Muller, W, E., Eckert, G, P. Isoprenoids, small GTPases and alzheimer's disease. Biochim Biophys Acta. 1801 (2010) 896-905.

45. Shafer, W. Protein prenylation: genes, enzymes, targets, and functions. Annual Rev Genet. 26 (1992) 209-237.

46. Liang, P., Ko, T., Wang, A, H. Structure, mechanism and function of prenyltransferases. Eur J Biochem. 269 (2002) 3339-3354.

47. Roskoski, R. Protein prenylation: A pivotal posttranslational process. Biochem Biophys Res Comm. 303 (2003) 1-7.

48. Benetka, W., Koranda, M., Eisenhaber, F. Protein prenylation: an (almost) comprehensive overview on discovery history, enzymology, and significance in physiology and disease. Monatshefte Für Chemie - Chemical Monthly. 137 (2006) 1241-1281.

49. Molnár, G., Dagher, M., Geiszt, M., Settleman, J., Ligeti, E. Role of prenylation in the interaction of Rho-family small GTPases with GTPase activating proteins. Biochemistry. 40 (2001) 10542-10549.

50. Auer, M., Hausott, B., Klimaschewski, L. Rho GTPases as regulators of morphological neuroplasticity. Annals Anat. 193 (2011) 259-266.

51. Spiering, D., Hodgson, L. Dynamics of the Rho-family small GTPases in actin regulation and motility. Cell Adhes Migr. 5 (2011) 170-180.

52. Ridley, A, J. Rho family proteins: Coordinating cell responses. Trends Cell Biol. 11 (2001) 471-477.

53. Degeer, J., Lamarche-Vane, N. Rho GTPases in neurodegeneration diseases. Exp Cell Res. 319 (2013) 2384-2394.

54. Govek, E, E., Hatten, M, E., Van Aelst, L. The role of rho GTPase proteins in CNS neuronal migration. Dev Neurobiol. 71 (2011) 528-553.

55. Samuel, F., Hynds, D, L. RHO GTPase signaling for axon extension: Is prenylation pmportant? Mol Neurobiol. 42 (2010) 133-142.

56. Musilli, M., Nicolia, V., Borrelli, S., Scarpa, S., Diana, G. Behavioral effects of rho GTPase modulation in a model of alzheimer's disease. Behav Brain Res. 237 (2013) 223-229.

57. Petratos, S., Li, Q, X., George, A, J., Hou, X., Kerr, M, L., Unabia, S, E., Francés, S., Santpere, G., Ferrer, I., Galea, E. The β-amyloid protein of Alzheimer's disease increases neuronal CRMP-2 phosphorylation by a rho-GTP mechanism. Brain. 131 (2008) 90-108.

58. Huesa, G., Baltrons, M, A., Gomez-Ramos, P., Moran, A., Garcia, A., Hidalgo, J., Francés, S., Santpere, G., Ferrer, I., Galea, E. Altered distribution of RhoA in Alzheimer's disease and AβPP overexpressing mice. J Alzheimer's Dis.19 (2010) 37-56.

59. Mendoza-Naranjo, A., Gonzalez-Billault, C., Maccioni, R, B. Aβ1-42 stimulates actin polymerization in hippocampal neurons through Rac1 and Cdc42 Rho GTPases. J Cell Sci. 120 (2007) 279-288.

60. Wang, P, L., Niidome, T., Akaike, A., Kihara, T., Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene. J Neurosci Res. 87 (2009) 2105-2114.

61. Desire, L., Bourdin, J., Loiseau, N., Peillon, H., Picard, V., De Oliveira, C., Bachelot, F., Leblond, B., Taverne, T., Beausoleil, E., Lacombe, S., Drouin, D., Schweighoffer, F. RAC1 inhibition targets amyloid precursor protein processing by γ-secretase and decreases aβ production *in vitro* and *in vivo*. J Biol Chem. 28045 (2005) 37516-37525.

62. Stankiewicz, T, R., Linseman, D, A. Rho family GTPases: Key players in neuronal development, neuronal survival, and neurodegeneration. Front Cell Neurosci. 8 (2014) 314.

63. Wang, G., Huang, Y., Wang, L., Zhang, Y., Xu, J., Zhou, Y., Lourenco, G, F., Zhang, B., Wang, Y., Ren, R, J., Halliday, G, M., Chen, S, D. MicroRNA-146a suppresses ROCK1 allowing hyperphosphorylation of tau in Alzheimer's disease. Sci Rep. 6 (2016) 26697.

64. Henderson, B, W., Gentry, E, G., Rush, T., Troncoso, J, C., Thambisetty, M., Montine, T, J., Herskowitz, J, H. Rho-associated protein kinase 1 (ROCK1) is increased in Alzheimers disease and ROCK1 depletion reduces amyloid-β levels in brain. J Neurochem. 138 (2016) 525-531.

65. Ma, Q., Yang, F., Calon, F., Ubeda, O, J., Hansen, J, E., Weisbart, R, H., Beech, W., Frautschy, S, A., Cole, G, M. P21-activated kinase-aberrant activation and translocation in Alzheimer disease pathogenesis. J Biol Chem. 283 (2008) 14132-14143.

66. Inoue, E., Deguchi-Tawarada, M., Togawa, A., Matsui, C., Arita, K., Katahira-Tayama, S., Sato, T., Yamauchi, E., Oda, Y., Takai, Y. Synaptic activity prompts γ-secretase–mediated cleavage of EphA4 and dendritic spine formation. J Cell Biol. 185 (2009) 551-564.

67. Posada-Duque, R, A., López-Tobón, A., Piedrahita, D., González-Billault, C., Cardona-Gomez, G, P. P35 and Rac1 underlie the neuroprotection and cognitive improvement induced by CDK5 silencing. J Neurochem. 134 (2015) 354-370.

68. Boo, J, H., Sohn, J, H., Kim, J, E., Song, H., Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch1. Biochem Biophys Res Comm. 372 (2008) 913-917.

69. Aguilar, B, J., Zhu, Y., Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease. Alzheimers ResTher. 9 (2017) 97.

70. Perez, S, E., Getova, D, B., He, B., Counts, S, E., Geula, C., Desire, L., Coutadeur, S., Peillon, H., Ginsberg, S, D., Mufson, E, J. Rac1b increases with progressive tau pathology within cholinergic nucleus basalis neurons in Alzheimers disease. Am J Pathol. 180 (2012) 526-540.

71. Manterola, L., Hernando-Rodríguez, M., Ruiz, A., Apraiz, A., Arrizabalaga, O., Vellón, L., Alberdi, E., Cavaliere, F., Lacerda, H, M., Jimenez, S., Parada, L, A., Matute, C., Zugaza, J, L. 1–42 β-amyloid peptide requires PDK1/nPKC/Rac 1 pathway to induce neuronal death. Transl Psychiatry. 3 (2013) e219.

72. Lee, M., You, H., Cho, S., Woo, C., Yoo, M., Joe, E., Kim, J. Implication of the small GTPase Rac1 in the generation of reactive oxygen species in response to β-amyloid in C6 astroglioma cells. Biochem J. 366 (2002) 937-943.

73. Sellers, K,J., Elliott, C., Jackson, J., Ghosh, A., Ribe, E., Rojo, A, I., Jarosz-Griffiths, H, H., Watson, I,A., Xia, W., Semenov, M., Morin, P., Hooper, N, M., Porter, R., Preston, J., Al-Shawi, R., Baillie, G., Lovestone, S., Cuadrado, A., Harte, M., Simons, P., Srivastava, D, P., Killick, R. Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil. Alzheimers Dement. 14 (2018) 306-317.

CHAPTER III

1. Carlo, M. D., Giacomazza, D., & Biagio, P. L. Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools, Journal of Physics: Condensed Matter. 24 (2012) 244102.
2. Alzheimer's Association. 2017 Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia 2017. 13 (2017) 325-373.
3. Serranzo-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological alterations in Alzheimer disease, Cold Spring Harbor Perspectives in Medicine. 1 (2011) a006189.
4. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer's disease, Progress in Neurobiology. 156 (2017) 189-213.
5. Curtis, I. D. Functions of Rac GTPases during Neuronal Development, Developmental Neuroscience. 30 (2007) 47-58.
6. Grabham, P. W. Microtubule and Rac 1-dependent F-actin in growth cones, Journal of Cell Science. 116 (2003) 3739-3748.
7. Martinez, L. A., & Tejada-Simon, M. V. Pharmacological inactivation of the small GTPase Rac1 impairs long-term plasticity in the mouse hippocampus, Neuropharmacology. 61 (2011) 305-312.

8. Govek, E. E., Hatten, M. E., & Van Aelst, L. The role of rho GTPase proteins in CNS neuronal migration, Developmental Neurobiology. 71 (2011) 528-553.
9. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders, Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.
10. Wang, P. L., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene, Journal of Neuroscience Research. 87 (2009) 2105-2114.
11. Boo, J. H., Sohn, J. H., Kim, J. E., Song, H., & Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch1, Biochemical and Biophysical Research Communications. 372 (2008) 913-917.
12. Posada-Duque, R. A., López-Tobón, A., Piedrahita, D., González-Billault, C., & Cardona-Gomez, G. P. P35 and Rac1 underlie the neuroprotection and cognitive improvement induced by CDK5 silencing, Journal of Neurochemistry. 134 (2015) 354-370.
13. Roberts, Patrick J., et al. Rho Family GTPase Modification and Dependence on CAAX Motif-Signaled Posttranslational Modification, Journal of Biological Chemistry. 283 (2008) 25150–25163.
14. Higgins, J. B., & Casey, P. J. The role of prenylation in G-protein assembly and function, Cellular Signalling. 8 (1996) 433-437.

15. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated, Cellular Signalling. 27 (2015) 630-637.
16. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease, Alzheimers Research & Therapy. 9 (2017).
17. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients, Pharmacopsychiatry. 42 (2009).
18. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain, Journal of Neurochemistry. 129 (2014) 732-742.
19. Iqbal, Khalid, et al. Cytosolic Abnormally Hyperphosphorylated Tau But Not Paired Helical Filaments Sequester Normal MAPs and Inhibit Microtubule Assembly, Journal of Alzheimers Disease. 14 (2008).
20. Iqbal, K., et al. Tau in Alzheimer Disease and Related Tauopathies, Current Alzheimer Research. 8 (2010) 656–664.
21. Papasozomenos, S. C., & Binder, L. I. Phosphorylation determines two distinct species of tau in the central nervous system, Cell Motility and the Cytoskeleton. 8 (1987) 210-226.

22. Busciglio, J., Gabuzda, D. H., Matsudaira, P., & Yankner, B. A. Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells, Proceedings of the National Academy of Sciences. 90 (1993) 2092-2096.
23. Zhang, Y., Thompson, R., Zhang, H., & Xu, H. APP processing in Alzheimer's disease, Molecular Brian. 4 (2011).
24. Wang, P., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene, Journal of Neuroscience Research. 87 (2009) 2105-2114.
25. Boo, J. H., Sohn, J. H., Kim, J. E., Song, H., & Mook-Jung, I. Rac1 changes the substrate specificity of γ-secretase between amyloid precursor protein and Notch, Biochemical and Biophysical Research Communications. 372 (2008) 913-917.
26. Inoue, E., Deguchi-Tawarada, M., Togawa, A., Matsui, C., Arita, K., Katahira-Tayama, S., et al. Synaptic activity prompts γ-secretase–mediated cleavage of EphA4 and dendritic spine formation, The Journal of Cell Biology. 185 (2009) 551-564.
27. Morgese, M. G., Schiavone, S., & Trabace, L. Emerging role of amyloid β in stress response: Implication for depression and diabetes, European Journal of Pharmacology. 817 (2017) 22-29.
28. Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. An Overview of APP Processing Enzymes and Products, NeuroMolecular Medicine. 12 (2009) 1-12.

29. Moon, M., Kim, H., Li, Y., Kim, J., Jeon, Y., Won, H., Kim, J., Kwon, H., Choi, I., Ro, E., Joe, E, Choe, M., Kwon, H., Kim, H., Kim, Y., & Park, J. Involvement of small GTPase RhoA in the regulation of superoxide production in BV2 cells in response to fibrillar Aβ peptides, Cellular Signalling. 25 (2013) 1861-1869.
30. Posada-Duque, Rafael Andrés, et al. Atorvastatin Requires Geranylgeranyl Transferase-I and Rac1 Activation to Exert Neuronal Protection and Induce Plasticity, Neurochemistry International. 62 (2013) 433–445.
31. Ostrowski SM, Wilkinson BL, Golde TE, Landreth G. Statins reduce amyloid-β production through inhibition of protein isoprenylation, J Biol Chem. 282 (2007) 26832–26844.
32. Cole SL, Grudzien A, Manhart IO, Kelly BL, Oakley H, Vassar R. Statins cause intracellular accumulation of amyloid precursor protein, β-secretase-cleaved fragments, and amyloid β-peptide via an isoprenoid-dependent mechanism, J Biol Chem. 280 (2005) 18755–1877

CHAPTER IV

1. Carlo, M. D., Giacomazza, D., & Biagio, P. L. Alzheimer's disease: Biological aspects, therapeutic perspectives and diagnostic tools, Journal of Physics: Condensed Matter. 24 (2012) 244102.
2. Alzheimer's Association. 2017 Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia 2017. 13 (2017) 325-373.
3. Brookmeyer, R., Gray, S., & Kawas, C. Projections of alzheimer's disease in the united states and the public health impact of delaying disease onset, American Journal of Public Health. 88 (1998) 1337-1342.
4. Serranzo-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. Neuropathological alterations in Alzheimer disease, Cold Spring Harbor Perspectives in Medicine. 1 (2011) a006189.
5. Curtis, I. D. Functions of Rac GTPases during Neuronal Development, Developmental Neuroscience. 30 (2007) 47-58.
6. Deng, Qiyue, et al. Small Nogo-66-Binding Peptide Promotes Neurite Outgrowth through RhoA Inhibition after Spinal Cord Injury, Brain Research Bulletin. 99 (2013) 140–144.
7. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders, Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.

8. Huesa, G., Baltrons, M. A., Gomez-Ramos, P., Moran, A., Garcia, A., Hidalgo, J., et., al. Altered distribution of RhoA in alzheimer's disease and AβPP overexpressing mice, Journal of Alzheimer's Disease: JAD. 19 (2010) 37-56.
9. Petratos, S., Li, Q. X., George, A. J., Hou, X., Kerr, M. L., Unabia, S. E., et., al. The β-amyloid protein of alzheimer's disease increases neuronal CRMP-2 phosphorylation by a rho-GTP mechanism, Brain: A Journal of Neurology. 131 (2008) 90-108.
10. Roberts, Patrick J., et al. Rho Family GTPase Modification and Dependence on CAAXMotif-Signaled Posttranslational Modification, Journal of Biological Chemistry. 283 (2008) 25150–25163.
11. Higgins, J. B., & Casey, P. J. The role of prenylation in G-protein assembly and function, Cellular Signalling. 8 (1996) 433-437.
12. Samuel, F., & Hynds, D. L. RHO GTPase Signaling for Axon Extension: Is Prenylation Important? Molecular Neurobiology. *42* (2010) 133-142.
13. Reddy, J. M., Samuel, F. G., Mcconnell, J. A., Reddy, C. P., Beck, B. W., & Hynds, D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated, Cellular Signalling. 27 (2015) 630-637.

14. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease, Alzheimers Research & Therapy. 9 (2017).
15. Eckert, G., Hooff, G., Strandjord, D., Igbavboa, U., Volmer, D., Müller, W., & Wood, W. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl-pyrophosphate is altered in male Alzheimer patients, Pharmacopsychiatry. 42 (2009).
16. Afshordel, S., Wood, W. G., Igbavboa, U., Muller, W. E., & Eckert, G. P. Impaired geranylgeranyltransferase-I regulation reduces membrane-associated Rho protein levels in aged mouse brain, Journal of Neurochemistry. 129 (2014) 732-742.
17. Iqbal, Khalid, et al. Cytosolic Abnormally Hyperphosphorylated Tau But Not Paired Helical Filaments Sequester Normal MAPs and Inhibit Microtubule Assembly, Journal of Alzheimers Disease. 14 (2008).
18. Iqbal, K., et al. Tau in Alzheimer Disease and Related Tauopathies, Current Alzheimer Research. 8 (2010) 656–664.
19. Papasozomenos, S. C., & Binder, L. I. Phosphorylation determines two distinct species of tau in the central nervous system, Cell Motility and the Cytoskeleton. 8 (1987) 210-226.
20. Bukhari, H., Glotzbach, A., Kolbe, K., Leonhardt, G., Loosse, C., & Müller, T. Small things matter: Implications of APP intracellular domain AICD

nuclear signaling in the progression and pathogenesis of Alzheimer's disease, Progress in Neurobiology. 156 (2017) 189-213.

21. Busciglio, J., Gabuzda, D. H., Matsudaira, P., & Yankner, B. A. Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells, Proceedings of the National Academy of Sciences. 90 (1993) 2092-2096.
22. Zhang, Y., Thompson, R., Zhang, H., & Xu, H. APP processing in Alzheimer's disease, Molecular Brian. 4 (2011).
23. Zhou, Y. Nonsteroidal Anti-Inflammatory Drugs Can Lower Amyloidogenic A 42 by Inhibiting Rho, Science. 302 (2003) 1215–1217.
24. Herskowitz, J. H., et al. Pharmacologic Inhibition of ROCK2 Suppresses Amyloid- Production in an Alzheimers Disease Mouse Model, Journal of Neuroscience. 33 (2013) 19086–19098.
25. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease, Alzheimers Research & Therapy. 9 (2017).
26. Hamano, Tadanori, et al. Pitavastatin Decreases Tau Levels via the Inactivation of Rho/ROCK, Neurobiology of Aging. 33 (2012) 2306–2320.
27. Perez, S. E., Getova, D. P., He, B., Counts, S. E., Geula, C., Desire, L., et al. Rac1b Increases with Progressive Tau Pathology within Cholinergic Nucleus Basalis Neurons in Alzheimers Disease, The American Journal of Pathology. 180 (2012) 526-540.

28. Park, J., Baik, S. H., Han, S., Cho, H. J., Choi, H., Kim, H. J., et al. Annexin A1 restores Aβ1-42-induced blood-brain barrier disruption through the inhibition of RhoA-ROCK signaling pathway, Aging Cell. 16 (2016) 149-161.
29. Sellers, K. J., Elliott, C., Jackson, J., Ghosh, A., Ribe, E., Rojo, A. I., et al. Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil, Alzheimers & Dementia. 14 (2018) 306-317.
30. Ostrowski SM, Wilkinson BL, Golde TE, Landreth G. Statins reduce amyloid-β production through inhibition of protein isoprenylation, J Biol Chem. 282 (2007) 26832–26844.
31. Cole SL, Grudzien A, Manhart IO, Kelly BL, Oakley H, Vassar R. Statins cause intracellular accumulation of amyloid precursor protein, β-secretase-cleaved fragments, and amyloid β-peptide via an isoprenoid-dependent mechanism, J Biol Chem. 280 (2005) 18755–18770.

CHAPTER V

1. Nadif Kasri, N., & Van Aelst, L. Rho-linked genes and neurological disorders. Pflugers Archiv: European Journal of Physiology. 455 (2008) 787-797.

2. Govek, E. E., Hatten, M. E., & Van Aelst, L. The role of rho GTPase proteins in CNS neuronal migration. Developmental Neurobiology. 71 (2011) 528-553.
3. Aguilar, B. J., Zhu, Y., & Lu, Q. Rho GTPases as therapeutic targets in Alzheimer's disease. Alzheimers Research & Therapy. 9 (2017).
4. Iba, M., Guo, J. L., Mcbride, J. D., Zhang, B., Trojanowski, J. Q., & Lee, V. M. Synthetic Tau Fibrils Mediate Transmission of Neurofibrillary Tangles in a Transgenic Mouse Model of Alzheimers-Like Tauopathy. Journal of Neuroscience. 33 (2013) 1024-1037.
5. Iqbal, K., Liu, F., Gong, C.X., & Grundke-Iqbal, I. Tau in Alzheimer disease and related tauopathies. Current Alzheimer Research. 7 (2010) 656-664.
6. Huesa, G., Baltrons, M. A., Gómez-Ramos, P., Morán, A., García, A., Hidalgo, J., Francés, S., Santpere, G., Ferrer, I., & Galea, E. Altered Distribution of RhoA in Alzheimers Disease and AβPP Overexpressing Mice. Journal of Alzheimers Disease. 19 (2010) 37-56.
7. Torres-Cruz, F. M., Rodríguez-Cruz, F., Escobar-Herrera, J., Barragán-Andrade, N., Basurto-Islas, G., Ripova, D., et al. Expression of Tau Produces Aberrant Plasma Membrane Blebbing in Glial Cells Through RhoA-ROCK-Dependent F-Actin Remodeling. Journal of Alzheimers Disease. 52 (2016) 463-482.

8. Matsui, C., Inoue, E., Kakita, A., Arita, K., Deguchi-Tawarada, M., Togawa, A., et al. Involvement of the γ-Secretase-Mediated EphA4 Signaling Pathway in Synaptic Pathogenesis of Alzheimers Disease. Brain Pathology. 22 (2012) 776-787.
9. Posada-Duque, R. A., López-Tobón, A., Piedrahita, D., González-Billault, C., & Cardona-Gomez, G. P. P35 and Rac1 underlie the neuroprotection and cognitive improvement induced by CDK5 silencing. Journal of Neurochemistry. 134 (2015) 354-370.
10. Reddy J.M., Samuel F. G., Mcconnell J. A., Reddy C. P., Beck B. W., Hynds D. L. Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated. Cell Signal. 27 (2015) 630-637.
11. Kremer, A. GSK3 and Alzheimer's disease: Facts and fictio. Frontiers in Molecular Neuroscience. 4 (2011).
12. Iqbal, K., Liu, F., Gong, C.X., & Grundke-Iqbal, I. Tau in Alzheimer disease and related tauopathies. Current Alzheimer Research. 7 (2010) 656-664.
13. Heinisch, J. J., & Brandt, R. Signaling pathways and posttranslational modifications of tau in Alzheimers disease: The humanization of yeast cells. Microbial Cell. 3 (2016) 135-146.

14. Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. An Overview of APP Processing Enzymes and Products. NeuroMolecular Medicine. 12 (2009) 1-12.
15. Wang, P., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. Rac1 inhibition negatively regulates transcriptional activity of the amyloid precursor protein gene. Journal of Neuroscience Research. 87 (2009) 2105-2114.
16. Inoue, E., Deguchi-Tawarada, M., Togawa, A., Matsui, C., Arita, K., Katahira-Tayama, S., et al. Synaptic activity prompts γ-secretase–mediated cleavage of EphA4 and dendritic spine formation. The Journal of Cell Biology. 185 (2009) 551-564.
17. Ripoli, C., Cocco, S., Puma, D. D., Piacentini, R., Mastrodonato, A., Scala, F., et, al. Intracellular Accumulation of Amyloid- (A) Protein Plays a Major Role in A -Induced Alterations of Glutamatergic Synaptic Transmission and Plasticity. Journal of Neuroscience. 34 (2014) 12893-12903.
18. Govek, E. E., Hatten, M. E., & Van Aelst, L. The role of rho GTPase proteins in CNS neuronal migration. Developmental Neurobiology. 71 (2011) 528-553.
19. Auer, M., Hausott, B., & Klimaschewski, L. Rho GTPases as regulators of morphological neuroplasticity. Annals of Anatomy. 193 (2011) 259-266.

APPENDIX

LIST OF ABBREVIATIONS

AD	Alzheimer disease
AICD	APP intracellular domain
ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated protein kinase
ANOVA	Analyses of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATCC	American Type Culture Collection
Aβ	β amyloid
BACE1 or β secretase	β-amyloid cleavage enzyme
BCA	Bicinchoninic Acid Protein Assay
BSA	Bovine serum albumin
CaaX	C= cysteine, a= two aliphatic amino acids, X= terminal amino acid
CaMK II	Calcium and calmodulin-dependent protein kinase-II
CDK 5	Cyclin-dependent kinase 5
Cdks	Cyclin dependent protein kinase-S
Ck-1	Casein kinase-1
$cPLA_2$	Cytosolic phospholipase A_2

CRMP-2	Collapsing response mediator protein-2
CTF	α-C terminal fragment
DAPI	4',6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle's Medium and Nutrient Mixture F12
EmGFP	Emerald Green Fluorescence Protein
ERK1/2	Extracellular regulated kinase 1/2
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
GAPs	GTPase activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine-nucleotide-exchange factors
GGPP	Geranylgeranylpyrophosphate
GGTase-I	Geranylgeranyl transferases
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
HD	Huntington's Disease
HG-SFDMEM	High glucose-serum free Dulbecco's Modified Eagle's Medium

HMG CoA ..... 3-hydroxy-3-methyl-glutaryl-coenzyme A

IACUC ..... Institutional Animal Care and Use Committee

IRDye ..... Infrared fluorescence dye

5-LO ..... 5- lipoxygenase

LSD ..... Least Significant Difference

MAP ..... Microtubule associated protein

NBM ..... Neurobasal media

NMDA ..... N-methyl-D-aspartate

PAK ..... p21-activated kinase

PBS ..... Phosphate buffered saline

PD ..... Parkinson's Disease

PDK1 ..... Phosphoinositol-dependent kinase 1

PDL ..... Poly-D-lysine

PGC-1α ..... Peroxisome proliferator-activated receptor γ coactivator 1-α

PHFs ..... Paired helical filaments

PI3K ..... Phosphatidylinositol 3-kinase

PIC ..... Protease inhibitor cocktail

PKA ..... Protein kinase A

PKC ..... Protein kinase C

PP-2A ..... Protein Phosphatase 2-A

PS1	Presenilin 1
PS2	Presenilin 2
PTEN	Phosphatase and tensin homolog
PTP1B	Protein tyrosine phosphatase 1B
Rac1	Ras-related C3 botulinum toxin substrate 1
ROCK 1	Rho-associated, coiled-coil containing protein kinase 1
sAPPβ	Soluble APPβ
SCM	Serum-containing medium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFM	Serum free media
SFs	Straight filaments
TBST	Tris buffered saline, 0.1% Tween-20