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Chanism Of G-βγ-Dependent Regulation Of Neuronal Differentiation And Neurodegeneration

Diana Olivas
University of Texas at El Paso

MECHANISM OF Gβγ-DEPENDENT REGULATION OF NEURONAL DIFFERENTIATION AND NEURODEGENERATION

DIANA ISUISS OLIVAS

Master's Program in Biology

APPROVED:

Sukla Roychowdhury, Ph.D., Chair

Siddhartha Das, Ph.D.

Mahesh Narayan, Ph.D.

German Rosas-Acostas, Ph.D.

Stephen L. Crites, Jr., Ph.D.
Dean of the Graduate School

Dedication

To be **Xzell** is to be **Xzellent**, you are my **eternal** inspiration.

MECHANISM OF Gβγ-DEPENDENT REGULATION OF NEURONAL DIFFERENTIATION AND NEURODEGENERATION

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The exemplary work of many accomplishes an extraordinary change. The pursuit of a goal is an individual task; achieving any goal takes many willing helping hands. As my mission concludes, I would like to thank all those that lent me a helping hand.

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Abstract:

Neurodegeneration is a pathological condition associated with a progressive loss of neurons, and occurs in many neurological disorders including Alzheimer's disease (AD), Parkinson's disease (PD) and Lou Gehrig's disease (ALS). It is the most common cause of dementia among people 65 and older. With the increasing of life expectancy, the prevalence of neurodegenerative diseases is also increasing rapidly. However, the cause of this disorder is largely unknown, and no effective drugs are available to treat the disease process. Therefore, it is crucial to identify new target(s) and strategies for therapeutic interventions. Cytoskeletal defects followed by dysfunction is a major characteristic of neurodegenerative diseases. During neurodegeneration microtubules (MTs) and proteins associated with MTs are severely altered. Tau is a stabilizing MT-associated protein (MAP) and in normal conditions, tau binds to MT, stabilizing neuron structure and integrity. In Alzheimer's disease (AD) and in other tauapathies, tau is hyper-phosphorylated leading to tau aggregation and NFT (neurofibriliary tangles) formation, and this pathway emerged as a major feature in the pathogenesis of AD and associated illness. Over activation of GSK3β, a downstream effector of PI3K pathway has been shown to play key roles in tau hyperphosphorylation. Previous results from our laboratory have shown that Gβγ, an important component of GPCR pathway promotes microtubule (MT) assembly and induces neuronal differentiation of PC12 cells and blocking the interaction between Gβγ and tubulin/MTs using Gβγ-blocking peptide GRK-CT, disrupted MTs, inhibited neurite outgrowth, and induced axonal damage indicating the possible involvement of Gβγ in neurodegeneration. Although Gβγ has been implicated in familial AD, and has been connected to APP (Amyloid precursor protein)-dependent toxicity, it has not been investigated as potential pathway for inducing neurodegeneration. In the current investigation we used human neuronal cells SHSY5Y to further understand the mechanism by which Gβγ is

involved in neuronal differentiation and neurodegeneration. I hypothesize that *Gβγ is involved in neurodegeneration by disrupting MTs, altering phosphorylation of tau protein, and Gβγ-mediated downstream PI3K/pAkt/pGSK3β signaling pathway*. This hypothesis was tested in the current study. Gallein, an inhibitor of Gβγ, and Tideglusib, a GSK3β inhibitor were used to elucidate the proposed signaling pathway. SHSY5Y cells are a neuroblastoma derived cell line that is commonly used in vitro to understand the process of neurodegeneration (ND) was included in the current thesis.

In Specific Aim 1, using biochemical, and immunoconfocal methodologies, I have demonstrated that Gallein (GAL) inhibited neurite formation and disrupted MT assembly. Surprisingly,the immunoblot analysis using p396 TAU (ser at 396 position) specific antibody, I observed the expression of phosphorylated tau was reduced in the presence of GAL, suggesting that MT disruption could also be associated with inhibition of tau phosphorylation (hypo-) similar to that observed in hyper-phosphorylation of Tau protein (Cortés-Gómez, M. Á., et al.,2020). In Specific Aim-2, I have demonstrated the PI3K pathway was affected by GAL as phosphorylation of Akt and GSK3β was inhibited. Similar to GAL, we found tideglusib also disrupted MTs and neurite formation. As found with GAL, tideglusib decreased expression of pAKT, pGSK3β, and ps396 Tau in SHSY5Y cells. Tideglusib has been used earlier in phase 2 clinical trial for AD patients. However, the primary outcome from the trial was negative. My results indicating that tideglusib disrupt MTs and neurite formation, could account for this outcome. The current results also suggest that that both Gβγ and its downstream effector GSK3β could work in concert to disrupt MTs, inhibit neuronal differentiation, and induce neurodegeneration. The study also indicates that more caution should be taken for developing drugs for the treatment of

neurodegeneration, and should first be tested for their effectiveness in vitro cell culture system for MT assembly/ neuronal differentiation.

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Introduction

1.1 Neurodegeneration

Neurodegeneration is characterized by the progressive loss of neurons and is seen in neurological diseases such as, Alzheimer's disease (AD), Parkinson's disease (PD), and other forms of dementia. At present the mechanism and the cause associated with these neurodegenerative disorders is unknown. There is no effective treatment to stop or slow down the disease process. Moreover, although there are treatments that produce some symptomatic relief, many become ineffective over time and can even produce disruptive symptoms of their own (Madireddy & Madireddy, 2019). Thus, it is urgent to develop more effective treatments for neurodegenerative disorders. A common characteristic for these disorders is disruption of the cytoskeleton and aggregation of microtubule associated proteins (Dave R.H et al., 2009).

Alzheimer's disease (AD) is currently the most common neurodegenerative disease (ND). The pathology of AD is characterized by two hallmarks, the presence of Amyloid beta plaques and neurofibrillary tangles (NFT). In normal neurons Tau protein participates in microtubule (MT) stabilization by binding to the MTs. In AD hyper- phosphorylated Tau is not able to bind to MT forming aggregates. Tau aggregates are disposed in the cell forming neurofibrillary tangles. Disruption of MT leads to axonal damage leading to an impairment in synaptic transmission. (Šimić et al., 2016)

Parkinson's disease hallmarks include Lewy bodies composed of alpha-synuclein aggregated proteins including tubulin, microtubule-associated protein 1 (MAP1), and MAP2.The Substantia Nigra is the brain region most affected by PD. The Substantia Nigra is the region of the brain where most dopaminergic neurons are found. PD predominately affects dopamine producing

neurons. Loss of neurons in these region and cytoskeletal impairment are thought to lead to the debilitating motor skill loss seen in PD. (Majounie et al., 2013)

1.2 Neuronal Cytoskeleton, Microtubule Dynamics and Neurodegeneration

Neurons are the brain cells responsible for receiving, processing, and transmitting information. During neuronal differentiation two separate domains extend from the cell body: multiple short projections called dendrites that serve to receive signals from adjacent neurons as well as a long slender axon which transmits information. The cytoskeleton provides structural organization of the neuron. It also serves as tracks for intracellular transport and comprises the core framework of cellular morphologies. The ability to achieve proper connections between the correct sets of cells, is fundamental for the proper functioning of the nervous system. This essential function is dependent on the polarized organization of axons and dendrites (Roychowdhury & Sierra-Fonseca, 2017). The main components of the cytoskeleton are MTs and actin filaments. MTs form dense, parallel arrays in axons and dendrites that are required for the growth and maintenance of neurites. In contrast, actin filaments are enriched in the growth cone and organized into long bundles forming filamentous protrusions (filopodia) or veil-like sheets of branched actin (lamellipodia). Microtubules are dynamic structures that undergo continual assembly and disassembly within the cell. These core cytoskeleton components are assembled through head to tail polymerization of alpha and beta tubulin heterodimers. Thus, resulting in two different asymmetric polarized polymers ends, referred to as plus and minus ends. Due to their highly dynamic structure Microtubules can rapidly go through cycles of assembly and disassembly. During neurodegeneration, the proper association and arrangement of cytoskeleton components is severely compromised. Several hallmark conditions, such as altered protein folding, amyloid plaques, neurofibrillary tangles, Lewy bodies, impaired cellular transport, and increased oxidative

stress among many others are observed during neurodegeneration (Fig.1). However, the mechanism by which ND diseases are triggered and further progress is poorly understood.

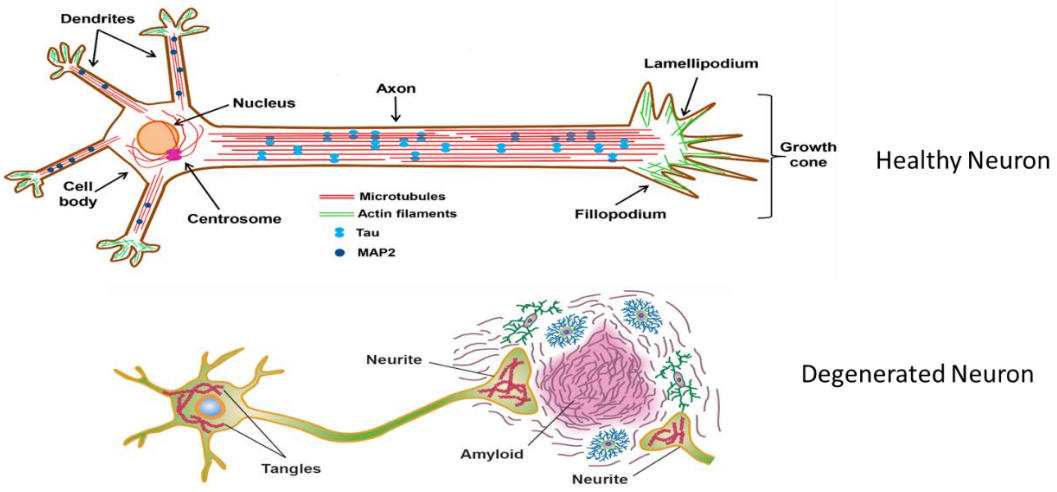

Figure. 1 Healthy and Degenerated Neuronal Cytoskeleton. Representation of the cytoskeletal organization in a healthy neuronal cell (upper panel). Microtubules form dense arrays mainly along the axon and dendrites of the cell (shown in red). The actin filaments, represented in green, localize primarily on the tips of the processes, forming lamellipodium, and fillipodium. Lastly, shown in blue are the microtubule-associated proteins, which play a role in the dynamic stability of the cell. (Roychowdhury & Sierra-Fonseca, 2017) In a degenerated neuron (lower panel) MAP protein TAU unbinds MTs leading to NFT formation in the neuron. Amyloid Beta plaques consists of several proteins including cytoskeletal proteins are found in extracellular region (Šimić et al., 2016).

1.3 Microtubule associated proteins, MAPs and Tau.

Microtubule dynamics in cells are regulated by various microtubule associated proteins (MAPs). Tau belongs to MAPs family of proteins, which was identified in 1975, by Weingarten *et al* (Bai & Burton, 2011) as a heat stable protein essential for microtubule assembly. Its role is important for the stabilization of microtubules, and the cytoskeleton integrity. Tau is normally found in axons. Aggregations of hyper phosphorylated Tau cause neurofibrillary tangles (NFT) (Fig.1) (Nobel W.et al., 2013). The presence of NFTs are a hallmark of AD and other NDs. In normal neuronal cells Tau will bind and stabilize MT. In abnormal neuronal cells Tau will disassociate from the microtubules and aggregate in the cell body and dendrites. The abnormal deposition of modified tau proteins in the neurons is a common aspect of many neurodegenerative disease referred to as tauopathies. Tauopathies are a class of neurodegenerative diseases occur by hyper phosphorylation and aggregation of the microtubule associated protein Tau, into paired helical filaments or straight filaments, forming NFTs in the brain (Chu D., & Liu, 2019). In a study using tau 441- transfected SHSY5Y human neuroblastoma cells, it was found, that hyperphosphorylated tau protein disrupts normal synaptic transmission of neurons independently of neurodegeneration or loss of synapse, suggesting that this is an early event in the progression of tauopathies (Löffler, T. et al. ,2012). Specific hyperphosphorylation sites on TAU have been shown in AD brain. The study confirmed high hyperphosphorylation of TAU at Ser396/Ser404/Thr181 as well as low hyperphosphorylation at Thr231/Ser235/Ser202 (Modi et al., 2014). Pharmacological interventions of tau phosphorylation are thought to present a new avenue in the treatment of tauopathies. There is a total of six isoforms of tau, due to alternative splicing of tau (MAPT) gene expressed in the adult human brain (Fig. 2) (Majounie et al., 2013).

Figure. 2 The human Tau gene

The six isoforms of the human CNS tau protein. Alternative splicing of MAPT gene produces the tau isoforms of 352-441 amino acids as indicated. Specific hyperphosphorylation sites on tau have been shown in Alzheimer's brain. Although there is evidence that phosphorylation of individual residues on tau significantly impacts its function; it remains to be established if the overall phosphorylation state of tau or phosphorylation at specific residues is important in ND pathogenesis (Majounie et al., 2013).

1.4 G protein-mediated signaling

G-proteins consist of several families of diverse cellular proteins that serve an equally diverse selection of cellular functions. These proteins derive their name, from the fact that they bind guanine nucleotides guanosine triphosphate (GTP) or guanosine diphosphate (GDP) and possess intrinsic GTPase activity. G proteins play a central role in signal transduction by transferring signals from cell surface receptor to intracellular effector molecules. (Gilman, 1987; Dohlman et al., 1991; Siman et. al 1991; Neves et al., 2002; McCudden et al., 2005) Heterotrimeric G-proteins consist of three elements, G-protein Coupled receptors (GPCRs), G Proteins and effectors. GPCR are the largest family of transmembrane receptors along, with their role in signal transduction. GPCRs regulate multiple physiological functions such as neurotransmission immune

system function, cell growth and proliferation, and hormonal signals. The involvement of GPCRs in complex signaling throughout the body has made them a target for about 40% of modern drug currently in the market. Despite their highly diverse nature all GPCRs consist of seven transmembrane domains with three extracellular and three intracellular loops. The extracellular domain is responsible for ligand binding and the intracellular domain is responsible for G-protein binding (Dohlman et al., 1991; Latek et. al 2012). G Protein heterotrimers, consisting of guanine nucleotide binding α plus βγ subunits, is inactive in cellular signaling. Agonist binding to GPCRs triggers structural rearrangements in the receptors such that the intracellular domain then catalyzes a nucleotide exchange in the α subunit of the heterotrimeric G proteins. Subsequently, activated Gα changes its association with Gβγ in a manner that permits both subunits to participate in the regulation of intracellular effector molecules (Fig.3). The typical effectors of Gα signaling include adenylyl cyclase, phospholipase C, phospholipase A_2, ion channels, and several kinases and transcription factors. Among the effector molecules interacting with Gβγ are phospholipases, K^+ and Ca^{2+} channels, GPCR kinases, members of the MAPK signaling pathway, monomeric G proteins, regulators of G protein signaling (RGS proteins), and phosphoinositide-3 kinase (PI3K) (Ueda et al., 1994; Wickman et al., 1994; Faure et al., 1994; Ford et al., 1998; Shi et al., 2001; Smrcka, 2008). Thus, G protein α and βγ subunits are capable of activating diverse downstream effectors. Termination of the signal occurs when the GTP bound to the Gα subunit is hydrolyzed by the GTPase activity, causing the functional dissociation from the effector and reassociation with the Gβγ subunit. Although G proteins are likely to membrane-bound when coupled to receptors, recent results from various laboratories suggest that G proteins associate with MTs and regulate assembly/dynamics of MTs and neuronal differentiation (Roychowdhury & Rasenick, 1997)

1.5 G-protein signaling pathway, Microtubule assembly and neurite outgrowth

The dynamic process of Microtubule assembly must be tightly regulated in order to accomplish the proper morphology and connectivity between neuronal cells. Microtubule assembly can be regulated by different pathways. Previous studies from our laboratory have indicated that MT assembly could be regulated by G-protein mediated signaling. As indicated in Fig.3 , binding of the agonist to GPCR leads to an activation of Gα and G. It is the Gβγ complex that has been shown to promote Microtubule assembly and induce neuronal differentiation (Fig.3) (Roychowdhury and Rasenick 1997; Sierra-Fonseca et. al 2014). It was observed that reconstructed heterotrimers, would not show signs of coupling with MTs nor take part in modulating MT assembly, concluding that G proteins activation was necessary for Gβγ dependent MT assembly (Fig.3) (Roychowdhury et. al 2006; Sierra-Fonseca et. al 2018). Using the antimiotic drug nocodazale, it has been demonstrated that tubulin Gβγ interaction is important for MT assembly in cultured PC12, NIH3T3 cells (Montoya et. al 2007). It was found that Gβγ-MT is critical for Nerve growth factor (NGF) induced neuronal of PC12 cells. (Sierra-Fonseca et al., 2014). Overexpression of Gβγ in PC12 cell in the absence of NGF produced neurites (Sierra-Fonseca et al., 2014) While Gβγ-sequestering peptide GRK2i inhibited neurite formation, disrupted MTs, and induced neurite damage; the Gβγ activator mSIRK stimulated neurite outgrowth, which indicates the involvement of Gβγ in this process (Sierra-Fonseca et al., 2014). These results suggest that the Gβγ initiated by the activation of the GPCR, interacts with microtubules and this interaction is important for MT assembly and neuronal differentiation and dysregulation of this process could lead to neurodegeneration. (Fig 3)

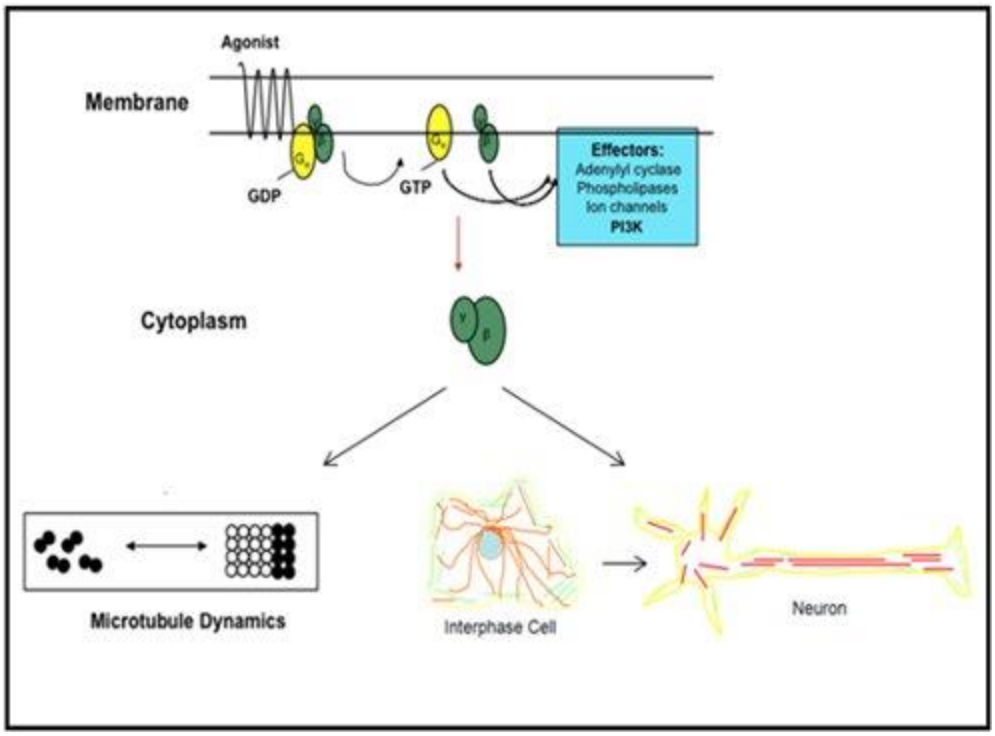

Figure. 3 Gβγ signaling, MT assembly and Neurite outgrowth. Activation of GPCR triggers the disassociation of the Gαβγ heterotrimer into Gα and Gβγ subunits. These subunits play a role in activation of several signaling pathways including adenylyl cyclase, phospholipases, and PI3-Kinase pathways. It has been previously found from our laboratory that Gβγ subunit interacts with αβ tubulin heterodimers to stimulate microtubule assembly and to induce neurite outgrowth in pheochromoctyoma (PC12) cells (Roychowdhury and Rasenick, 1997; Montoya et al., 2007; Sierra-Fonseca et. al 2014).

1.6 Gβγ signaling and PI3K/AKT/GSK3B pathway: Regulation of MT assembly and neurite outgrowth

Although results from our laboratory indicate an important role of Gβγ in NGF-induced neuronal differentiation in PC12 cells (sierra-Fonseca et.al 2014), NGF is also known to induce neurite outgrowth in PC12 cells by activating the receptor tyrosine kinase, TrkA [Knight et al., 2006] and subsequent activation of downstream phosphatidylinositol 3-kinase (PI3K) pathway. Two downstream effectors of PI3K that have been shown to participate in the regulation of neurite outgrowth and are particularly associated with MT remodeling, are AKT (a serine/threonine-specific protein kinase) and the glycogen synthase kinase 3β (GSK-3β) (Zhou et.al 2004; Zhou and Snyder 2005, 2006; Dill et.al 2008). PI3K is known to phosphorylate and activate AKT, which is followed, by phosphorylation and inactivation of GSK-3β. Unlike AKT or many other kinases, GSK-3β is usually active in resting cells. Upon activation of the PI3K signaling pathway, GSK-3β is inactivated through phosphorylation at the serine 9 residue in its amino terminal region (Doble and Woodgett 2003). Phosphorylation of GSK3β leads to inhibition of glycogen synthesis. (Brunet, Datta & Greenberg, 2001). Recently GSK3β has emerged as an important signaling mediator in the regulation of axon extension and axon branching by transducing downstream signaling to reorganize axonal cytoskeleton and MTs. (Kim, Hur, Snider & Zhou, 2011). GSK3β dysregulation has been implicated in AD and PD. In AD, GSK3β is thought to attribute to hyperphosphorylation of TAU. In a recent study TAU hyperphosphorylation was induced by overexpressing GSK3β in SHSY5Y cell line (Cortez-Gomez et al.,2020). Another study determined that dysregulation of GSK3β contributed to parkinsonian dopaminergic neuronal cell death (Li, J et al, 2020). Recent studies suggest that the activation of PI3K/AKT pathway by NGF

is, in part, mediated through the βγ subunit. (Wang & Wong, 2009). These findings correlate with previous findings from our laboratory that Gβγ overexpression in PC12 cells was sufficient to promote neurite outgrowth as well as increasing levels of PAKT/pGSK3β. (Verela J, MS thesis UTEP 2016) These findings suggest that the co-ordination of Gβγ and PI3K/AKT/GSK3β pathway may regulate neurite morphology and outgrowth (Fig 4). Manipulation of the Gβγ and PI3K pathway can help understand the regulation for neuronal differentiation and neurodegeneration (Roychowdhury & Sierra-Fonseca 2017).

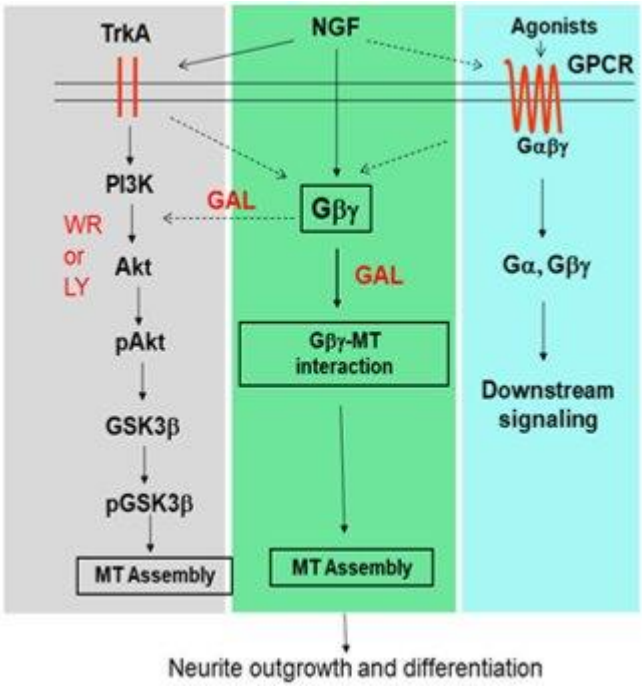

Figure. 4 Gβγ and PI3K signaling pathway in regulating MT assembly and neurite outgrowth: A proposed Model. NGF acts through TrkA receptors to activatePI3K and its downstream effectors Akt and GSK3β (left panel). The results from our laboratory demonstrate the NGF utilizes Gβγ, a key component of the GPCR pathway (right panel), to alter MT assembly and neurite outgrowth (central panel). PI3Ky is a downstream effector of Gβγ in GPCR signaling.

Gβ overexpression in PC12 cells was sufficient to promote neurite formation, as well as to increase levels of PAKT and pGSK3β.

1.7 Gallein, Tideglusib, and Retinoic acid

Previous studies have used inhibitors to further understand the signaling mechanism, interaction and function. Gallein (GAL), a small molecule found to bind to the Gβγ subunit with high affinity blocking Gβγ dependent cellular activities. In a recent study GAL was used in amphetamine induced cultured dopamine neurons. The study concluded that there is an interaction between Gβγ and dopamine transporter (DAT) signaling. GAL has been shown to block Gβγ-dependent activation of the PI3Kγ isoform (Mariana 2019). In a recent study using gallein, it was found that APP-dependent toxicity is mediated by Gβγ complex signaling. p38MAPK was identified as a downstream target of Gβγ complex (Bignonte, et al.,2019). Tideglusib (TIDE) , an ATP non-competitive GSK-3β inhibitor from the tiadiazolidinone family, has been tested clinically in several neurological disorders and was shown to reduce hyperphosphorylation of Tau. It was tested in phase II clinical trial for the treatment of AD (Lovestone S. et al., 2015). Both GAL and TIDE were used in the current study to further understand the mechanism by which Gβγ and GSK3β are involved in the regulation of neurite outgrowth, MT assembly, and neurodegeneration. Retinoic Acid (RA) is a vitamin A derivative known to possess powerful growth inhibiting and cellular differentiation promoting properties. It has been used extensively in differentiation of SHSY5Y cells. Usage of RA treatment for the differentiation of SHSY5Y leads to neurons exhibiting more cholinergic properties since the differentiation induces the expression of cholinergic markers (De Medeiros et al., 2019). Dysfunctions in cholinergic signaling have been linked with the pathophysiology of several disorders and diseases, including depression, schizophrenia, cancer, Huntington's, Parkinson's and Alzheimer's disease (Madireddy & Madireddy, 2019). RA has been shown to promote cellular differentiation and survival in SHSY5Y cell line by utilizing the PI3K/AKT pathway. (Cheung et al., 2009). RA has been used in the

current study to differentiate SHSY5Y cells to further understand the mechanism of neuronal differentiation by Gβγ.

1.8. Hypothesis and specific aims

The goal of this proposal is to determine the mechanism by which Gβγ interferes with neuronal differentiation and induces neurodegeneration. Gβγ has been shown to play a critical role in neuronal differentiation in NGF-differentiated PC12 cells through its interaction with tubulin/MTs, and subsequent stimulation of MT assembly (Sierra-Fonseca 2013). Blocking the interaction between Gβγ and MTs was shown to disrupt MTs and inhibit neurite outgrowth, suggesting a role of Gβγ in neurodegeneration, More recently, PI3K/PAKT/GSK3β pathway was shown to be involved in Gβγ dependent regulation of MTs and neurite outgrowth (Varela J, MS thesis, UTEP 2016). In the present study we will address this issue using neuronal cells SHSY5Y. I hypothesize that ***Gβγ is involved in neurodegeneration by disrupting MTs, inducing hyperphosphorylation of tau proteins, and altering Gβγ-mediated downstream signaling pathway.*** We plan to test our hypothesis by focusing on the following aims in this study.

Specific Aim-1: Determine the role of Gβγ in MT disruption, degeneration of neurites, and tau phosphorylation.

Specific Aim-2: Delineate the mechanism of Gβγ-dependent microtubule disruption/neuronal damage by downstream PI3K/pAKT/pGSK3β pathways.

Chapter 2: Materials and Methods

2.1. Cell culture and RA Treatment for SHSY5Y

Human neuroblastoma cells SHSY5Y cells were grown in T-25 or T-75 culture flasks at 37°C in Dulbecco's modified of Eagle's medium (DMEM)/Ham's F-12 50/50 mix (DMEM/F-12 50/50), supplemented with 10 % fetal bovine serum and penicillin (100U/mL)-streptomycin (100um/mL) in 5% CO_2. For Retinoic acid treatment cells where grown in 10μM RA (stock 10 mM) diluted in DMSO, 2.5% FBS, 1% penicillin and 2mM Glutamic acid (stock 20mM) per ml.

2.2 Preparation of whole cell lysates

SHSY5Y cells were grown under the conditions described in section 2.2. For Gallien treatment, cells were grown on 100 mm culture dishes until they reached ~80% confluence. Cells were treated with the drug (10 μM) for 4 hours. Whole cell lysates were also prepared. After treatment culture media was removed, cells were washed with PBS, and then lysed by incubation in 500 μL lysis buffer (10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl2, 0.3 M sucrose, 0.1% Triton X-100, 1 mM DTT, and 10 μM GTP) supplemented with protease inhibitor cocktail, for 10 min at room temperature. Cells were then scraped and sonicated for 1 min, followed by centrifugation at 10,000 × g for 10 min. The supernatants constituted whole cell lysates, and the protein concentration was determined by the Bradford assay.

2.3 Electrophoresis and immunoblotting

Samples for immunoblotting were subjected to SDS-polyacrylamide gel (10%) electrophoresis, followed by ElectroTransfer onto nitrocellulose membranes (find distributer). The membranes were blocked in 5% nonfat dry milk in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) for 2 h at room temperature, followed by overnight incubation at 4 °C with mouse monoclonal anti-tubulin

, rabbit polyclonal anti-Gβ (check company check ratio), or rabbit monoclonal anti-AKT and anti-pAKT (Cell Signaling Technology, 1:500) antibodies in TBS containing 0.01% BSA. The membranes were washed three times with 0.05% Tween-20 in TBS (TBST) and incubated with the appropriate Conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit, or donkey anti- goat from check ratio check man..) in TBST containing 0.01% BSA for 1 h. For sensitive detection, the chemiluminescence (ECL) technique (check how it was made we make it but where do we get the reagents) was used according to the manufacturer's instructions. Quantitative analysis of the protein bands was performed with the I-Bright image acquisition and analysis software (who make I-bright where is the software from).

2.4 Immunoprecipitation

For immunoprecipitation experiments, 100 µL aliquots of cellular fractions (~0.25–1 mg/mL of protein) were incubated with or without anti-Gβ antibody (5-10 µl), or non-specific rabbit IgG for 1 hr at 4 °C, followed by an overnight incubation (4 °C) with 100 µL of 50% protein A-sepharose (where did we get the beads), Samples were then centrifuged at 10,000 × g for 10 min, and the supernatants (SUP) were saved. The pellets (immunocomplex) were washed with PBS and eluted with 3% SDS Laemmli sample buffer containing 0.15 M dithiothreitol (DTT) and boiled in a water bath for 5 min. Samples were then clarified by centrifugation. Both IP and SUP fractions were then subjected to immunoblotting using anti-tubulin or anti-actin antibodies as described in section 2.3

2.5 Immunofluorescence and confocal microscopy

For confocal microscopic analysis, cells were attached to immunocytochemistry slides seeded on glass coverslips using 12-well plates and were grown overnight as described in section 2.1. Cells were then treated with or without RA and/or inhibitors as indicated and subsequently fixed by the addition of ice-cold 100% methanol (previously cooled to -20 °C) and incubated at -20 °C for 5 min (). The cells were then rinsed three times in PBS, blocked for 1 h at room temperature in 5% normal goat serum (NGS) (Sigma-Aldrich) in PBS, followed by overnight incubation at 4 °C with primary antibodies (anti-tubulin, anti-Gβ, and anti-tau1:100 dilution) in 1% NGS in PBS.(check primary and secondary) The slides were rinsed as before and incubated with the appropriate secondary antibodies (tetramethyl rhodamine (TMR)-conjugated goat anti-mouse IgG, AlexaFlur488-conjugated goat anti-mouse IgG, Alexa Fluor 647-conjugated donkey anti-rabbitIgG; for 2 h at room temperature in the dark to diminish photo-bleaching effects. The slides were then mounted with DAKO mounting media (DAKO Corporation, Carpenteria, CA), or with ProLong Gold anti-fade reagent with DAPI (Invitrogen, for nuclear staining), and covered with coverslip. High-resolution, digital, fluorescent images were captured by employing inverted, confocal laser-scanning microscopy (model LSM 700; Zeiss, Thornwood, NY), utilizing a Plan-Apochromat 63x/1.40 immersion-oil DIC objective and assisted with 2009 ZEN software (Zeiss, Thornwood, NY). DAPI and Alexa Fluor 350 (blue), FITC (green), and TMR (red) were excited with laser emissions of 405-, 488-, and 555-nm wavelengths.

2.6 Differential nuclear staining (DNS) assay for cytotoxicity

To determine the levels of cytotoxicity caused by the experimental compounds a previously described DNS assay adapted for high-throughput screening was used (Lema et al., 2011). This assay uses two fluorescent nucleic acid intercalators, Hoechst 33342 (Hoechst) and propidium

iodide (PI). SHSY5Y cells were seeded in a 96-well plate format and incubated with GAL inhibitor. One hour before image capturing, a staining mixture of Hoechst and PI at a final concentration of 1 μg/mL for each dye was added to the cells. Subsequently, cells were imaged in live-cell mode using a BD Pathway 855 Bioimager system (BD Biosciences, Rockville, MD). Montages (2x2) from four adjacent image fields were captured per well to acquire an adequate number of cells for statistical analysis, utilizing a 10x objective. To determine the percentage of dead cells from each well, both image acquisition and data analysis were performed using the BD AttoVision v1.6.2 software (BD Biosciences), and each experimental condition was assessed with 8 replicates.

2.7 Preparation of inhibitor

GAL was purchased from Sigma Aldrich CAS 2103-64-2. GAL was diluted in DMSO as instructed by manufacturer. GAL stock concentration is 12.5 mM after dilution in 1ml of DMSO. To dilute GAL concentration to 10 uM 3.2 ul of original stock / 4 ml of DMEM media.

Tideglusib was purchased from Sigma Aldrich CAS 865854-05-03. Tideglusib was diluted in DMSO as instructed by manufacturer. Final stock concentration of Tideglusib is 30 mM. To bring Tideglusib concentration to 10 uM by diluting 3.33 ul of 30 mM stock per 10 ml of DMEM media.

Chapter-3: Results

3.1 Specific Aim-1: Determine the role of Gβγ in MT disruption, tau phosphorylation, and degeneration of neurites,

3.1.1 Objective and overview

We have shown previously that βγ subunits of heterotrimeric G proteins promote MT assembly *in vitro* and in cultured PC12 cells (Roychowdhury 1997; Montoya 2007). Recently, we found that the interaction of Gβγ with tubulin and MTs were increased during NGF-induced neuronal differentiation of PC12 cells. In addition, the sequestration of Gβγ by GRK2i, a Gβγ-inhibitory peptide, inhibited neurite outgrowth, disrupted MTs and induced cellular aggregation (Sierra Fonseca 2014), thus, indicating a possible role of Gβγ in neuronal disruption and inducing neurodegeneration. Since PC12 cells are non-neuronal cells, in this aim we will determine if Gβγ-tubulin/MT interaction occurs in neuronal cells SHSY5Y, and whether blocking this interaction leads to MT disruption and neuronal damage as observed in PC12 cells. We will use gallein (GAL), a small molecule inhibitor of Gβγ which is known to inhibit Gβγ-dependent cellular activities, to conduct this study. SHSY5Y cells are a neuroblastoma derived cell line that is commonly used in vitro to further understand the process of neurodegeneration (ND). SHSY5Y cells were further differentiated in the presence RA, into a more mature neuron-like phenotype that is characterized by neuronal markers. The goal of this aim is to determine the role of Gβγ in MT disruption, and neuronal damage in neuronal cells SHSY5Y. Specifically, we will determine if blocking Gβγ by gallein (GAL), an inhibitor of Gβγ, interferes with Gβγ-tubulin interaction, MT organization and neurite formation. Tau hyperphosphorylation, which is a signature event occurs during neurodegeneration will also be examined in the presence of GAL.

3.1.2 Results:

Gallein disrupts MT organization and inhibits neurite formation in SHSY5Y cells:

To test the effect of GAL on neurite formation and MT assembly/organization, immunofluorescence and confocal microscopic analysis were carried out (Sierra-Fonseca 2014). Briefly, SHSY5Y cells were attached to cover slips placed in 12 wells plate (25000 cells/well). The samples were then treated with 1uM, 5uM, or 10μM GAL for 1h, and processed for confocal microscopy as described in the methods. MT organization and neurite outgrowth were examined. Gβγ (red) was colocalized with MTs in the cell body and neuronal processes in control SHSY5Y cells (Fig. 1A). As the concentration of GAL increases, neurite formation is inhibited, the disruption of MTs is increased, and MT assembly/organization was drastically disrupted as evident by the tubulin staining (green). Gβγ becomes localized more towards the ends of the neurites as evident by Gβγ staining (red) where tubulin immunoreactivity was not observed. This is consistent with the recent observation that Gβγ was found to be colocalized with actin filaments at the distal end of the neurites and growth cones (Sierra-Fonseca, 2021). The neurites begin to thin as the concentration of GAL increase. At 1μM GAL the neurites resemble control neurites, only slightly thinner. At 5μM GAL, the filaments are thinner than both control and in the presence of 1μM GAL, but still connected to the cell body of the neuron. Neurite formation was inhibited at 10 μM GAL. The neurites become very thin causing it to break off from the cell body of the neuron (Fig.5). MT assembly/organization was drastically disrupted as evident by the tubulin staining (green). Both MTs and Gβ localization was disrupted by GAL suggesting a clear relationship between Gβ and MT organizations. In addition to SHSY5Y cells, we tested the effect of GAL in RA-differentiated SHSY5Y cells. As shown in Fig.6. RA induced differentiation of SHSY5Y cells

which is exhibited by stabilized neuronal processes and interconnectivity among neurons. We found that similar damaging effect of GAL in RA-differentiated SHSY5Y cells treated with 10 μM GAL (Fig.6). When both SHSY5Y cells and RA-treated SHSY5Y cells were incubated with 10 μM GAL for 72 hours, it was observed that GAL caused the cells to aggregate in both treatment groups. Loss of neurites were evident in both SHSY5Y and differentiated SHSY5Y (Fig 5 & Fig 6).

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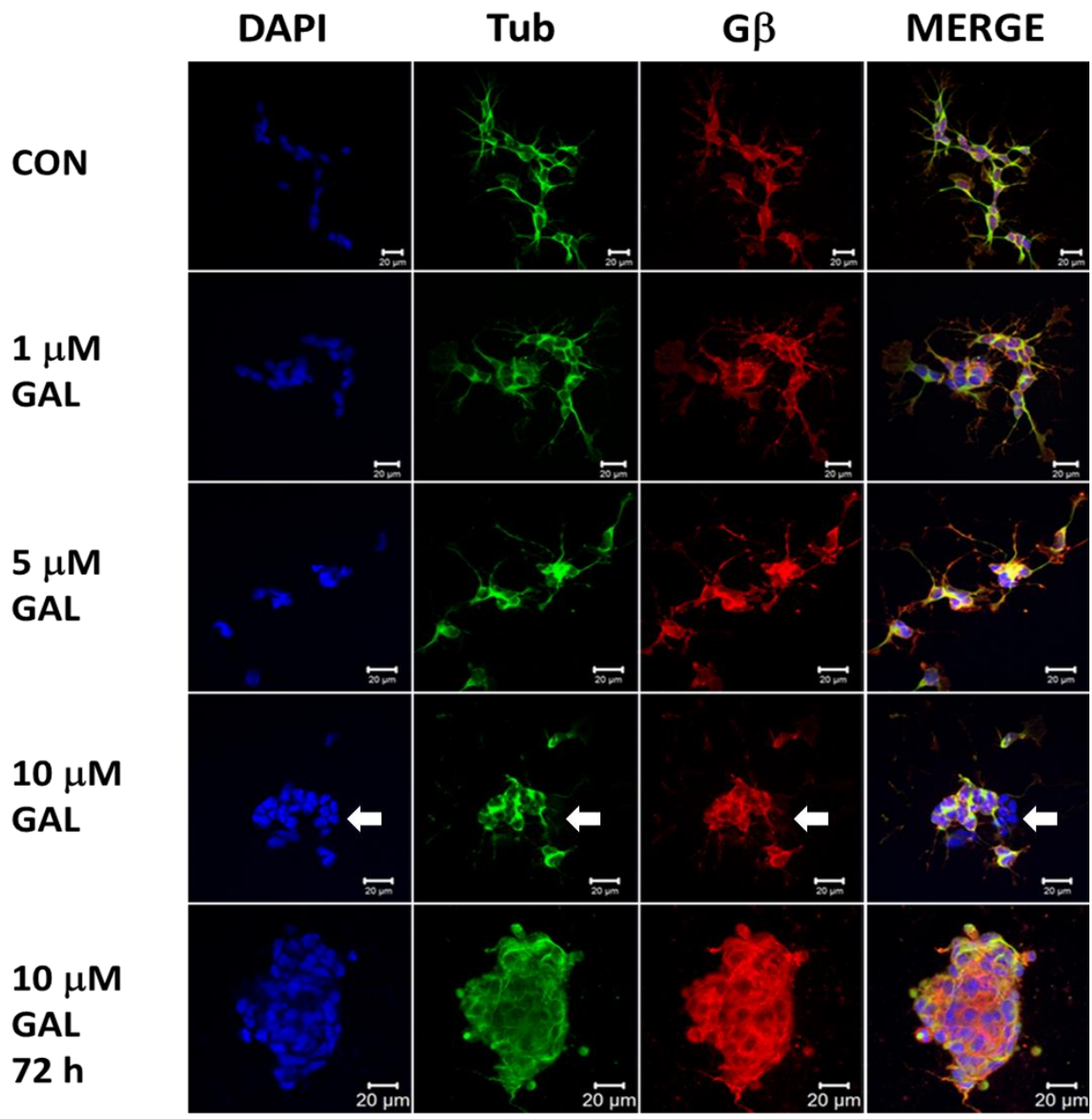

Figure 5. Effect of GAL on MTs and Gβγ organization, and neurite formation.

SHSY5Y were seeded on glass coverslip in 12 well plate at a density of 35,000/well. Once cells fully adhered GAL treatment was administered at a concentration of 1μM, 5μM, or 10μM for 1h (A) or 72h (B). The cells were then fixed and double labeled with Alexa-Fluor 488 conjugated anti-tubulin (1-100 dilution) (green) and Alexa-Fluor 546 conjugated anti-Gβ (red) antibodies

(1:100 dilutions) as indicated in the methods. Nucleus was labelled with DAPI (blue). Areas of overlay (merge) appear yellow.

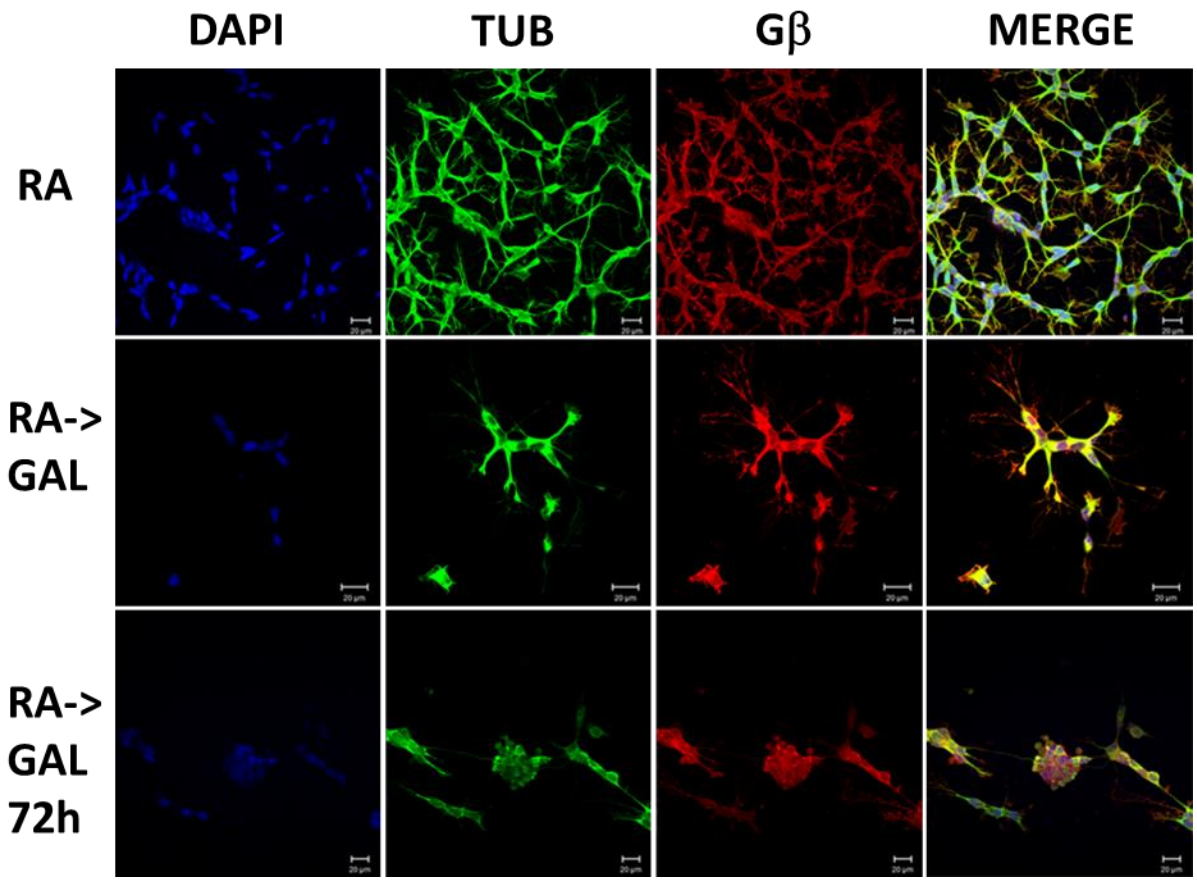

Figure 6. GAL disrupts MT assembly in RA differentiated SHSY5Y

SHSY5Y Cells were treated with 10 μM RA for 3 days or until visible differentiation occurred. Subsequently, cells were treated with or without 10 μM GAL and processed for confocal microscopy using Alexa-Fluor 488 conjugated anti-tubulin (1-100 dilution) (green and Alexa-Fluor 546 conjugated anti-Gβ (red) antibodies (1:100 dilutions) as indicated in the methods. Nucleus was labelled with DAPI (blue). Areas of overlay (merge) appear yellow.

GAL inhibited Gβ expression in both SHSY5Y and RA-differentiated SHSY5Y cells while tubulin expression was not affected by GAL.

Because GAL affected MT/Gβγ organization, and neurite formation in both SHSY5Y and RA-differentiated SHSY5Y cells, I tested the effect of GAL on tubulin and Gβγ (Gβ) expression in these cells. SHSY5Y cells were seeded on 100 mm plates. SHSY5Y cells were prepared for RA differentiation. Cells were treated with 10 uM RA for three days or until visible differentiation occurred. Both control and RA-differentiated cells were treated with GAL for one hour. Whole-cell lysates were prepared as indicated in the methods, subjected to gel electrophoresis followed by immunoblotting with anti-Tubulin and anti-Gβ antibodies. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as percent control. As shown in the figure 6, GAL did not alter tubulin expression significantly, but the expression of Gβ appears to be inhibited in the presence of GAL both in SHSY5Y and RA-differentiated SHSY5Y

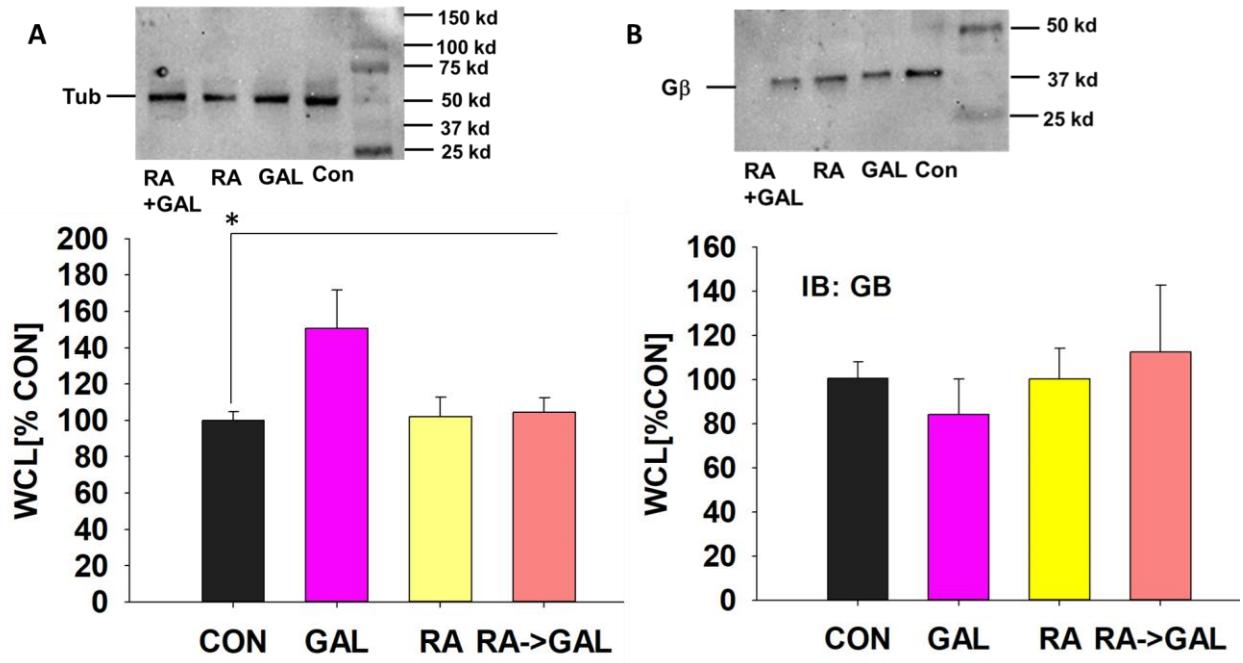

Figure.7 Effect of GAL on Gβ and tubulin expression in SHSY5Y and differentiated SHSY5Y cells.

SHSY5Y cells were seeded on 100 mm plates. SHSY5Y cells that were prepared for differentiation were treated with RA for 3 days or until differentiation was visible. Cells were then treated with 10 μM GAL. Cells were then prepared for WCL and IB analysis. Anti-Tubulin (Fig.7A) and Anti-Gβ (Fig.7B) were used to incubate IB overnight. Protein bands were detected by ECL plus reagent. Protein analysis was done using iBright fl100, statistical analysis was done using Sigma plot. One-way anova analysis was performed on 5 experiments showed a p-value of 0.034 of tubulin expression of all treatment groups when compared to control.

Tau expression was reduced in the presence of GAL

Since Tau is a microtubule-associated protein found in axons and known to regulate MT assembly and neurite outgrowth, I examined whether the tau protein is affected by GAL. Specifically I determined whether Tau expression is altered in the presence of GAL. Both SHSY5Y cells and RA-differentiated SHSY5Y cells were used for this experiment. Cells were seeded in 100mm plates, followed by treatment with GAL as indicated in the figure (Fig, X). Whole-cell lysates (WCL) were prepared, and subjected to immunoblotting using Tau-specific antibodies. Tau expression in SHSY5Y cells was found to be slightly decreased in the presence of GAL. However, in RA-differentiated cells, Tau expression was significantly decreased in the presence of GAL.

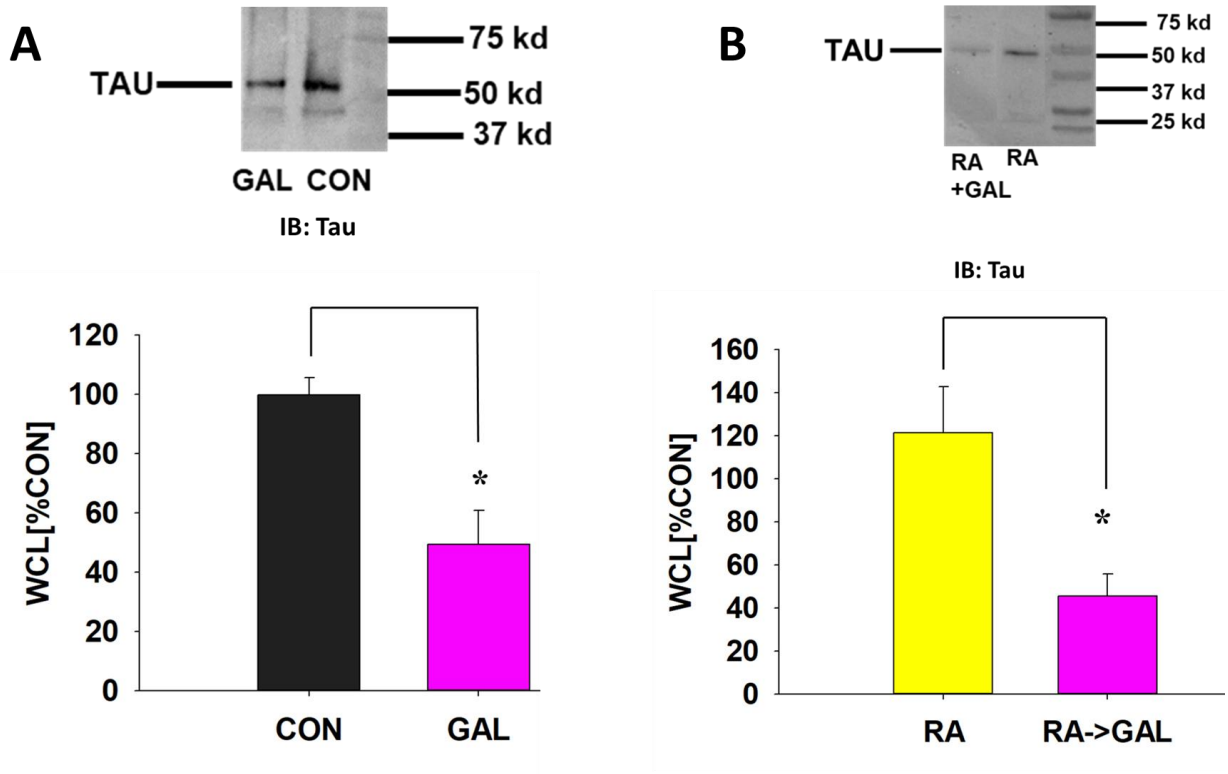

Figure 8. GAL decreases TAU protein expression in SHSY5Y cells and differentiated SHSY5Y cells.

SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with 10uM RA until differentiation followed by GAL for 1 hour. Equal amounts of proteins from each sample were subjected to immunoblot analysis using the anti-Tau antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure. The experiment was repeated three times with duplicates and statistical analysis was carried out using one-way ANOVA. Controls represent 100%. Tau expression was decreased after GAL.T-test Analysis of 5 experiments showed statistical significance of p=0.004 between CON and GAL in Tau expression; Statistical Significance of 3 experiments p=.033 in Tau expression was found in RA and GAL.

GAL decreases phosphorylation of TAU in the SER site 396

Since the hyperphosphorylation of tau is a signature event of neuronal damage and neurodegeneration, and GAL has been shown to affect Tau expression, I determined if GAL changes the phosphorylation status of tau. The tau protein has 70 potential phosphorylation sites that almost span the entire protein structure. Based on the literature, several phosphorylation sites are known to be pathologically relevant. We tested the effect of GAL on the phosphorylation of tau at Ser396 using anti-ps396 Tau. Both SHSY5Y cells and RA-differentiated SHSY5Y cells were used for this study. After treatment with GAL for one hour, whole-cell lysates were subjected to immunoblotting with anti-ps396 Tau (abeam). Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as percent control. As indicated in the figure (Fix), GAL decreased the expression of phosphorylated Tau in both SHSY5Y and RA-differentiated SHSY5Y cells.

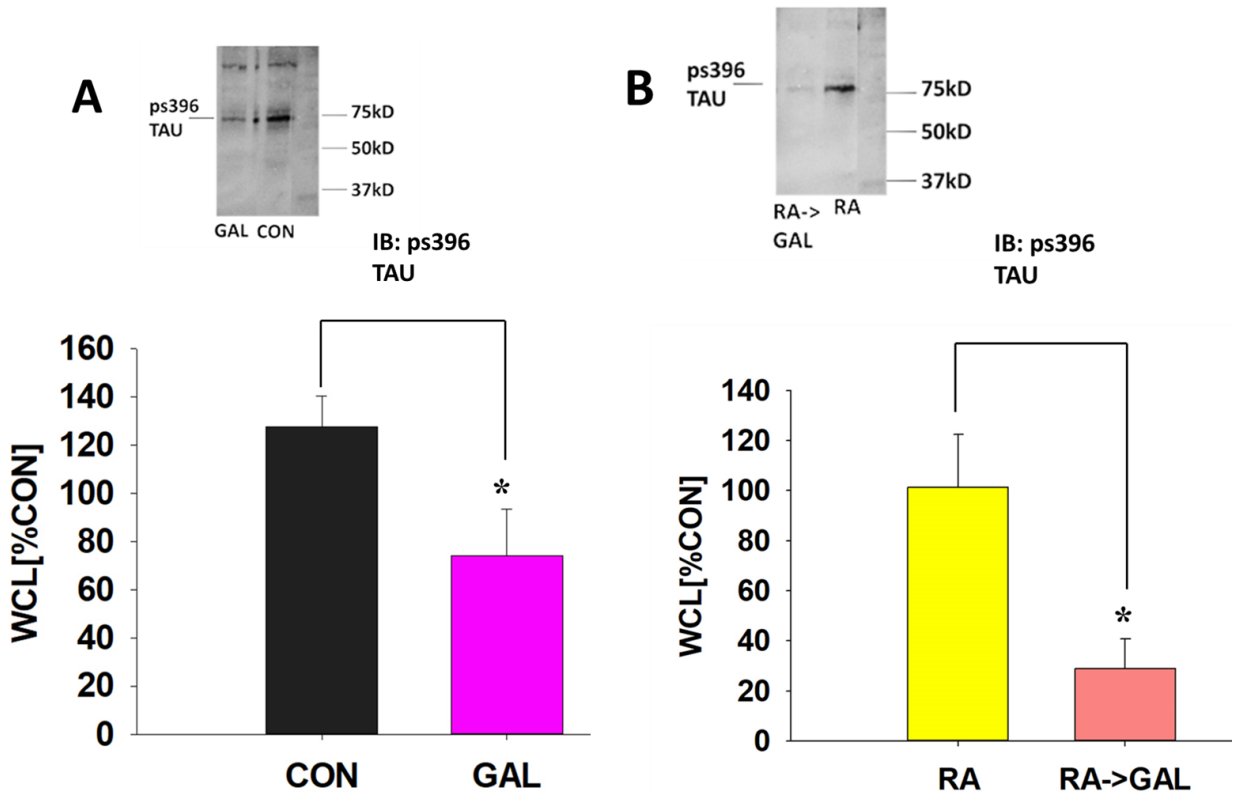

Figure 9. GAL on phosphorylation of TAU in the SER site 396 in SHSY5Y and differentiated SHSY5Y cells

SHSY5Y Cells where seeded on 100 mm plates. SHSY5Y cells that were prepared for differentiation were treated with RA for 3 days or until differentiation was visible. Cells were then treated with GAL one-hour followed by WCL extraction and immunoblotting using anti-ps396 TAU. Protein detection was done using ECL plus reagent. Quantification and analysis were done by I-bright fl1000. Statistical analysis was performed by Sigma plot. T-test Analysis of 6 experiments showed statistical significance of p=0.042 between CON and GAL in ps396 Tau expression; Statistical Significance of 4 experiments p=.025 in ps396 Tau expression was found in RA and GAL.

3.2 Specific Aim-2: Delineate the mechanism of Gβγ-dependent microtubule disruption/neuronal damage by downstream PI3K/pAKT/pGSK3β pathways.

3.2.1 Objective and overview

The goal of this aim is to determine the signaling pathway by which Gβγ interferes with MT assembly and neurite outgrowth, and induces neurodegeneration. One of the downstream effector of Gβγ, which is known to known to participate in the regulation of neurite outgrowth and MT assembly is Phosphatidyl inositol-3-kinase (PI3K). Activation of PI3K has been shown to promote the phosphorylation of downstream AKT and GSK-3β, which subsequently participate in the regulation of neurite outgrowth, and are particularly associated with microtubule remodeling. It has been shown earlier from our laboratory that the overexpression of Gβγ in PC12 induces neurite outgrowth (Sierra-Fonseca et al.,2014) and phosphorylate Akt and GSK3β supporting the notion that Gβγ and PI3K pathway co-ordinate to regulate neurite outgrowth. Therefore, in the current aim, I will dissect the interplay among Gβγ, GSK3β, and MTs in inducing neurodegeneration. Gβγ inhibitor gallein, and GSK3β inhibitor tideglusib will be used to conduct the study. Because over activation of GSK3β, has been shown to play key roles in tau hyperphosphorylation, we will determine if GAL or tideglusib interferes with Tau phosphorylation.

The Following sub aims will be addressed

1) Determine how GAL and/or tideglusib interferes with the phosphorylation of Akt and GSK3β in SHSY5Y cells.

2) Does tideglusib interferes with Gβγ localization/expression and neurite formation?

3) How does GSK3β inhibitor affect expression of TAU, and ps396 TA

3.2.2 Results:

Gβγ and GSK3β inhibitors decrease phosphorylation of Akt and GSK3β

To examine the increasing effect of gallein and tideglusib SHSY5Y cells were treated with inhibitors at 1μM, 5μM, and 10 μM concentrations for one hour. Previously I found Gβγ decreased phosphorylation of Akt and GSK3β. I wanted to see the effect GAL had at a lower concentration on these molecules. At 1 μM GAL slightly decreased phosphorylation Akt and had a greater decrease of expression of GSK3β. 5 μM GAL had slightly greater decreasing effect of expression of molecules. A confocal experiment done in this study showed that SHSY5Y cells treated with 1 μM GAL morphology was similar or better then control. Previous studies have suggested that GAL may have a positive effect on MT dynamics (Bignante, E., et al., 2018). To further understand Gβγ signaling involvement with PI3K/AKT/GSK3β signaling pathway in MT assembly and organization; SHSY5Y cells were treated with 5 μM and 10 μM Tideglusib. Inhibition of GSK3β lead to a decrease of both pAKT and pGSK3β. Inhibition of GSK3β is thought to promote MT assembly (Noble, W., et al, 2013). Previous studies from our laboratory have shown that pGSK3β is increased in Gβγ overexpressed cells (Sierra-Fonseca, et al.,2014). Suggesting Gβγ may have a direct effect on GSK3β.

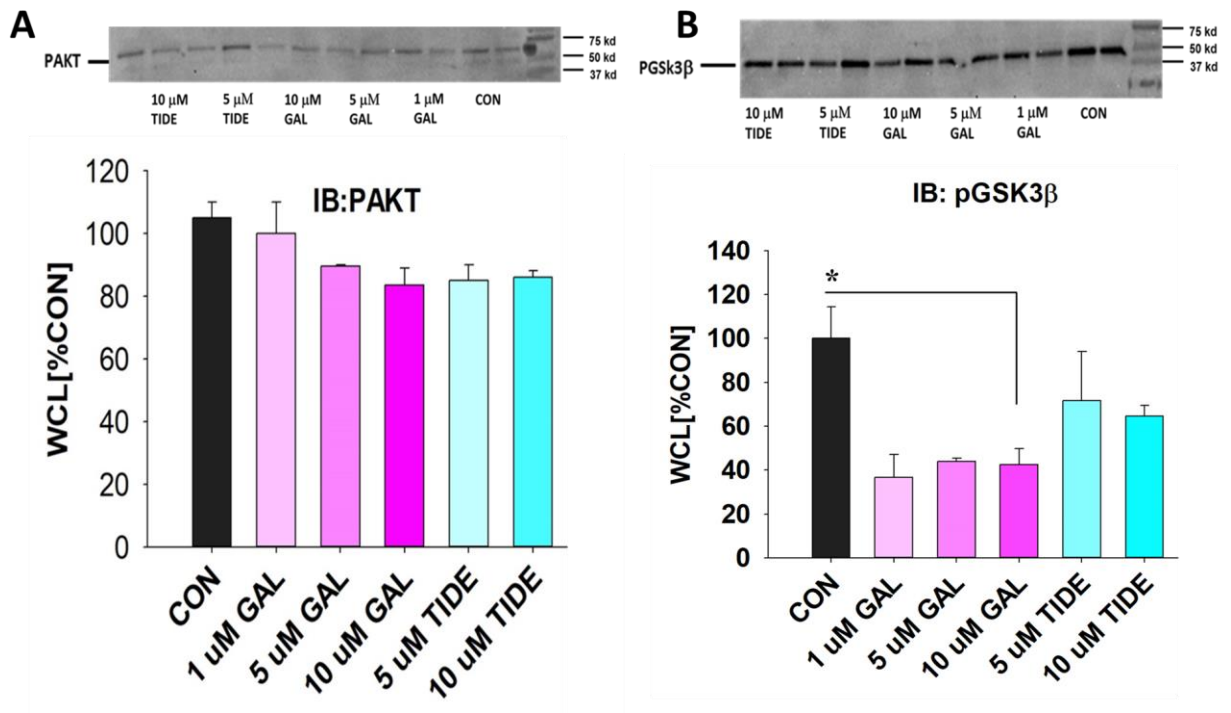

Figure 10. Increasing Concentrations of Gβγ and GSK3β inhibitors leads to decreasing expression of pAKT and pGSK3β

Inhibition of Gβγ and GSK3β lead to a decrease expression of pAKT and pGSK3β in SHSY5Y cells. SHSY5Y cells were plated in 100 mm. Once cells fully adhered treatment of GAL and Tideglusib at varying concentrations (1µM,5µM, and 10µM) was administered. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti-pAKT and anti-pGSK3β antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure above. One way anova analysis using sigma plot software showed a p=.005 for pGSK3b expression for all concentrations of GAL n=4.

3.2.3 Results:

Tideglusib interferes with MT organization and alters neurite formation in SHSY5Y cells:

To test the effect of TIDE, on neurite formation, MT assembly/organization, and localization/co-localization of Gβγ and MTs, immunofluorescence and confocal microscopic analysis were carried out as described before (Sierra-Fonseca 2014). Briefly, SHSY5Y cells were attached to cover slips placed in 12 wells plate (25000 cells/well). The samples were then treated with 10μM GAL for 1h. MT organization and neurite outgrowth were examined. As shown in the Fig.10, neurite formation was altered by TIDE. Cells became aggregated. MT assembly/organization was disrupted as the concentration of TIDE increased as evident by the tubulin staining (green). Gβγ (red) was co-localized with MTs in the cell body and neuronal processes in control SHSY5Y cells. Both MTs and Gβ localization was disrupted by TIDE suggesting a clear relationship between TIDE a GSK3β inhibitor and Gβ in MT organizations. In addition, the additive/synergistic effect of GSK3β inhibitor and GAL was tested to delineate Gβ and GSK3β mechanism in MT assembly and organization. Fig.11. Neurites formation was inhibited in both low and high combined concentrations of inhibitors as evident by the tubulin staining (green). At the lower combined concentration of inhibitors, the nucleus staining (blue) shows the nucleus swollen when compared to control. At the higher concentration the combined effect of GAL and TIDE shows some change in the nucleus staining (blue). Gβγ staining (red) localization in both treatments is evident at the ends of the process near the growth cone. Further indicating Gβ involvement in MT assembly.

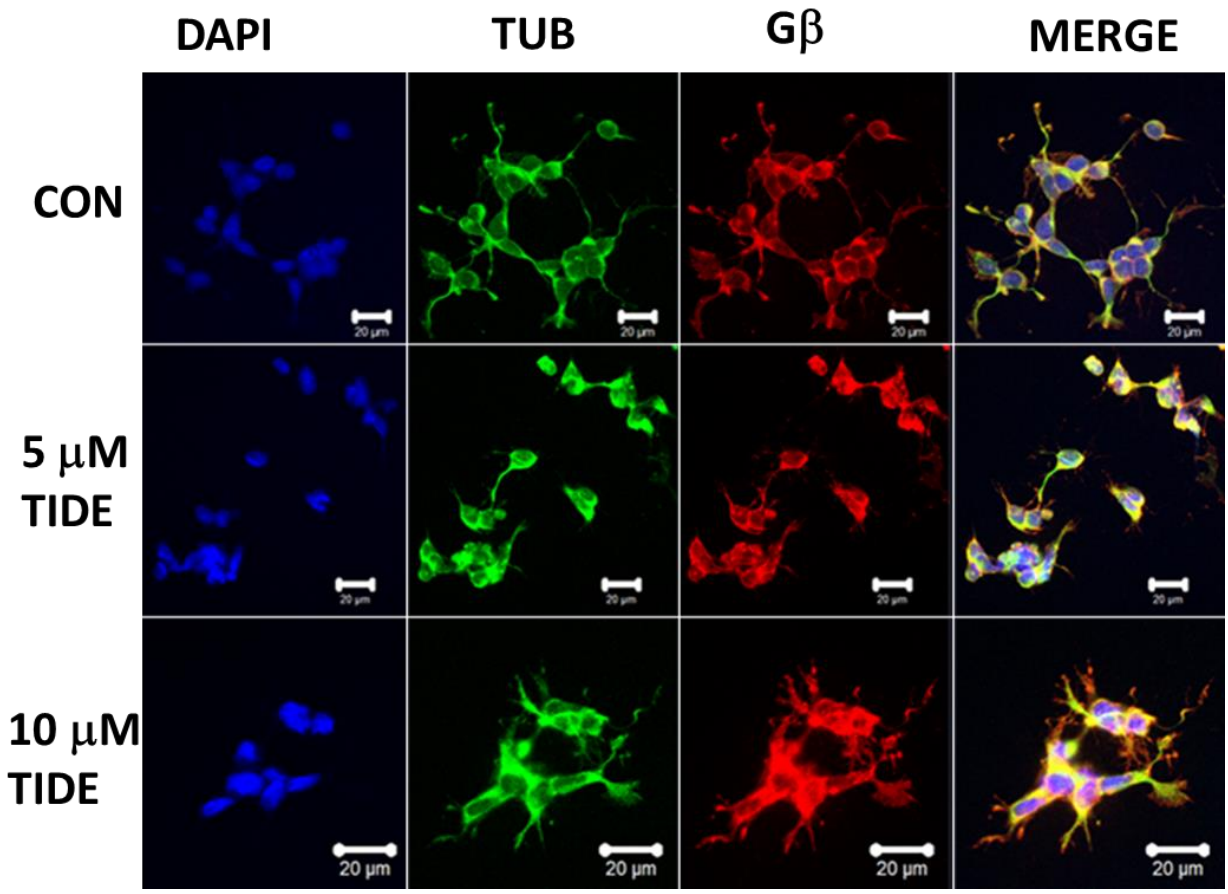

Figure 11. GSk3β inhibitor alters neurites and Gβγ expression in SHSY5Y

SHSY5Y were seeded on glass coverslip in 12 well plate at a density of 35,000/well. Once cells fully adhered Tideglusib treatment was administered at a concentration of 5μM, or 10μM for 1h. The cells were then fixed and double labeled with Alexa-Fluor 488 conjugated anti-tubulin (1-100 dilution) (green) and Alexa-Fluor 546 conjugated anti-Gβ (red) antibodies (1:100 dilutions) as indicated in the methods. Nucleus was labelled with DAPI (blue). Areas of overlay (merge) appear yellow.

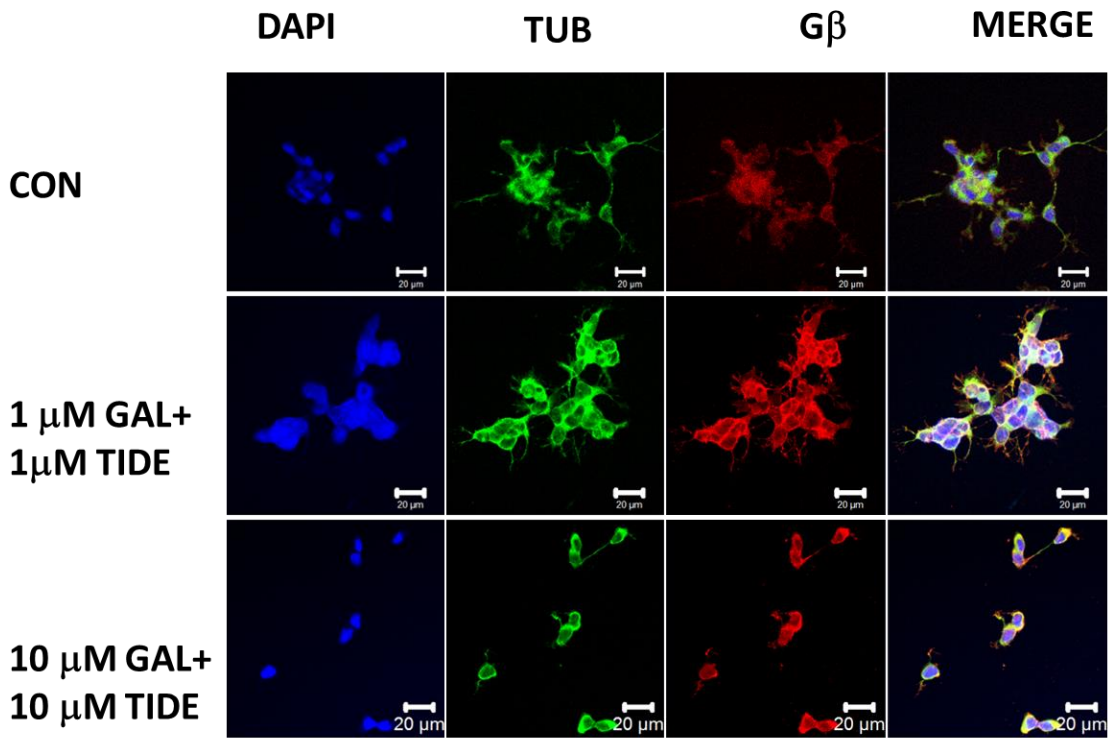

Figure 12. Combined inhibition of Gβγ and GSK3β leads to neuronal disruption.

SHSY5Y were seeded on glass coverslip in 12 well plate at a density of 35,000/well. Once cells fully adhered combined treatment of Gallein and Tideglusib treatment was administered at concentration of 1μM, or 10μM for 1h. The cells were then fixed and double labeled with Alexa-Fluor 488 conjugated anti-tubulin (1-100 dilution) (green) and Alexa-Fluor 546 conjugated anti-Gβ (red) antibodies (1:100 dilutions) as indicated in the methods. Nucleus was labelled with DAPI (blue). Areas of overlay (merge) appear yellow.

Gβγ and GSK3β inhibitors lead to a decrease of phosphorylation of Tau at serine 396 site in SHSY5Y cells.

In previous preclinical studies, tideglusib was reported to reduce a range of disease outcomes, including tau phosphorylation, amyloid deposition, neuron loss, and gliosis in mouse entorhinal cortex and hippocampus, and to reverse a spatial memory deficit in transgenic mice. Neuroprotective, anti-inflammatory, and neurogenesis-inducing effects of tideglusib have been reported in animal models, as well. (Sereno et al., 2009; Morales-Garcia et al., 2012, Wang et al., 2016). In our current study Inhibition of Gβγ lead to a decrease of Tau phosphorylation in SHSY5Y and differentiated SHSY5Y cells. Similarly, Tideglusib also reduced the expression of ps396 tau in SHSY5Y. As previously mentioned, a study on mice concluded that Amyloid Precurser protein/Go protein Gβγ complex signaling mediates Ab degeneration and cognitive impairment in AD models (Bignante, E. A., et al.,2018) Thus, indicating Gβγ inhibition may also have a positive effect on Tau phosphorylation, NFT, amyloid deposition, and neuronal loss.

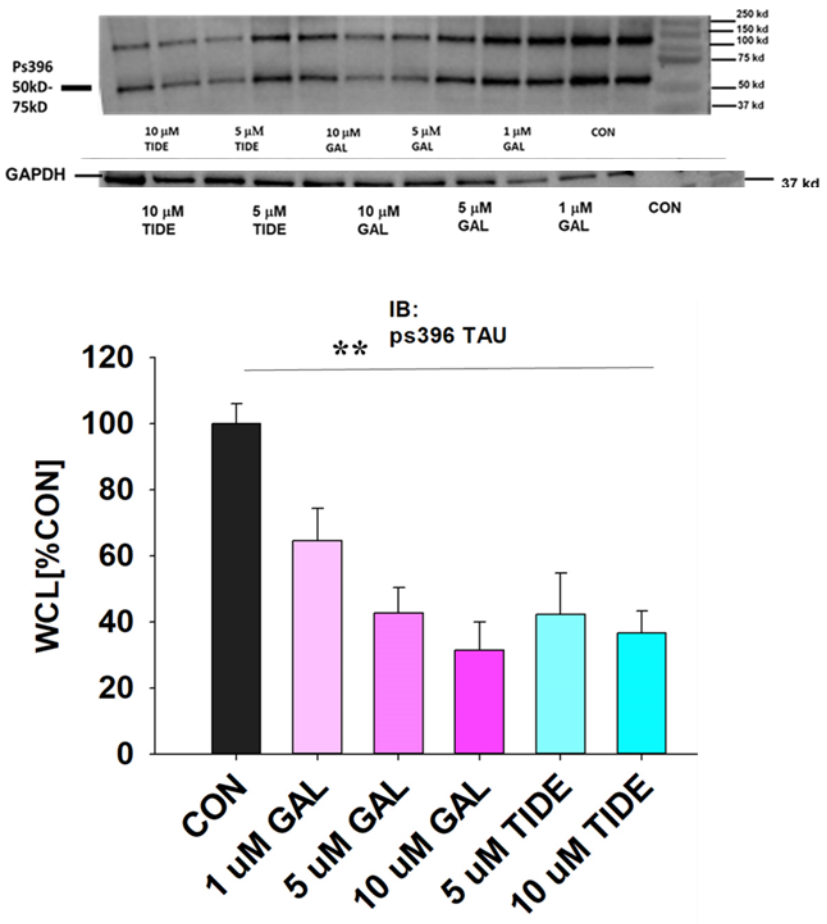

Figure 13. Increase in the concentrations of Gβγ and GSK3β inhibitors lead to a significant decrease of phosphorylation of Tau at serine 396 site in SHSY5Y cells.

Inhibition of Gβγ and GSK3β lead to a decrease expression of ps396 TAU in SHSY5Y cells. SHSY5Y cells were plated in 100 mm. Once cells fully adhered treatment of GAL and tideglusib at varying concentrations (1µM,5µM, and 10µM) was administered. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti-ps396 antibody as indicated. GAPDH was used as a loading control anti-GAPDH was used. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure above. One way anova analysis using

sigma plot software showed a p=.001 for ps396 Tau expression for all concentrations of GAL and tideglusib n=4.

The effects of Tideglusib on RA differentiated SHSYSY.

In our current study to further understand how inhibition of GSK3β affects neuronal differentiation and MT assembly Tideglusib was used in RA differentiated SHSY5Y cells. Surprisingly, in the presence of RA Tideglusib increased phosphorylation of Akt, GSK3β, Tau. It also increased expression of Tau and Gβγ. Previous results from our laboratory indicated that NGF differentiation, and overexpression of Gβγ in PC12 cells lead to an increase of pGSK3β (Varela J, MS thesis, UTEP 2016). RA is thought to bind TrkB receptor to phosphorylate AkT/GSK3β signaling pathway to induce further differentiation in SHSY5Y cells (Masia S., et al., 2007). NGF binds to TrkA receptor activating the PI3K/AkT/GSK3β signaling cascade, to promote neuronal differentiation of PC12 cells. Tideglusib inhibition of GSK3β is thought to lead to pGSK3β promoting MT assembly (Greene, L. A., & Tischlert, A. S,1976). Surprisingly our study saw that Tideglusib not only increases phosphorylation of AKT and GSk3β, but phosphorylated Tau as well. Phosphorylation of Tau is thought to contribute to Tau hypothesis of ND pathology (Morales-Garcia J.A., et al.,2012). Indicating that up regulated promotion of inhibition of GSK3β may not be ideal to treating ND.

Phosphorylation of Akt and GSK3β are not significantly affected by Tideglusib in RA-Differentiated SHSY5Y cells

To further understand Gβγ signaling involvement with PI3K/AKT/GSK3β, Tideglusib was used to treat RA differentiated cells. Tideglusib is known to increase phosphorylation of GSK3β. Dysregulation of GSK3β is attributed to tau hyperphosphorylation. In a recent study overexpression of GSK3β lead to increase in the phosphorylation of Tau and GSK3β modulated acetylcholinesterase expression (Cortés-Gómez, M. et al., 2020). As previously mentioned, RA differentiated SHSY5Y cells express cholinergic markers. Gallein had a decrease of expression in pGSK3β and no change in pAKT. Indicating may have a Gβγ direct effect on phosphorylation of GSK3β.

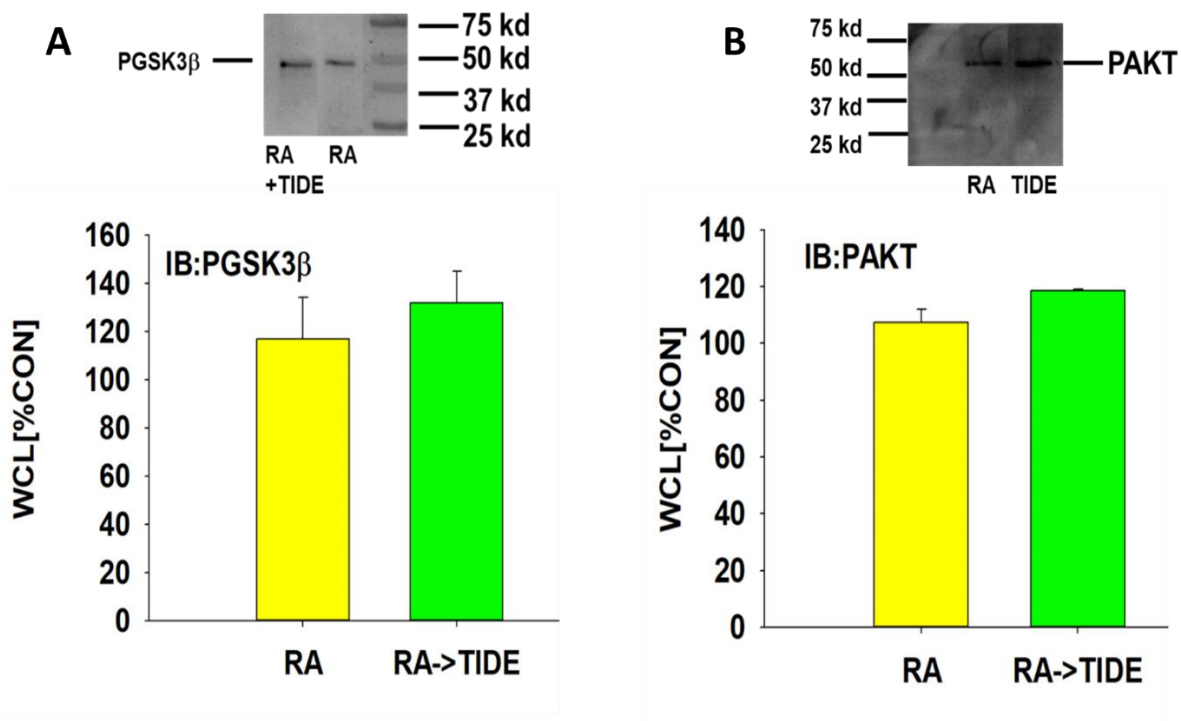

Figure 14. Effect of GSK3β inhibitor on the phosphorylation of AKT and GSK3β in RA differentiated SHSY5Y cells

Effect of TIDE on the expression of pAKT and pGSK3β in differentiated SHSY5Y. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with RA at 10 uM until differentiated, TIDE at 10uM, one hour after cells treated for differentiation occurred. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti-pAKT and anti-pGSK3β antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure above.

Inhibitor of GSk3β increases expression of Tau, ps396 Tau, and Gβγ in RA-differentiated SHSY5Y cells

Dysregulation of GSK3β is believed to lead to phosphorylation of Tau. Tideglusib increased expression Tau in SHSY5Y and differentiated SHSY5Y. Indicating that inhibition of GSK3β leads to an increase of expression of TAU. This finding in my study resonates previous studies indicating Tideglusib positive effect on Tau protein expression. In preclinical studies Tideglusib had a neuroprotective effect in animal models. Tideglusib failed in phase II human clinical trial. GSK3β inhibition in differentiated SHSY5Y cells lead to an increase in the phosphorylation of Tau at serine 396 site. Hyperphosphorylation of Tau at serine 396 site is thought to attribute to AD pathology by inducing tau aggregations, leading to NFT. In our current study inhibition of GSK3β lead to an increase in tau phosphorylation in RA differentiated SHSY5Y cells. As previously mentioned, RA is thought to induce differentiation by binding to TrkB receptor. Binding to receptor leads to cascading signal activation of PI3K/Akt/GSK3β pathway. Tideglusib is known to inhibit phosphorylation of TAU in mice studies. Few studies were done on human neuronal cells with Tideglusib. Tideglusib increased Gβγ expression in SHSY5Y differentiated cells. Simiallerly our lab showed that NGF differentiated PC12 cells increased expression of Gβγ (Sierra-Fonseca,2014). NGF is thought to cause neuronal differentiation by PI3K/AKT/GSK3β pathway by binding to TRkA receptor (Greene, L. A., & Tischlert, A. S. 1976) Our laboratory showed the PC12 cells overexpressing Gβγ lead to neuronal differentiation and increased expression of pGSK3β (Varela J, MS thesis, UTEP 2016). Inhibition or promotion of Gβγ could regulate GSK3β. Gallein a Gβγ inhibitor decreased phosphorylation of Tau in both SHSY5Y and differentiated SHSY5Y. Gallein decreased Tau expression. It is possible that Gβγ is needed to

regulate GSK3β neuroprotective role as well as MT assembly and organization. (Sereno et al., 2009; Morales-Garcia et al., 2012, Wang et al., 2016).

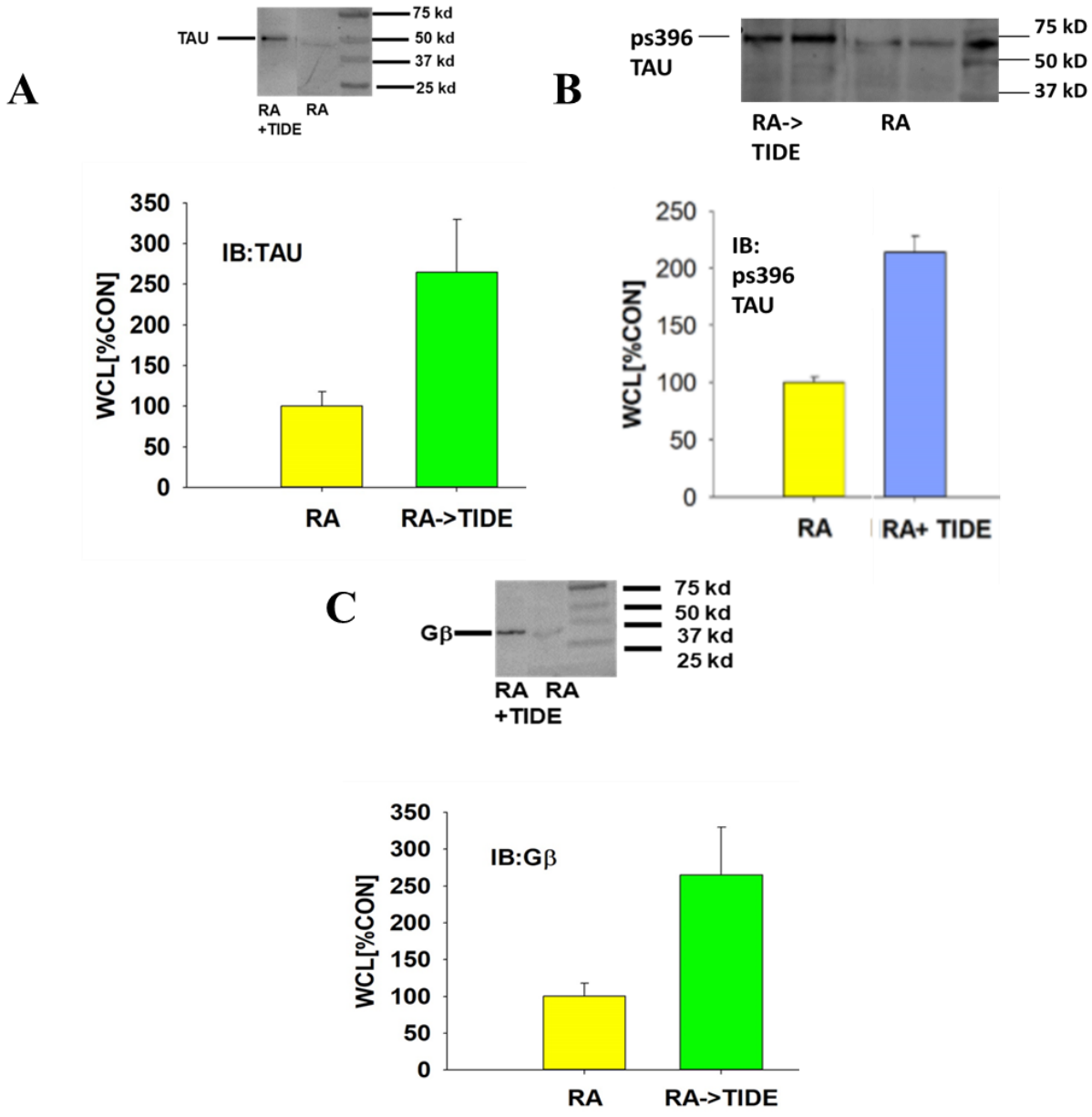

Figure 15. Tideglusib in retinoic acid differentiated SHSY5Y leads to an increase of expression of Tau, ps396 Tau, and Gβγ.

Tau expression decreased in SHSY5Y cells and in differentiated SHSY5Y treated with Tideglusib. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with RA at

10 uM until differentiated, TIDE at 10uM, one hour after cells treated for differentiation occurred. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti-Tau, anti-ps396Tau, and anti-Gβ antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure above.

Gallein does not induce neuronal cell death in SHSY5Y cells

To determine if Gallein induced neuronal cell death at the concentrations used, cytotoxicity assay was performed. This assay uses two fluorescent dyes —Hoechst, which is membrane-permeable and stains the nuclei of both living and dead cells, and propidium iodide (PI), which is only able to enter cells with compromised plasma membranes, thus selectively staining the nuclei of dead cells.

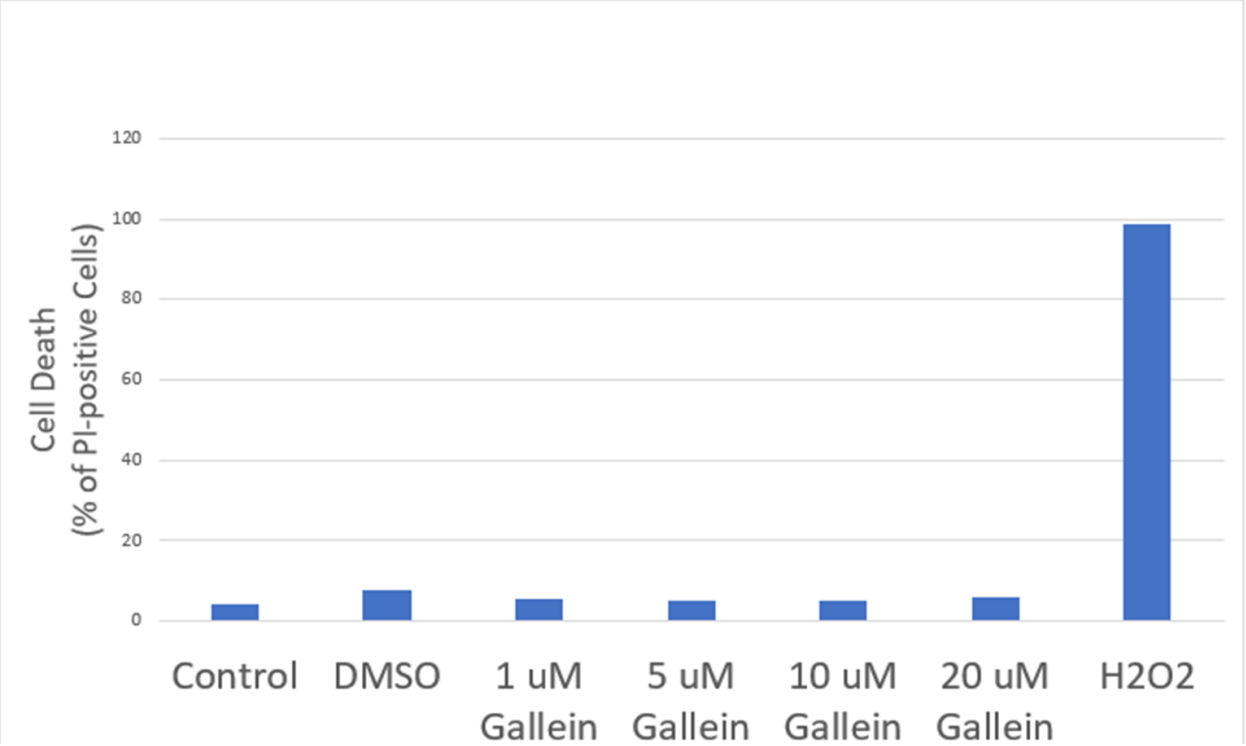

Figure 16. Gallein Dose not induce cell death.

Cytotoxicity was performed using the BD AttoVision v1.6.2 software (BD Biosciences), and each experimental condition was assessed with 8 replicates.. SHSY5Y were seeded on a 96-well plate at a density of 10,000 cells per well and placed in 5% CO2 water jacket incubator at 37 degree Fahrenheit. Cells where then treated with dyes Hoschet (penetrates cell membrane in viable cells) and Pi (penetrates dead cells membrane) for 1 hour along with Gallein.

Chapter 4 Discussion:

Our laboratory has previously shown that Gβγ promotes MT assembly and induces neurite outgrowth in NGF-differentiated PC12 cell. The interaction between Gβγ and TUB was increased in the presence of NGF. Blocking the interaction by Grk2i, a Gβγ inhibitory peptide disrupted MTs and inhibited neurite outgrowth. Furthermore, overexpression of Gβγ prompted neurite outgrowth in the absence of NGF. These results clearly suggested a role of Gβγ in neurodegeneration. In the current study, Gallein, a small molecule inhibitor of Gβγ, was found to inhibit neurite outgrowth, and disrupt MTs both in SHSY5Y and RA-differentiated SHSY5Y cells. The results from microscopic analysis (Fig. 5) indicate that as the concentration of GAL increases colocalization of TUB/Gβγ decreases, with Gβγ gradually moving towards the neurite ends and in many cells Gβγ is solely found in the ends of the neurites at the growth cone. At 10 μM GAL the neurites start to cut off, some neurons have completely lost their process, and many cells were only stained in blue (DAPI) suggesting that MT structure is completely lost. Since Gβγ is known to binds to MTs and stabilize MTs and neurites, the result indicates that Gβγ association with MTs and its localization are disrupted by increasing concentration of GAL leading to its release from the cell body as well as from neurites allowing disruption of MTs. thinning of neurites and eventually loss of neurites, and cellular aggregation. Similar results were observed in RA-differentiated SHSY5Y cells (Fig.6) where GAL disrupted MT assembly, induced loss of neurites and cellular aggregation.

Recently, GAL has been shown to inhibit amyloid-β (Aβ)-induced dendritic and axonal dystrophy, abnormal tau phosphorylation, synaptic loss, and neuronal cell death in hippocampal neurons, and reversed the memory-impairment in mice (Bigante et. al 2018). This study appears

to contract our results which indicates that GAL induces MT disruption and inhibits neuronal differentiation. However, it is likely both data sets are correct, and caution should be taken to interpret the results. In our study we used 1h incubation with GAL while 30 min incubation with GAL was used in earlier studies. Previously, we also used 30 min incubation with GAL, and found that GAL stimulated neurite outgrowth slightly (Sierra-Fonseca PhD dissertation, UTEP, 2013). Because we demonstrated earlier (Sierra-Fonseca 2014) that Gβγ (which is the target for GAL) stimulated MT assembly and induced neurite outgrowth, we increased time for incubation with GAL from 30 min to 1h to determine if cells exposed with GAL for a longer period of time is able to disrupt MT network and neurite outgrowth. As expected, we found that neurite outgrowth as well as MT assembly was disrupted by 1h incubation with GAL (Figs.5 and 6). Thus, it likely that when cells were exposed minimally with GAL, it was able to influence multiple effector systems (which includes adenylyl cyclase, phospholipases, ion channels, and PI3K) regulated by Gbg, and positively influence neuronal cell differentiation. However, the result presented here clearly indicates that GAL has the ability to directly modulate MTs and neurite formation, and should be taken into account when examining the effect of GAL.

In this study, we found TIDE (Tideglusib) , an inhibitor of GSK3β also disrupted MTs and inhibited neurite formation similar to that observed with GAL. The relationship between Gβγ and GSK3β has been shown earlier. Overexpression of Gβγ has been shown to phosphorylate GSK3β (Jose Varela, Thesis UTEP 2016). Confocal analysis indicate that the nucleus of SHSY5Y cells treated with a GSK3β inhibitor appeared swollen. Recently, Tideglusib was in clinical trial against AD, and the trial was failed. Tideglusib was selected mainly based on studies done on mice. Our results clearly indicate the damaging effect of Tideglusib on MTs and neurite formation in cultured neuronal cells, and could be the reason why it was not successful in clinical trial. It should be noted

that before Tideglusib was chosen for clinical trial, hardly any studies utilizing confocal analysis were done to see how TIDE affected the morphology of human neuronal cells.

Another study administered GAL to see the effects of inhibiting Gβγ in dopaminergic neurons. Abnormal levels of dopamine are thought to contribute to several neurological disorders including PD. The study found Gβγ-Dopamine transporter interaction was affected by GAL ((Li, J., et al.,2020). Our findings along with the studies mentioned further elucidates Gβγ involvement in MT assembly and organization and neurodegeneration.

Effect of GAL and TIDE on phosphorylation of TAU

In our study, when SHSY5Y cells were treated with GAL, expression of both TAU and phosphorylated TAU were decreased. The result was not anticipated based on the fact that GAL disrupted MTs in SHSY5Y cells. Because neurodegeneration is usually associated with cytoskeletal disruption and Tau hyperphosphorylation, the result is intriguing, and suggests that cytoskeletal disruption could also be associated with hypo-phosphorylation of Tau protein instead of hyper-phosphorylation. In addition to GAL, TIDE was found to inhibit phosphorylation of TAU. It might be the reason why TIDE was in clinical trial for Alzheimer disease.

Interestingly, however, It was found that TIDE increased Tau expression and phosphorylation in RA-differentiated SHSY5Y cells as opposed to SHSY5Y cells (Fig.15). Binding of RA triggers downstream activation of PI3K/AKT signaling (Kaplan DR, et al.,1993; Edsio A, et al. 2003). PI3K is a major pathway that promotes neuronal survival (Wu,C.,et al., 2019). Overexpression of GSK3β in SHSY5Y was used to induce phosphorylation of TAU and was successfully done in a previous study (Cortés-Gómez, M., et al., 2020). In comparison previous results from our laboratory have shown that overexpression of Gβγ induced neuronal

differentiation in PC12 cells in the absence of NGF. Further understanding of how these pathways interact with each other may lead to a target for ND treatment.

Understanding Gβγ signaling pathway interaction with PI3K/AKT/GSK3β

The relationship amongst Gβ-MT and PI3K/AKT/GSK3β remains to be understood. Previous results from our laboratory indicate both pAKT and pGSK3β expression is increased in the presence of NGF differentiated PC12 cells. In the presence of GAL, NGF differentiated PC12 cells decreased expression of both pAKT and pGSK3β. Overexpression of Gβγ in PC12 cells increased expression of pGSK3β, suggesting Gβγ directly interacts with GSK3β promoting phosphorylation. In our current study GAL significantly decreased expression of pGSK3β in SHSY5Y and increased expression in RA-differentiated SHSY5Y. Similarly, TIDE also decreased pGSK3β expression in SHSY5Y cells and increased expression in RA-differentiated SHSY5Y cells. This event may explain why TIDE a GSK3β inhibitor increased expression of pGSK3β in SHSY5Y cells.

Microscopic analysis of combined low and high concentrations of Gβγ and GSK3β inhibitors show that SHSY5Y cells seem to loss neuronal properties Colocalization of TUB/Gβγ is seen throughout the cells. Synergistic use of inhibitors seems to have canceling effects on neuronal differentiation. More studies need to be done to conclude. Furthermore, a study focusing whether GSK3α and GSK3β are activated during dopaminergic neuronal loss, found that GSK3β is activated during dopaminergic cell death (Li, J., et al.,2020). As previously mentioned, another study concluded that inhibition of Gβγ blocked Gβγ-DAT interactions (Garcia-Olivares, J., et al.,2017). GAL decreased extracellular dopamine.

Our study along with the studies mentioned above suggest that manipulation of Gβγ-MT and PI3K/AKT/GSK3β pathway may lead to an understanding of how neurodegeneration occurs and may lead to a drug model to treat ND.

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Curriculum Vita

Diana Isuiss Olivas was born in El Paso TX. After graduating with her bachelor's degree from The University of Texas at El Paso, she joined the master's program at The University of Texas at El Paso where she worked as a teaching assistant for the course of General Biology and Anatomy and physiology one. This thesis was typed by Diana Isuiss Olivas.