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# G $\beta\gamma$ Dependent Modulation of Cytoskeleton and Neurite Outgrowth Involves the PI3K/AKT/GSK-3 $\beta$ Pathway

Jose L. Varela

University of Texas at El Paso, [jlvarela@miners.utep.edu](mailto:jlvarela@miners.utep.edu)

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Gβγ DEPENDENT MODULATION OF CYTOSKELETON AND NEURITE OUTGROWTH  
INVOLVES THE PI3K/AKT/ GSK-3β PATHWAY

JOSE LUIS VARELA

Master's Program in Biological Sciences

APPROVED:

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Sukla Roychowdhury, Ph.D., Chair

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Siddhartha Das, Ph.D.

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Laura Diaz-Martinez, Ph.D.

---

Mahesh Narayan, Ph.D.

---

Charles Ambler, Ph.D.  
Dean of the Graduate School

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Jose Luis Varela

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Gβγ DEPENDENT MODULATION OF CYTOSKELETON AND NEURITE OUTGROWTH  
INVOLVES PI3K/AKT/ GSK-3β PATHWAY

by

JOSE LUIS VARELA, B.S.

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## Abstract

Assembly and disassembly of microtubules is critical for axon and dendrite formation and neurite outgrowth. Previous studies have shown that  $\beta\gamma$  subunits of heterotrimeric G proteins promote microtubule (MT) assembly *in vitro* and in cultured PC12 cells. Recently, it was found that the interaction of  $G\beta\gamma$  with MTs is important for nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells and that the blocking of the interaction between  $G\beta\gamma$  and tubulin/MT disrupted MTs, inhibited neurite outgrowth and induced axonal damage indicating the involvement of  $G\beta\gamma$  in these processes. A different pathway involving the receptor tyrosine kinase (TrkA) and its downstream effector Phosphatidylinositol-3-kinase (PI3K) has been shown to modulate neuronal differentiation. PI3K downstream effectors AKT and GSK-3 $\beta$ , have been shown to participate in the regulation of neurite outgrowth, and are particularly associated with microtubule remodeling. The current investigation is based on our hypothesis that  $G\beta\gamma$  and PI3Kinase pathway co-ordinate to regulate neurite outgrowth by modulating MT assembly. Overexpression of  $G\beta\gamma$ , and inhibitors of  $G\beta\gamma$  (Gallein) and PI3K (LY294002) were used to identify the role of  $G\beta\gamma$ /PI3K signaling in regulating neuronal differentiation and morphology. Neuronal differentiation of PC12 cells induced by NGF or  $G\beta\gamma$  overexpression promoted the activation of PI3k/AKT/GSK-3 $\beta$  pathway supporting the idea that  $G\beta\gamma$  and PI3K pathway works in concert. Both gallein and LY294002 inhibited the phosphorylation of AKT. However, stimulation of phosphorylation of GSK-3 $\beta$  was only partially inhibited by LY294002 while gallein was



ineffective in reversing the effect of  $G\beta\gamma$  overexpression. Both gallein and LY294002 disrupted neurite formation and altered cellular morphology. Our result suggests that  $G\beta\gamma$ /PI3K pathway is involved in neuronal differentiation by regulating assembly and organization of microtubules. Because neurodegenerative and neuropsychiatric diseases are associated with alterations in neuronal cytoskeleton, these results will increase our understanding of the molecular basis of neurological diseases and strategies to develop novel drugs to treat them.

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## **Chapter 1: Introduction**

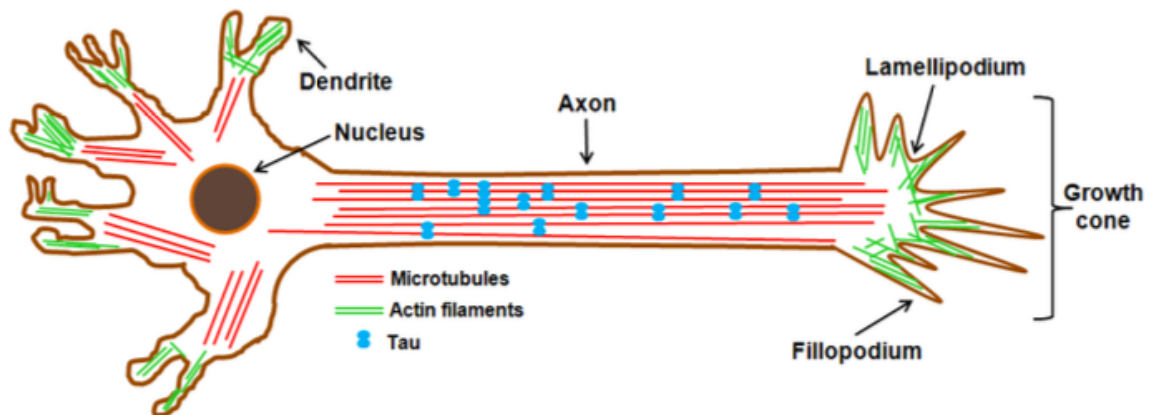
### **1.1 Neuronal cytoskeleton and microtubule dynamics**

Neurons are the brain cells responsible for receiving, storing 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 a long and thin axon that serves to transmit signals to adjacent neurons. The morphology achieved after full axon and dendrite elongation allow the neurons to achieve precise connectivity with other specific adjacent neurons.

The assembly and disassembly of cytoskeletal structures embodied within neurite extension and growth cone formation are responsible for the intricate connections between neurons. The neuronal cytoskeleton is composed of microtubules (MTs), actin filaments (AFs) and intermediate filaments (IFs). MTs are formed by the GTP-dependent polymerization of alpha and beta tubulin dimers [Murphy and Borisy, 1975; Kowalski and Williams, 1993]. MTs form dense parallel arrays in axons and dendrites that are required for the growth and maintenance of such neurites. Unlike MTs, actin filaments in neurons are enriched in growth cones and organized into long bundles [Witte and Bradke, 2008; Geraldo and Gordon – Weeks, 2009]. This arrangement provides structural support and stabilization, making the interaction between these two crucial cytoskeletal filaments for the growth and maintenance of these neurites (Fig.1). With this morphology it is

possible to create intricate signaling networks among neurons fundamental for the proper functioning of the nervous system.

During neuronal differentiation, the organization of MTs needs to be remodeled rapidly, and MT stability changes depending on their cellular localization. In the axon, MTs are bundled by tau, a microtubule-associated protein (MAP), with their plus end oriented toward the nerve terminal. MAP2, a group of high molecular weight MAPs, participates in MT bundling in the dendrites. However, the precise signaling pathways that lead to this unique organization of the microtubules are not clearly understood (Li and Gundersen, 2008).



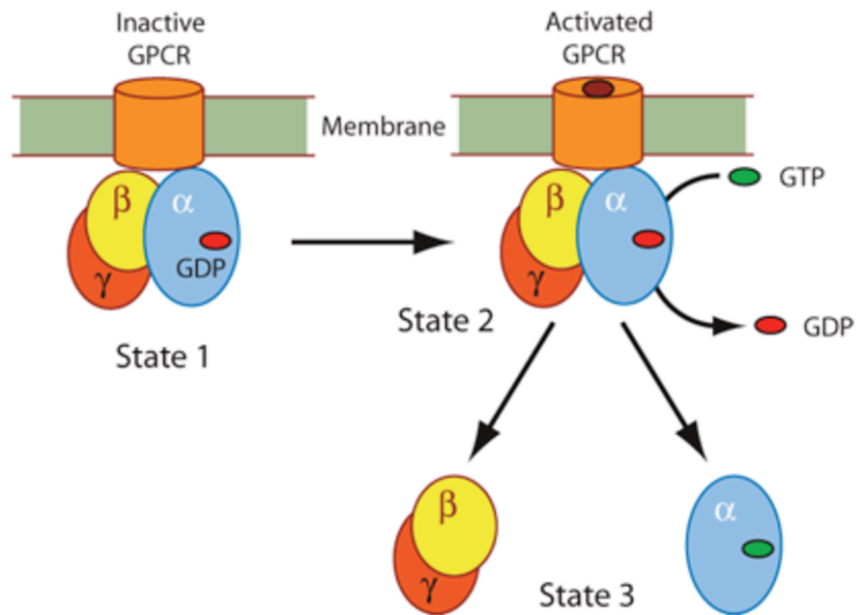
**[Figure 1. Neuronal cytoskeleton.** Microtubules (red) are shown arranged in parallel along the axon, and dendrites. MAP Tau (blue) is found along the length of the axon with MTs where these two interact. Actin filaments (green) found in the growth cone and dendrites form specialized structures used for neurite differentiation.] (Figure adapted from Serra-Fonseca, 2014, with permission)

## 1.2. G protein-mediated signaling

Heterotrimeric G proteins function as signal transducers in transmembrane signaling pathways that consist of three elements: G protein-coupled receptors (GPCRs), G proteins, and effectors. GPCRs are the largest family of transmembrane receptors and regulate multiple physiological functions such as neurotransmission, immune system function, cell growth and proliferation, and hormonal signaling. Because of the complex signaling pathways in which GPCRs are involved, these have been found to be involved in many diseases and have been the target for about 40% of modern drugs in the market (Filmore, 2004). GPCR family of proteins is highly diverse in nature and encoded by almost 1000 genes in human. However, all consist of seven transmembrane domains with three extracellular and three intracellular loops, with the extracellular domain responsible for ligand binding, and the intracellular domain responsible for G-protein binding (Latek et al., 2012). Current models of G proteins favor a heterotrimeric structure composed of the guanine-nucleotide binding  $\alpha$ , plus  $\beta$  and  $\gamma$  subunits, the latter two forming a tight association under nondenaturing conditions. Heterotrimers are inactive in GPCR signaling and do not interact with the receptors functionally. Upon activation of the GPCR by the agonist binding, GTP binds to the  $\alpha$  subunit of the heterotrimer, thus changing the association between the  $G\alpha$ , and  $G\beta\gamma$  subunits in a way (either by subunit dissociation or by changing the mode of association) that allows both subunits to initiate complex intracellular signaling pathways (Figure 2). Termination of the signal occurs when the GTP bound to the  $G\alpha$  subunit is hydrolyzed by the GTPase activity, causing the functional dissociation from the effector and reassociation with

the G $\beta\gamma$  subunit (Gilman, 1987; Dohlman et al., 1991; Neves et al., 2002; McCudden et al., 2005). In humans, there are 21 isoforms of G $\alpha$  subunits, 6 G $\beta$  isoforms, and 12 isoforms of G $\gamma$  [31]. Typical effectors of G $\alpha$  signaling include adenylyl cyclase, phospholipase C, phospholipase A<sub>2</sub>, ion channels, and several kinases and transcription factors. Among the effector molecules interacting with G $\beta\gamma$  are phospholipases, K<sup>+</sup> and Ca<sup>2+</sup> 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, there is a great deal of flexibility in the system and G protein  $\alpha$  and  $\beta\gamma$  subunits are capable of activating diverse downstream effectors.





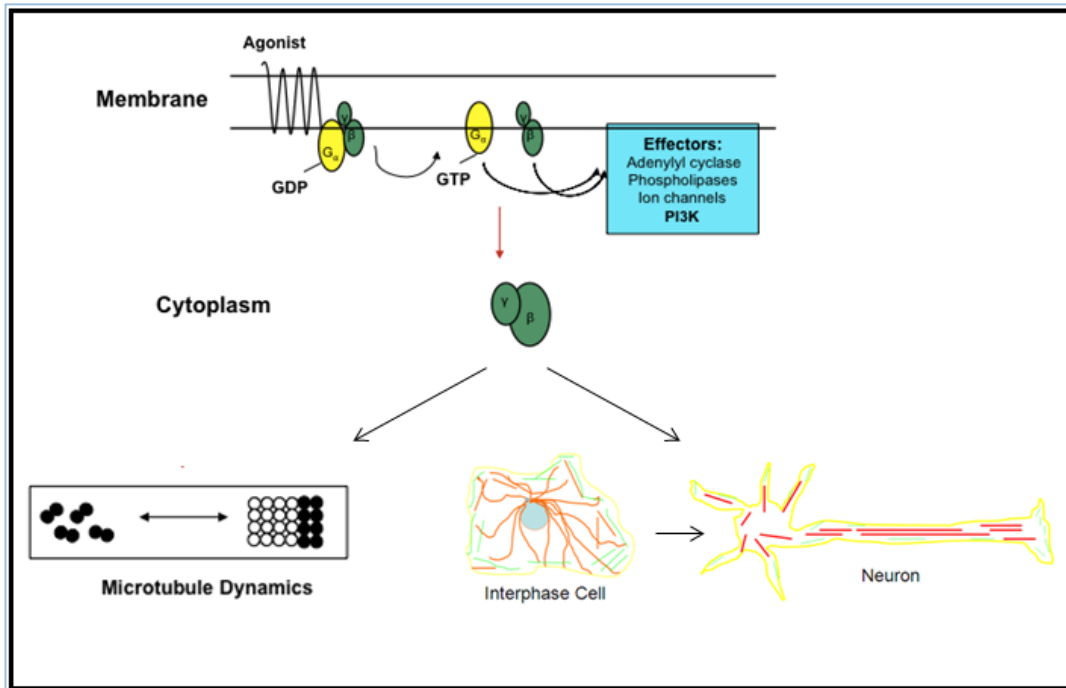
**[Figure 2. G-protein activation cycle.** State 1: G-protein is in an inactive state where the G-protein subunits ( $G\alpha$  and  $G\beta\gamma$ ) keep its heterotrimeric structure. State 2: An agonist binds to the GPCR receptor activating the G-protein,  $G\alpha$  and  $G\beta\gamma$  subunits disassociate through a GDP/GTP exchange mechanism. State 3: Association of  $G\alpha$  and GTP causes the heterotrimer to disassociate; both  $G\alpha$  and  $G\beta\gamma$  subunits activate a variety of effector molecules.] (Adapted from German Research School for simulation Sciences, grs-sim.de.)

### **1.3 Interaction of g proteins with microtubules**

Although the role of heterotrimeric G proteins in transducing signals from extracellular receptors to intracellular effector molecules, is well established, association of G protein subunits ( $G\alpha$  and  $G\beta\gamma$ ) with subcellular compartments, such as MTs have been reported both in interphase and mitotic cells by many investigators (Cote et al., 1997; Williard and Crouch, 2000; Wu et al., 2001; Sarma et al., 2003). In addition, G protein subunits ( $\alpha$  and  $\beta\gamma$ ) have been shown to bind to tubulin and modulate MT assembly/dynamics (Wang et al., 1990; Roychowdhury and Rasenick, 1997; Roychowdhury et al., 1999; Roychowdhury et al., 2006; Montoya et al 2007). Using the MT-depolymerizing drug nocodazole, it has been demonstrated that  $G\beta\gamma$ -MT interaction is important for MT assembly in cultured NIH3T3 and PC12 cells (Montoya et. al 2007).  $G\alpha$  inhibits MT assembly and increases MT disassembly by increasing the GTPase activity of tubulin (Roychowdhury et.al 1999); on the other hand  $G\beta\gamma$  promotes tubulin polymerization (Roychowdhury and Rasenick, 1997). It was noticed that reconstituted heterotrimers would not show signs of coupling with MTs nor taking part in modulating MT assembly, coming to the conclusion that G protein activation was necessary for both  $G\alpha$  and  $G\beta\gamma$  to interact with MTs and modulate MT assembly (Figure 3) [Roychowdhury et. al 2006].

#### **1.4. G $\beta$ $\gamma$ -microtubule interactions and neuronal differentiation**

The involvement of G $\beta$  $\gamma$  in neuronal development and differentiation has been reported previously (Sanada and Tsai 2005; Sachdev et. al 2007). G $\beta$ 1-deficient mice have been shown to have neural tube defects (Okabe and Iwakura 2010) and G $\beta$ 5-knockout mice have been shown to display abnormal behavior and develop multiple brain abnormalities (Zhang et. al 2011). Recent results from our laboratory indicate that, G $\beta$  $\gamma$ -MT interaction is critical for the formation of neuronal-like projections in rat pheochromocytoma (PC12) cells after addition of nerve growth factor (NGF) (Sierra-Fonseca et. al 2014). NGF-induced neurite outgrowth in PC12 cells is a well-established model to study neurite formation in vitro (Green and Tishler, 1976), and therefore used in this study (Sierra-Fonseca et. al 2014). It was found that NGF promoted the interaction of G $\beta$  $\gamma$  with microtubules and stimulated MT assembly. Overexpression of G $\beta$  $\gamma$  induced neurite outgrowth in the absence of NGF, further suggesting the importance of G $\beta$  $\gamma$  in neuronal differentiation. Furthermore, overexpressed G $\beta$  $\gamma$  exhibited similar pattern of association with MTs as observed in NGF-differentiated PC12 cells. GRK2i, a G $\beta$  $\gamma$ -inhibitory peptide, inhibited neurite formation, disrupted MTs and induced neurite damage organization, indicating an important role of G $\beta$  $\gamma$  in this process (Sierra-Fonseca et. al 2014). The result suggests that the interaction of G $\beta$  $\gamma$  with tubulin/MTs and its ability to stimulate MT assembly may provide a mechanism by which G $\beta$  $\gamma$  regulates neuronal differentiation.

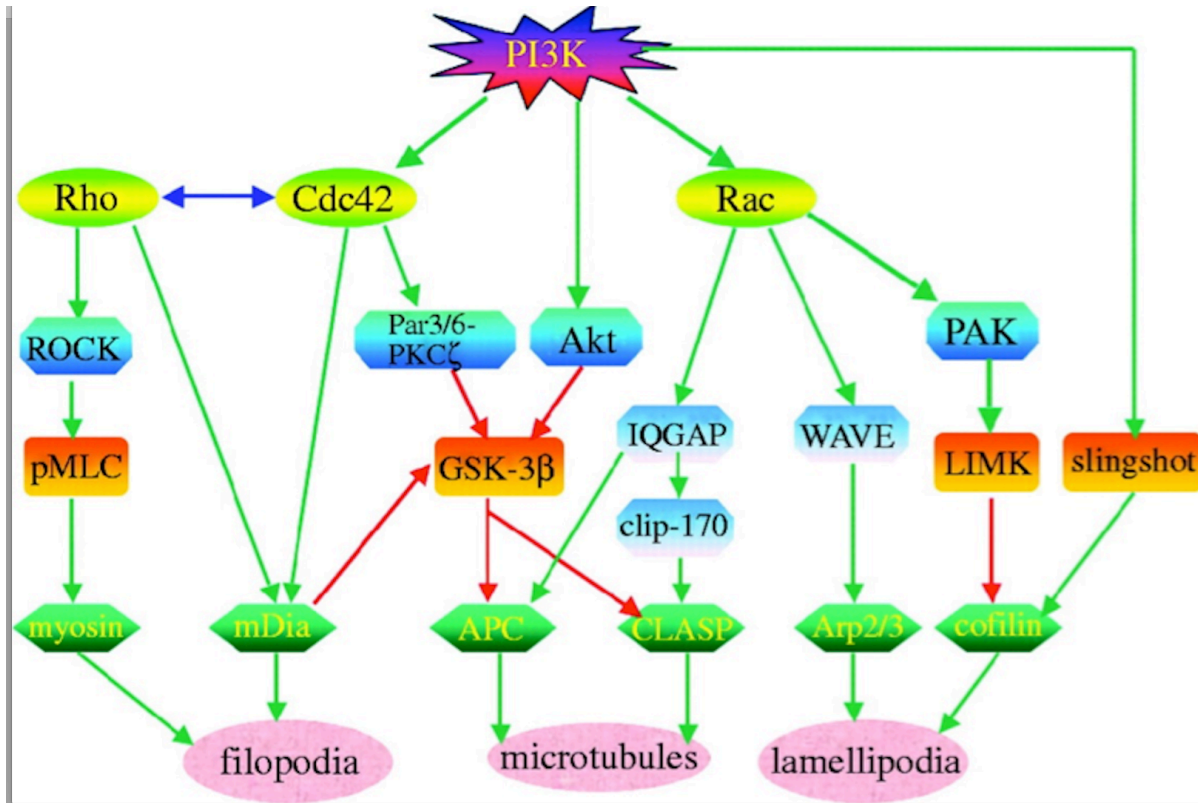


**[Figure 3. Gβγ – MT dependent neurite outgrowth.** Activation of GPCR triggers the disassociation of the  $G\alpha\beta\gamma$  heterotrimer into  $G\alpha$  and  $G\beta\gamma$  subunits. These subunits play a role in activation of several signaling pathways such as adenylyl cyclase, phospholipases, and PI3-Kinases. It has been previously found from our laboratory that  $G\beta\gamma$  subunit interacts with  $\alpha\beta$  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] (Figure adapted with permission)

### 1.5 The phosphatidyl inositol-3-kinase (PI3K) signaling pathway

Although results from our laboratory indicate an important role of  $G\beta\gamma$  in NGF-induced neuronal differentiation in PC12 cells (sierra-Fonseca et.al 2014), NGF is known to induce neurite outgrowth in PC12 cells by activating the receptor tyrosine kinase, TrkA [Knight et al., 2006]. TrkA is a receptor for NGF that directly acts upon PI3K triggering its activation, being part of the downstream signaling of TrkA pathway, PI3K appears to take part in regulating the assembly of MTs/actin filaments via further downstream signaling [Cantley, 2002; Zhou et al., 2004]. 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 3 $\beta$  (GSK-3 $\beta$ ) (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-3 $\beta$ . Unlike AKT or many other kinases, GSK-3 $\beta$  is usually active in resting cells. Upon activation of the PI3K signaling pathway, GSK-3 $\beta$  is inactivated through phosphorylation of the serine 9 residue in its amino terminal region (Doble and Woodgett 2003). It has been shown that the inactivation of GSK-3 $\beta$  downstream of PI3K controls axon morphogenesis as well as MT polymerization via the regulation of multiple MT binding proteins. PI3K is also a downstream effector of  $G\beta\gamma$  in GPCR signaling (Stephens et al., 1997), and recent results suggest that the activation of PI3K/AKT pathway by NGF is, in part, mediated through the  $G\beta\gamma$  subunit (Wu and Wong, 2005a and 2005b; Wang and Wong, 2009).

As discussed above, results from our laboratory indicates that  $G\beta\gamma$  directly interacts with tubulin/MTs, stimulates MT assembly, and induces neurite outgrowth, Therefore, it is likely that  $G\beta\gamma$  and PI3k/AKT/ GSK-3 $\beta$  pathways coordinates to regulate neurite outgrowth and morphogenesis, and my research focuses on understanding this process.



**[Figure 4. PI3K/AKT/PI3K $\beta$  Signaling pathway].** Schematic representation of the PI3K/AKT/PI3K $\beta$  signaling pathway. The PI3K signaling pathway involves many signaling events leading in different actions within a cell. Activation of PI3K causes the phosphorylation of phosphatidylinositol 3,4-bisphosphate (PIP<sub>2</sub>) to phosphatidylinositol 3,4-triphosphate (PIP<sub>3</sub>) and activation of its downstream effector AKT. Activation of AKT promotes the inactivation of the pGSK-3 $\beta$  enzyme leading to cell growth and proliferation [Matsouka and Yashiro, 2014]. (Figure adapted with permission)

## **1.6 The inhibitors of PI3K and Gβγ signaling pathways**

Several small molecules inhibitors/promoters have been used earlier to study the function of Gβγ signaling as well as PI3k pathways. Gallein has been found to bind to the Gβγ subunit with high affinity to block Gβγ dependent cellular activities, including the Gβγ-dependent activation of the PI3K-γ isoform [Lehmann et al., 2008]. A morpholine-containing chemical compound LY294002 has been found to be very effective in inhibiting PI3K [Maira et al., 2009]. It has shown remarkable growth inhibition as well as apoptosis-inducing effects in colon cancer cell lines, by decreasing the expression of phosphorylated AKT. These compounds and chemicals have sparked great interest in our research being that they have shown to alter the two pathways discussed. By altering the activity of either one or both at a time differences in the MT assembly/organization and neurite outgrowth can be studied. We have used these tools, a better understand the mechanism of Gβγ /PI3K dependent regulation of MT assembly/neurite outgrowth,



## **1.7 Microtubules and neurodegeneration**

Neurodegeneration is a pathological condition that leads to the progressive loss of neuronal structure and function due to neuronal cell death (Jellinger, 2009). This pathology occurs in many neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Frontotemporal dementia (FTD), and prion diseases among others. Each disease affects and damages neurons in its own specific way, but the ultimately the latter outcome is the same, progressive neural cell death. The presence of abnormal protein aggregates in neuronal cells is general for most if not all neurodegenerative diseases; these proteins can be related to cytoskeletal proteins such as tubulin and MAPs. (Goedert et al., 2001; Al-Chalabi and Miller, 2003; Skovronsky et al., 2006; Jellinger, 2010).

The pathology of the most common neurodegenerative disease Alzheimer's disease is composed of extra cellular deposits of Ab peptides called Amyloid plaques and neurofibrillary tangles. In normal neurons tau participates in MT stabilization by binding to MT's, in AD Ta becomes hyperphosphorylated not being able to bind to MTs forming aggregates that are deposited in the cells. MTs get disrupted and eventual axonal damage occurs (Drechsel et al., 1992; Ittner and Gotz, 2010).

Lewy bodies composed of a-synuclein aggregated proteins, tubulin, MAP1 and MAP2 proteins, are major markers for Parkinson's disease. (Alonso et al., 2008; Amniai et al., 2008; Gustav et al., 2010). The area affected by PD is the Substantia Nigra Pars Compacta located in the midbrain and responsible for regulating the role of the striatum on fine motor skills (Pioli Et. Al., 2008). Along with a-synuclein

induced neurotoxicity, disruptions in the microtubule cytoskeleton may also play an important role on the progression of this disease (Cardoso et al., 2012).

Other neurodegenerative diseases induce similar cytoskeletal alterations by affecting specific signaling and mechanistic pathways in neuronal cells, but the cause of most disorders is still unknown, there also are no effective drugs to treat these neurodegenerative disorders. With the recent discovery that MT assembly is severely compromised in AD and PD patients, it could be possible to find an approach to treat these diseases by the regulation of MT assembly.

## 1.8 Hypothesis and specific aims

The goal of this study is to relate the  $G\beta\gamma$ /PI3K signaling pathway with the MT cytoskeleton and neurite outgrowth. Both the tyrosine kinase A and the G protein-coupled receptor pathways have shown to regulate the MT assembly and neurite outgrowth (Sierra-Fonseca et. al. 2014). Also, NGF-induced activation of the PI3K/AKT/GSK-3 $\beta$  pathway is thought to be mediated in part by the  $G\beta\gamma$  subunit given that PI3K is a downstream effector in GPCR. The goal of this project is to determine whether these two signaling pathways interact with one another to regulate microtubule assembly and neurite outgrowth. We have hypothesized that:  *$G\beta\gamma$ -Microtubule dependent regulation of neuronal differentiation involves the PI3K/AKT/GSK-3 $\beta$  pathway.* We plan to test our hypothesis by focusing on the following aims in this study.

### Specific Aim 1

Do the  $G\beta\gamma$ -Microtubule dependent pathway and the PI3K/AKT/GSK-3 $\beta$  pathway coordinate to regulate NGF-induced neuronal differentiation?

### Specific Aim 2

Does the  $G\beta\gamma$  subunit modulate the PI3K/AKT/GSK-3 $\beta$  pathway to regulate microtubule assembly/organization and neuronal differentiation?

## **Chapter 2: Materials and methods**

### **2.1 Cell culture and NGF treatment**

*Cell culture and NGF treatment:* PC12 cells (pheochromocytoma cells derived from the adrenal gland of *Rattus norvegicus*) were grown in T-25 and T-75 culture flasks at 37 °C in Dubelcco's modified Eagle's Medium (DMEM), supplemented with 10% bovine calf serum, and antibiotics (100 U/mL Penicillin and 100 µg/mL streptomycin) in 5% CO<sub>2</sub>. For NGF treatment PC12 cells were grown on 6-well plates (500,000 to 800,000 cells per well) to 85% confluence over 1 day, then treated with 100 ng/ml of nerve growth factor (NGF) dissolved in complete media for 2 consecutive days. Control cells were grown without NGF for the same length of time.

### **2.2 Gallein and PI3K Inhibitors**

The small molecule gallein was obtained from Tocris Bioscience; additionally LY294002 was obtained from Sigma-Aldrich. Stock solutions of the two inhibitors were prepared in DMSO (20mM gallein, LY294002 100mM); inhibitors were diluted in complete media (gallein 10mM, LY294002 100mM) the same fashion as NGF. Control experiments were conducted in the presence of only NGF. Treatment lengths were of 30 minutes for gallein, and 1 hour for LY294002 exactly prior to cell extraction.

### **2.3 Overexpression of $G\beta_1$ and $G\gamma_2$**

PC12 cells were transiently co-transfected with yellow fluorescent protein (YFP)-tagged and mCherry-tagged pcDNA3.1 plasmids encoding for the  $G\beta_1$  and  $G\gamma_2$  subunits respectively. Dr. N Gautam (Washington University, St. Louis, MO) generously provided the expression plasmids via the Addgene online plasmid repository. The plasmids were transfected separately ( $G\beta_1$  and  $G\gamma_2$ ) or co-transfected to generate the  $G\beta_1\gamma_2$  combination, a plasmid encoding only YFP pcDNA3.1-YFP, Addgene, Cambridge, MA) was used as a control. Cells were transfected with the plasmids using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PC12 cells were seeded on glass coverslips using 12-well plates at a density of 30,000 cells/well and incubated overnight for under normal growth conditions for confocal microscopy analysis, a density of 500,000 cells were seeded on 6-well plates under the same conditions for whole cell lysate extraction and immunoblotting analysis. The following day the cells were transfected with a mixture of the Lipofectamine 3000 containing 1.5  $\mu$ g of the desired plasmid (3.0 mg total plasmid for co-transfection) and incubated overnight in normal growth media. Cells were monitored for YFP (YFP fluorescence) and mCherry (mCherry fluorescence) expression as well as morphological changes using differential interference contrast (DIC) images at different time points (24, 48, and 72h), with a Zeiss Axiovert 200 fluorescence microscope equipped with GFP and dsRED filters.

## **2.4 Preparation of whole cell lysates:**

After treatments of PC12 cells with NGF and inhibitors, or transient transfection and inhibitors, culture media was removed, washed with PBS, and lysed by incubation of 55 ml lysis buffer (10mM Tris-HCL, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 0.3M sucrose, 0.1% Triton X-100, 1mM DTT, 10 mM GTP) supplemented with protease inhibitor cocktail for 10 minutes over ice. Cells were scraped and sonicated for 1 minute (3 sonications of 20 seconds over ice to prevent heating of the samples), centrifuged at top speed for 10 minutes to finally analyze protein concentration by the Bradford assay. Protein was leveled to optimal levels and loading dye was added as required for SDS-PAGE.

## **2.5 Electrophoresis and immunoblotting:**

Samples for immunoblotting were subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose membranes [Leammli 1970; Towbin et al., 1970]. Membranes were then blocked with 5% nonfat dry milk in TBS (10mM Tris-HCl and 150 mM NaCl, pH 7.4) for 1 hour at room temperature, followed by overnight incubation at 4 C with mouse monoclonal anti- $\alpha$ -tubulin (DMIA, Sigma Aldrich, 1:500), Rabbit polyclonal anti G $\beta$  (Santa Cruz Biotechnology, 1:500), rabbit monoclonal anti-AKT and anti-pAKT (Cell signaling Technology, 1:100), rabbit monoclonal anti GSK-3 $\beta$  and anti-pGSK-3 $\beta$  (Cell Signaling Technology, 1:100) antibodies in TBS. Membranes were washed three times with 0.05% Tween-20 in TBS (TBST) and incubated with the appropriate HRP-conjugated secondary antibodies (goat anti-mouse or goat anti-

rabbit from Promega, Madison WI; 1:5000) in TBS for one hour. Chemiluminescence (ECL) technique (SuperSignal West Pico Chemiluminescence Substrate) was used according to the manufacturers instructions (Pierce Biotechnology, Rockford, IL). Quantitative analysis of the protein bands was performed with the LabWorks image acquisition and analysis software (UVP Laboratory Products, Upland, CA).

## **2.6 Immunofluorescence and confocal microscopy**

For confocal microscopic analysis, cells were allowed to attach to glass coverslips using 12-well plates, and were grown and treated as described above. Media was then removed and cells were fixed using 100% methanol previously cooled to -20 C incubated for 6 minutes at this same temperature (Montoya et al., 2007), washed three times in PBS, blocked for 1 hour at room temperature in 5% normal goat serum (NGS) (Sigma-Aldrich) in PBS. Primary antibody incubation followed for 1 hour at room temperature (anti- $\alpha$ -tubulin and anti G $\beta$ , 1:100) in 1% NGS in PBS (detailed description of these antibodies provided above).

Cells were washed in PBS before continuing with secondary antibody incubation (Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 568-conjugated donkey anti-mouse IgG; Molecular Probes-Invitrogen, Carlsbad, CA) for 2 hours at room temperature and in the dark to diminish photo-bleaching effects. Coverslips were then mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen, for nuclear staining), and covered with a glass slide. Transiently transfected cells were fixed, washed and mounted, omitting the NGS blocking or the antibody incubation. High-resolution, digital, fluorescent images were captured by employing inverted,

confocal laser-scanning microscopy (LSM 700; Zeiss, Thornwood, NY), utilizing a Plan-Apochromat 40x/1.40 immersion-oil DIC objective and assisted with 2009 ZEN software (Zeiss, Thornwood, NY). DAPI (blue), Alexa Fluor 488 (green), and Alexa Fluor 568 (red) were excited with laser emissions of 405-, 488-, and 555-nm wavelengths, respectively; as well as YFP (green) and mCherry (red) were excited with laser emissions of 488-, and 55-nm wavelengths respectively for transiently transfected cells.

## **2.7 Differential nuclear staining (DNS) assay for cytotoxicity**

To determine the levels of cytotoxicity caused by the experimental compounds (gallein, LY294002), a previously described NDS assay adapted for high-throughput screening was used (Lerma et. Al, 2011). This assay uses two fluorescent nucleic acid intercalators, Hoechst 33342 (Hoechst) and propidium iodide (PI). Briefly cells were seeded in a 96-well plate format incubated with NGF and inhibitors. One hour before image capturing, a staining mixture of Hoechst and PI at a final concentration of 1  $\mu$ g/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 (3x3) from four adjacent image fields were captured per well in order to acquire an adequate number of cells for statistical analysis, utilizing a 10x objective. To determine the percentage of dead cells from each individual well, both image acquisition and data analysis were performed using the BD AttoVision v 1.6.2 software (BD Biosciences), and each experimental condition was assessed in triplicate.



## **2.8 Statistical analysis**

All statistical analysis was performed using Sigma Plot 11 software (Systat Software Chicago, IL, USA). In case of Western-blot quantitative analysis, the differences between controls and treatments were assessed by means of the Student's paired t-test. For comparisons between two groups, the Student's paired t-test was employed, and in all cases, a value of  $P < 0.05$  was considered to be statistically significant.

## **Chapter 3: Results**

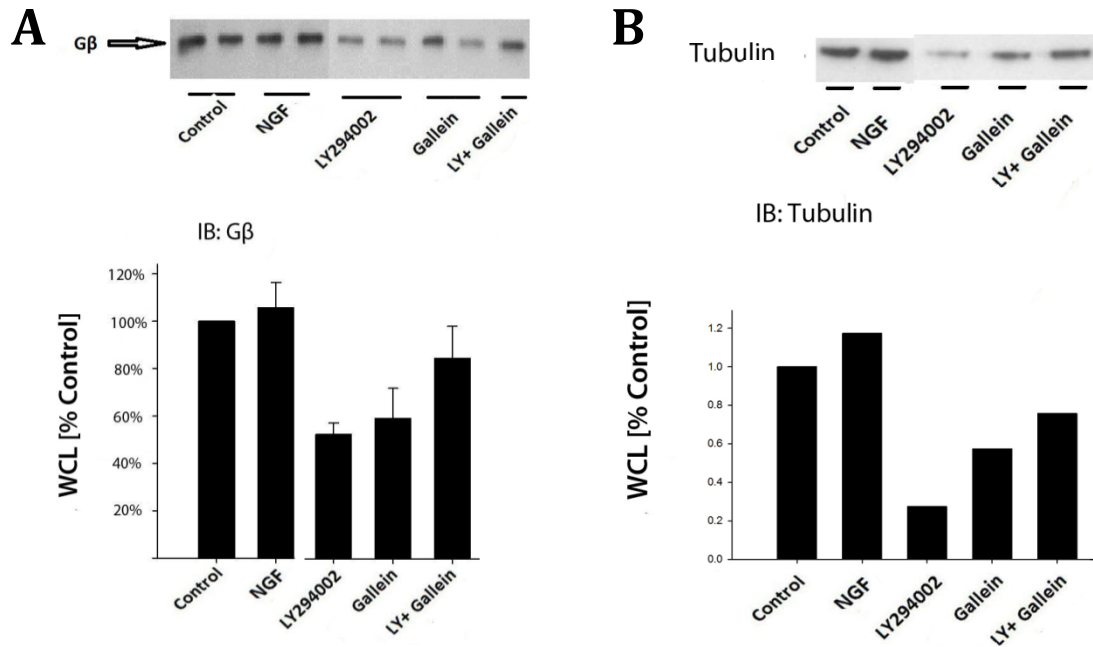
### **3.1 Specific Aim 1: Do the $G\beta\gamma$ -Microtubule dependent pathway, and the PI3K/AKT/GSK-3 $\beta$ pathway coordinate to regulate NGF-induced neuronal differentiation?**

#### **3.1.1 Objective and overview**

The goal of this aim is to identify the relationship between  $G\beta\gamma$ -MT pathway and the PI3K/AKT/GSK-3 $\beta$  signaling pathway in NGF-induced differentiation of PC12 cells. These two pathways appear to play key roles in the neurite outgrowth and differentiation. However, the mechanism by which these two pathways regulate the neuronal differentiation has not been fully understood. NGF has been shown to act on the tyrosine kinase receptor TrkA that regulates cytoskeletal rearrangement in neurons involving the PI3K and its downstream signaling components AKT and GSK-3 $\beta$ . The results from our laboratory indicate that NGF also utilizes  $G\beta\gamma$  to induce neurite outgrowth of PC12 cells (Sierra-Fonseca 2014). PI3K being a downstream effector of  $G\beta\gamma$  in GPCR signaling suggest that the  $G\beta\gamma$  subunit in part mediates the activation of the TrkA pathway. Therefore it is hypothesized that  $G\beta\gamma$  in conjunction with PI3K coordinates signals from the TrkA and GPCR pathways to regulate MT assembly and neurite outgrowth. In this specific aim we used inhibitors of  $G\beta\gamma$  signaling (gallein) and PI3K signaling (LY294002) to understand the mechanism of neurite outgrowth using NGF differentiated PC12 cells.

### **3.1.2 Gallein, and LY294002 inhibit tubulin and G $\beta$ (G $\beta$ $\gamma$ ) expression in NGF-differentiated PC12 cells**

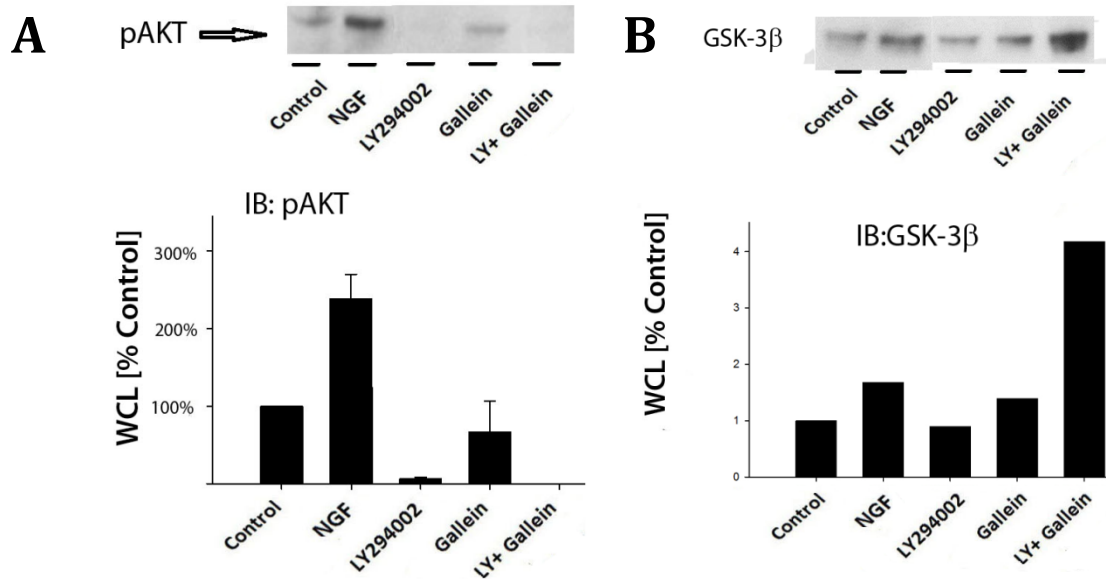
To determine the role of PI3K inhibitor LY294002 and gallein in Tubulin and G $\beta$  $\gamma$  expression, PC12 cells were allowed to differentiate in the presence of NGF (100ng/ml) for 3 days as indicated in the methods. Subsequently cells were treated with or without 10 $\mu$ M gallein and/or 100  $\mu$ M LY294002 as indicated in the figure (Fig.5). The treatments were followed by cell lysis and immunoblotting using anti-tubulin or anti-G $\beta$  antibodies as indicated in the figure (Fig.5). We found that NGF did not alter tubulin and G $\beta$  $\gamma$  expression alone, but tubulin and G $\beta$  expression was decreased in PC12 cells after treatment with 10 $\mu$ M gallein and/or 100 $\mu$ M LY294002 (Fig. 5) G $\beta$  expression was decreased by 52% and 45% when subjected to LY294002 and Gallein treatment respectively (Figure 5A). Tubulin expression decreased by 84% and 51% when subjected to LY294002 and Gallein treatment respectively (Figure 5B). Previous studies demonstrated that tubulin-G $\beta$  $\gamma$  interaction was increased significantly in NGF differentiated PC12 cells; an increased interaction could be responsible in part for the extended neurites in these cells (Sierra-Fonseca et. al 2014).



**[Figure 5. Effect of Gallein and LY294002 on  $G\beta$  and tubulin expression in NGF-differentiated PC-12 cells.** PC12 cells were treated with 100ng/ml NGF for two consecutive days to allow for neuronal differentiation. This was followed by treatments with gallein (10mM for 30 min) and /or LY294002 (100 mM for 1 h) as indicated in the figure. Cell lysates were prepared as described in the methods. Equal amounts of proteins from each sample were then to western blot analysis using anti- $G\beta$  (A, upper panel) or anti-tubulin antibody (B, upper panel). Protein bands were detected using the ECL-plus reagent (upper panels) and quantitated (lower panels, A and B). As indicated in the figure, both LY294002 and Gallein inhibited the expression of  $G\beta$  and tubulin suggesting that both  $G\beta\gamma$  and PI3K inhibitors affected their expressions.

### **3.1.3 Gallein and Ly294002 inhibits NGF-induced stimulation of pAKT and pGSK-3 $\beta$ expression**

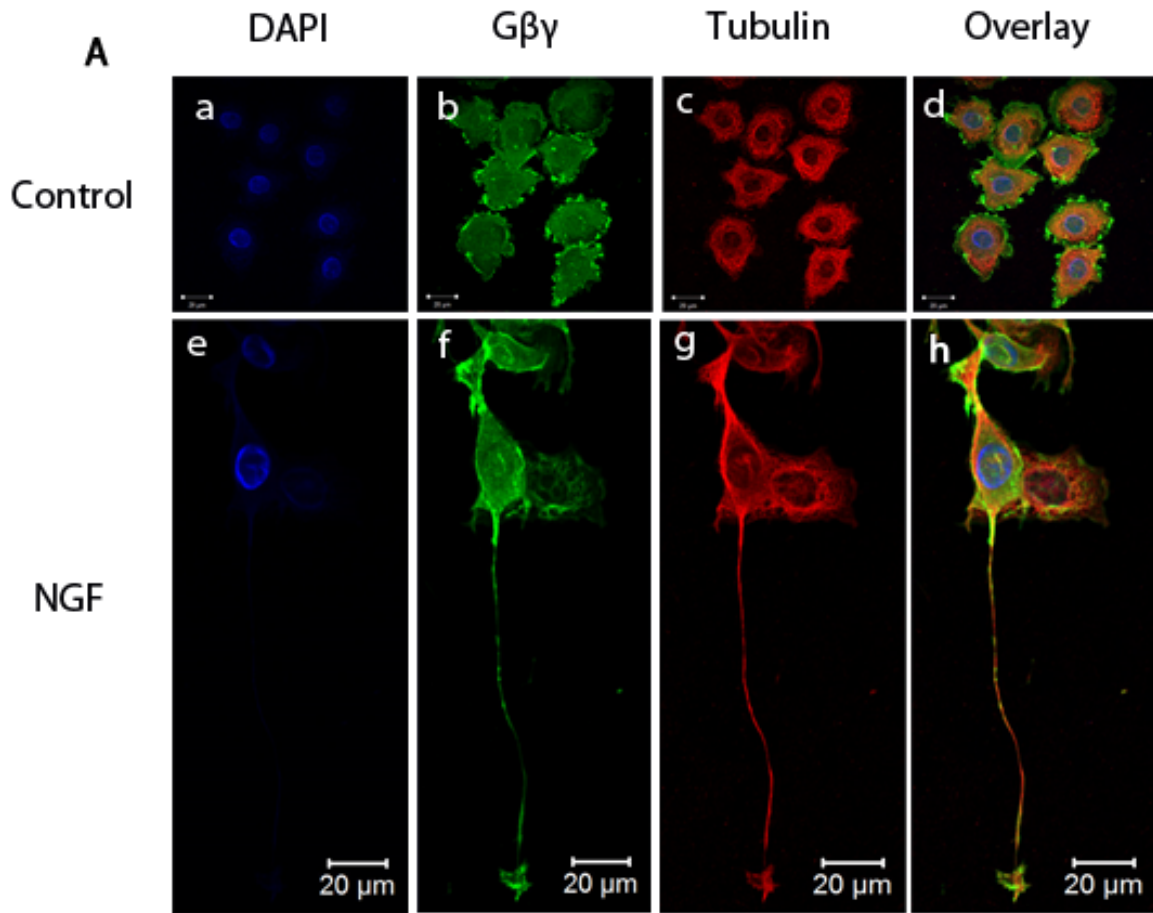
NGF-induced differentiation of PC12 cells increased the phosphorylation of AKT and GSK-3 $\beta$ , as demonstrated by the increased phosphorylation of AKT and GSK-3 $\beta$  (Fig.6). AKT phosphorylation was increased by 150% in the presence of NGF, while AKT phosphorylation was completely inhibited by LY294002. Gallein also inhibited the expression below the control level. NGF increased GSK-3 $\beta$  phosphorylation by 52% and LY294002 inhibited the phosphorylation to control level. Gallein also inhibited GSK-3 $\beta$  expression as indicated in the fig (Figure 6B). Stimulation of pGSK-3 $\beta$  is known to inactivate GSK-3 $\beta$  and this inactivation is necessary for NGF stimulation and axon formation.



**[Figure 6. Effect of Gallein and LY294002 on pAKT and GSK-3β expression in NGF-differentiated PC12 cells.** PC12 cells were treated with 100ng/ml NGF for two consecutive days to allow for neuronal differentiation. Cells were then treated with gallein at a concentration of 10mM for 30 min., and LY294002 at a concentration of 100 mM for 1 hr. After incubation cell lysate extraction was performed and subjected to immunoblotting. Protein bands were detected using the ECL-plus reagent and quantitated. A) WCLs were subjected to western blot analysis using anti-pAKT antibody as indicated in the methods to determine the phosphorylation of AKT in the presence of gallein or LY294002. B) WCLs were subjected to western blot analysis using anti- pGSK-3β antibody as indicated in the methods to determine the phosphorylation levels in the presence of gallein or LY294002.]

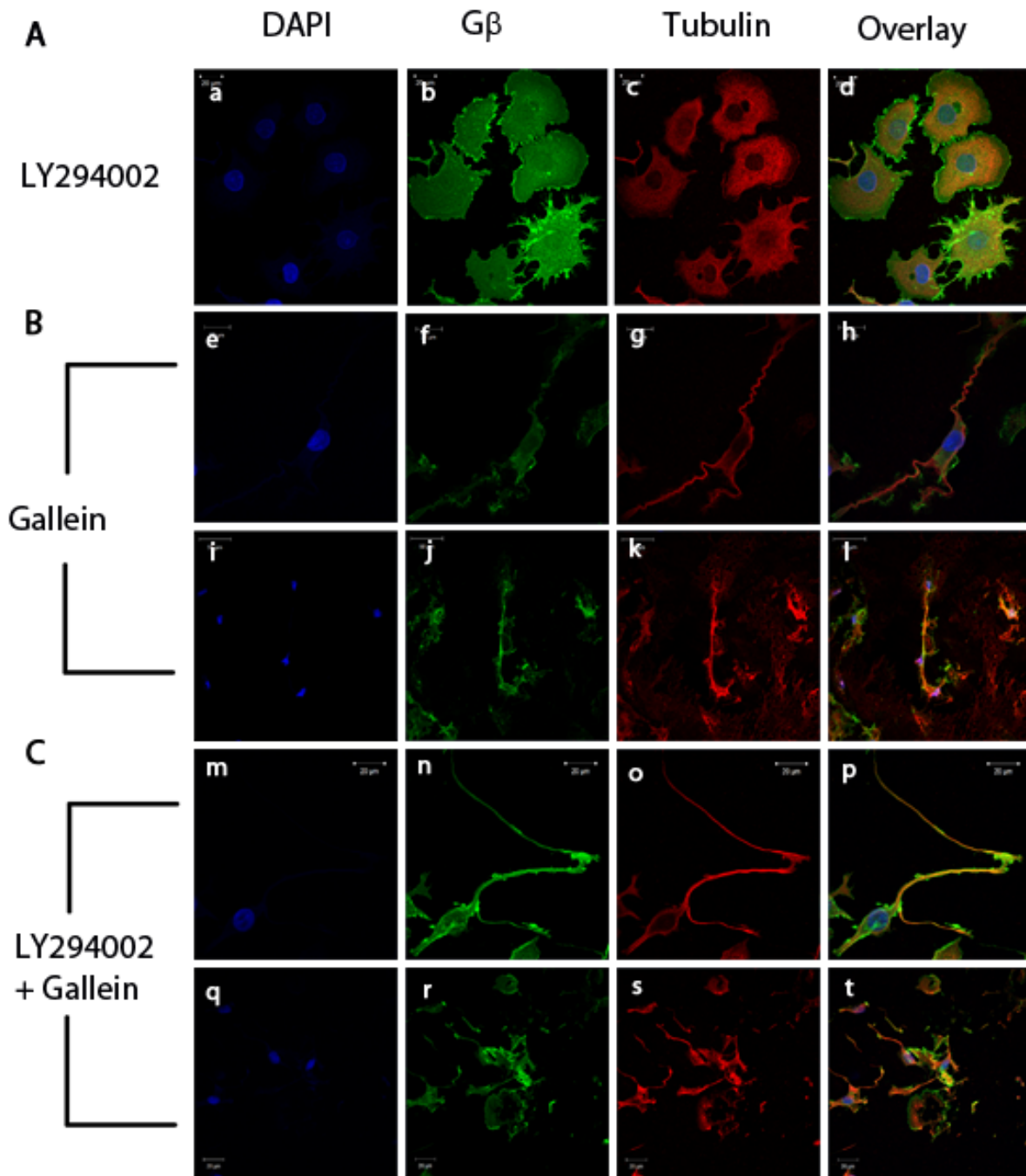
### **3.1.4 Confocal microscopic analysis of the effect of gallein and LY294002 on NGF-induced differentiation of PC12 cells**

The co-localization of G $\beta$  $\gamma$  with MTs in NGF-differentiated cells was also assessed by immunofluorescence microscopy. PC12 cells that were treated with or without NGF were examined for G $\beta$  and tubulin immunostaining by confocal microscopy. Tubulin was detected with a monoclonal anti-tubulin (primary antibody) followed by a secondary antibody (goat-anti-mouse) that was labeled with tetramethyl rhodamine (TMR). Similarly, G $\beta$  $\gamma$  was identified with rabbit polyclonal anti-G $\beta$  followed by FITC-conjugated secondary antibody (goat-anti-rabbit), and their cellular localizations and co-localizations were recorded by laser-scanning confocal microscopy. In control cells G $\beta$  $\gamma$  co-localized with MTs mainly in the perinuclear region (Figure 7A, a-d). After NGF treatment neurite outgrowth was detected in the majority of cells (Figure 7A, e-h). G $\beta$  $\gamma$  was detected in the neurites, cell bodies, as well as the tips of the axonal growth cones. Cell treated with PI3K inhibitor LY294002 inhibited neurite outgrowth and affected cellular morphology (Figure 8A, a-d). The effect of gallein is not completely clear, although it appears that the cell body is shrunk and the processes are not of normal morphology as indicated in Figure 8B, e-h. Join treatments of gallein and LY294002 showed very similar results when compared to gallein, for the most part the gallein morphological change was dominant in these cells. (Figure 8C, m-t)



**[Figure 7. NGF-induced neuronal differentiation in PC12 cells.** PC12 cells were treated with 100ng/ml NGF for three days to induce neuronal differentiation. Samples were then fixed and stained with mouse monoclonal anti-tubulin and rabbit polyclonal anti-G $\beta$  antibodies. Co-localization of G $\beta$  $\gamma$  (green) and tubulin (red) are shown by double immunofluorescence labeling. Blue represents DAPI for nuclear labeling. In NGF treated cells (e-h), neurite outgrowth was observed. Co-localization between MTs and G $\beta$  $\gamma$  (yellow) was observed mainly in the neuronal processes. G $\beta$  was detected with Alexa Fluor 488, and tubulin/MTs was detected with Alexa Fluor 568. Scale bars are 20  $\mu$ m.]

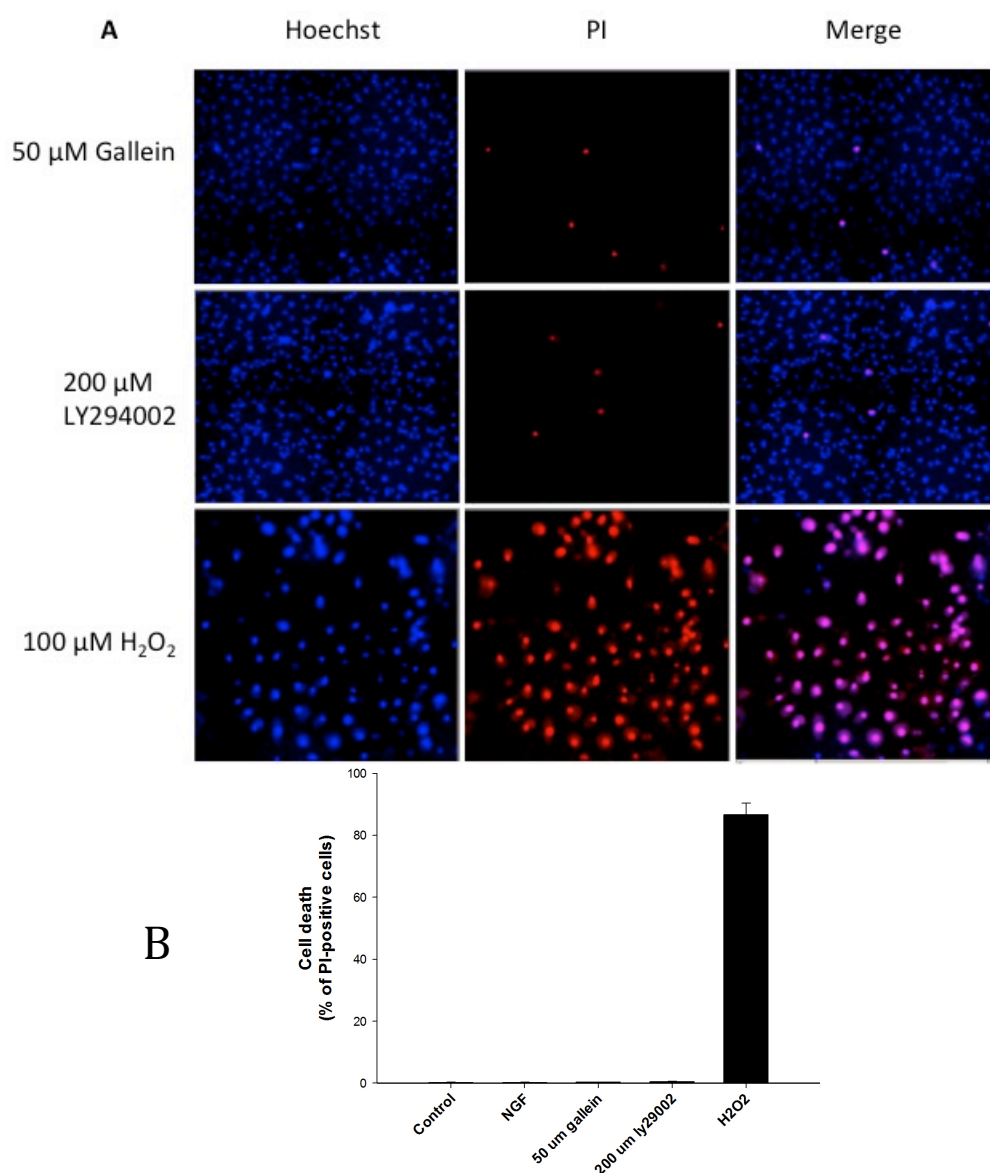




**[Figure 8. Gallein and LY294002 disrupt neurite outgrowth of NGF differentiated PC12 cells.** PC12 cells were induced to differentiate by treatment with NGF for one day, cell were then treated with or without gallein (40 mm for 30 min)(B and C), and LY294002 (100 mm for 1 h)(A and C). Cells were then fixed and processed for confocal microscopy. Gβγ was detected with Alexa Fluor 488, and tubulin/MTs was detected with Alexa Fluor 568. Scale bars are 20 mm.]

### **3.1.5 Inhibitors of PI3K and G $\beta$ $\gamma$ -dependent activation do not cause cell death in PC12 cells**

To determine if the PI3K inhibitor LY294002 or the G $\beta$  $\gamma$ -dependent activation inhibitor gallein induced PC12 cell death at the concentrations used, a previously described differential nuclear staining (DNS) assay (Lema et al., 2011) was performed as described in section 2.7. Briefly, PC12 cells were grown on 96-well plates and incubated with NGF and inhibitors as indicated, followed by 1hr incubation with a mixture of Hoechst/propidium iodide (PI). Hoechst has the ability to cross cell membranes of both healthy and dead cells to stain nuclear DNA, labeling the total number of cells, PI is only able to stain the cells whose plasma-membrane has been compromised, thus showing only the number of dead cells. Subsequently, cells were imaged in live mode using a BD Pathway Bioimager system as described in the methods. Representative images corresponding to the same section of 3 x 3 image montages from several conditions are shown (a-i) (Figure 6A). The percentage of dead cells in the presence of inhibitors was determined by using the BD AttoVision v1.6.2 software (BD Biosciences), and the result was plotted as shown in Fig 6B. As indicated in the figure either gallein or LY294002 did not cause cytotoxicity on NGF-differentiated PC12 cells. Hydrogen peroxide (100  $\mu$ M) was used as a positive control. This means that what looked like cell death induced from gallein in figure 29C, q-t, was in fact due to the greatly altered morphology induced, and not cell death.



**[Figure 9. Inhibitors of PI3k and G $\beta$  $\gamma$  do not induce neuronal cell death.** PC12 cells were grown on 96-well plates and treated with NGF and inhibitors as indicated in methods. Afterwards, cells were incubated with Hoechst/PI mixture for DNS cytotoxicity assay. The images were captured using live-cell-image mode using the confocal automated microscope BD Pathway Bioimager system and a 10x objective, assisted by AttoVision Software. (A) Representative images corresponding to the same section of 3 x 3 image montages from several conditions are shown. H<sub>2</sub>O<sub>2</sub> (100

$\mu\text{M}$ ) was used as a positive control. Cell nuclei stained with Hoechst provided the total number of cells; cell nuclei stained with PI indicate the number of dead cells; merged Hoechst and PI images indicate the number of dead cells in the image. Cell death was plotted as the percent of PI-positive cells, denoting the total number of dead cells for each condition.

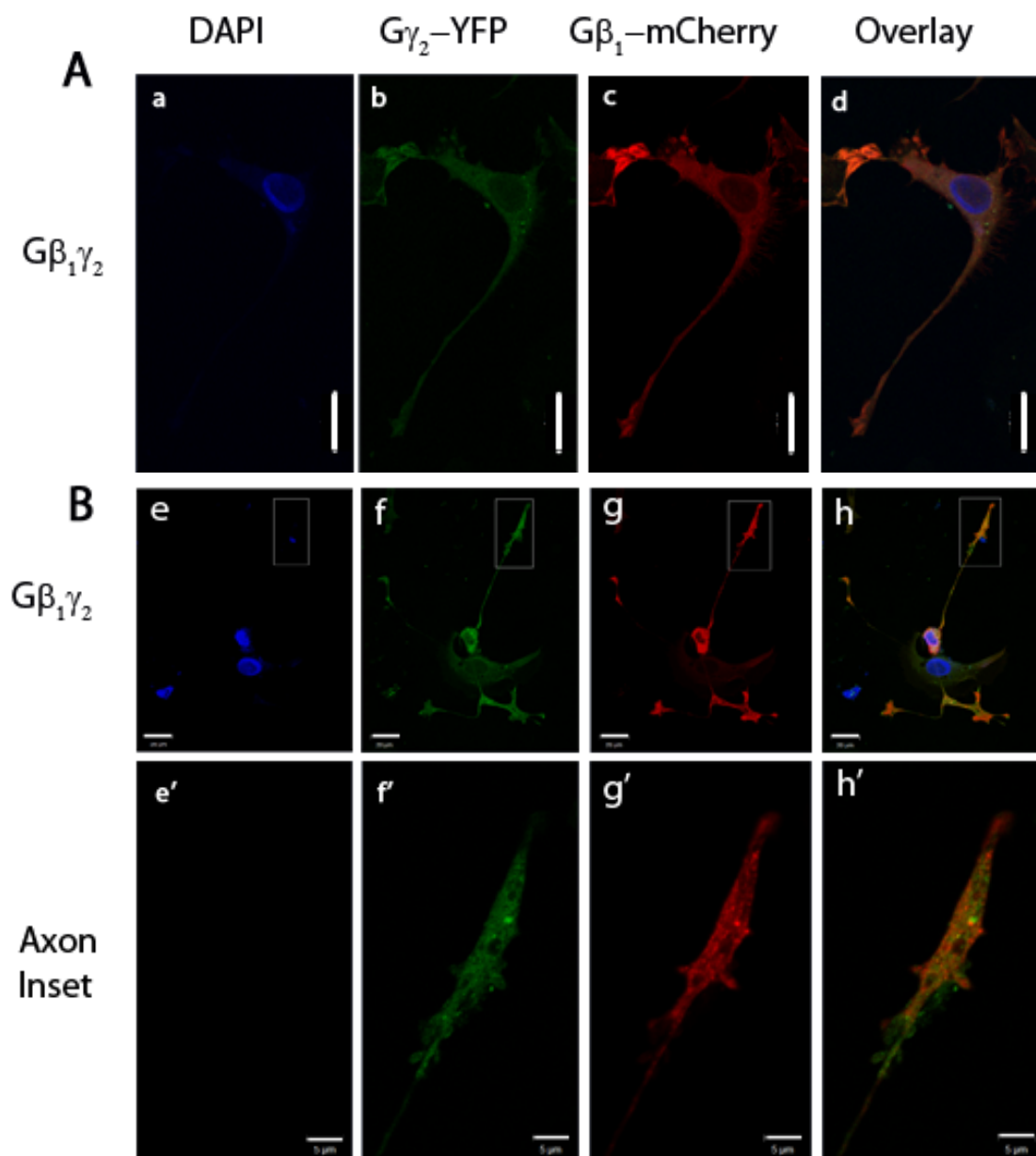
## **3.2 Specific aim 2: Does $G\beta\gamma$ subunit modulates the PI3K/AKT/GSK-3 $\beta$ pathway to regulate microtubule assembly/organization and neuronal differentiation?**

### **3.2.1 Objective and overview**

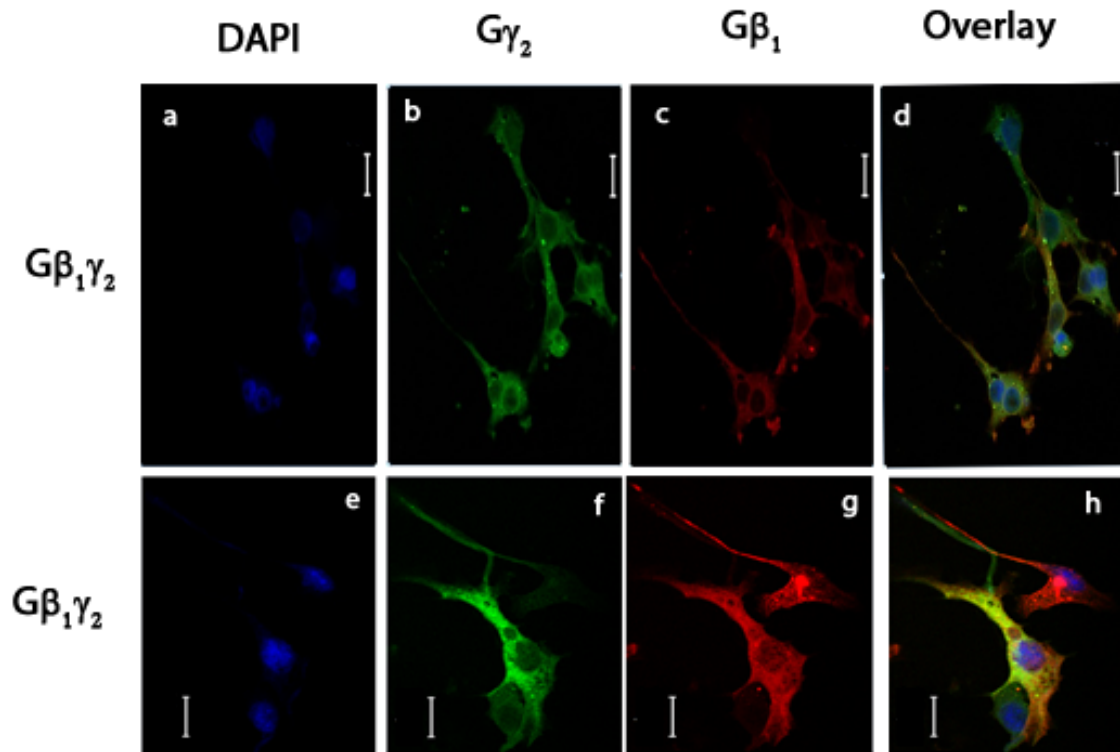
In order to better assess the role of  $G\beta\gamma$  during neurite outgrowth,  $G\beta\gamma$  was overexpressed in PC12. Earlier studies from our laboratory have shown that  $G\beta\gamma$  overexpression induces neurite outgrowth in PC12 cell in the absence of NGF (Sierra-Fonseca et. al 2014). In many instances it also induced morphological alterations in overexpressed cells along with neurite outgrowth, these included cell body bulging as well as a possible inhibited cytokinesis resulted in multinucleated cells. In that study YFP-tagged  $G\beta_1$  and  $G\gamma_2$  constructs were used. Therefore, it was not possible to determine the localization of overexpressed proteins ( $G\beta_1$  and  $G\gamma_2$ ) in cells. In the present study, PC12 cells were co-transfected with mCherry- $\beta_1$  (red) and YFP- $\gamma_2$  (green) to better understand the  $G\beta\gamma$ -induced neuronal differentiation. The goal of this aim is to determine if neuronal differentiation induced by overexpression of  $G\beta\gamma$  (in the absence of NGF) modulate PI3K/AKT/GSK-3 $\beta$  pathway.

### **3.2.2 Overexpression of $G\beta\gamma$ induces neuronal differentiation as well as morphological alterations in the absence of NGF in PC12 cells.**

Transfected cells were monitored for protein expression at 48 and 72 h, it was after 72 that neurite outgrowth was widely noted in the overexpressed cells. We found that overexpressed proteins localized in the neurite processes, growth cones, and cell bodies as shown by fluorescent labeling (Figure 10). We observed that  $\beta_1$  tends to localize in the neurites and the edges of the cells (Figure 10B, g and g'), while  $\gamma_2$  is localized mainly in the cytoplasm (Figure 10B, f and f'). Co-localization of  $\beta_1$  and  $\gamma_2$  was also observed (yellow) in cell bodies as well as neuronal processes. We found that many of the  $G\beta\gamma$ -overexpressed cells produced altered morphology with wider neurites and expanded growth cones as shown by  $G\beta_1$  (red) and  $G\gamma_2$  (green) labeling (Figure 10, e'-h'). The result suggests an important role of  $G\beta\gamma$  in neuronal development. Upon transfection of PC12 cells with  $G\beta_1$  and  $G\gamma_2$  not only neuronal differentiation was seen but also altered cell morphology was noted, two phenotypes were present in many of the cells. First many of the cells with confirmed fluorescence and expression for  $G\beta\gamma$  had a tendency to divide into equal halves at the tips of neurites (Figure 11, a-d). Secondly overexpressed cells were also seen to bear two nuclei, as well as overgrown neurites when compared to NGF treated cells (Figure 11, e-h). The effect of this phenomenon could be in part due to the role of excess  $G\beta\gamma$  in microtubule assembly in a disorganized fashion where a cell grows in unspecified directions failing to fully differentiate as well as altering the normal cell division cycle.



**[Figure 10. Overexpression of  $G\beta\gamma$  induced neurite outgrowth in PC12 cells.** PC12 cells were co-transfected with YFP- and mCherry-tagged constructs encoding  $G\gamma_2$  and  $G\beta_1$  ( $G\beta_1\gamma_2$ ), respectively, in the absence of NGF, using Lipofectamine 3000 reagent according to manufacturer instructions. Cells overexpressing fluorescent proteins were monitored at different points for protein expression and morphological changes using a fluorescence microscope.  $G\beta$ -mCherry was found in higher concentration at the growth cones of the neurites. Scale bars: 20  $\mu$ m in a-h, 5  $\mu$ m in e'-h'.

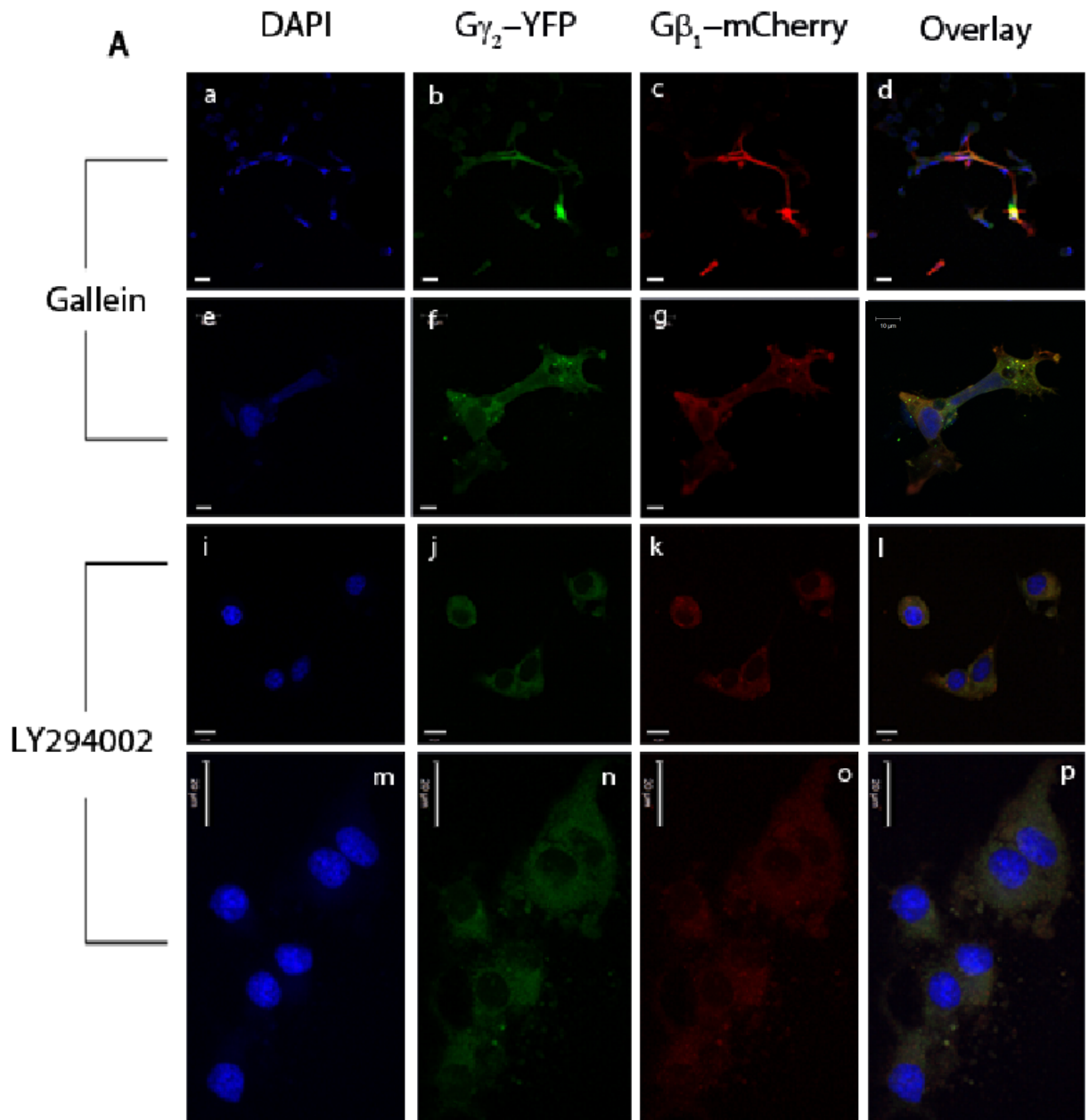


**[Figure 11. Overexpression of  $G\beta_1\gamma_2$  Induces morphological alterations and affects cytokinesis in PC12 cells.** PC12 cells were co-transfected with YFP- and mCherry-tagged constructs encoding  $G\gamma_2$  and  $G\beta_1$  ( $\beta_1\gamma_2$ ), respectively, in the absence of NGF, using Lipofectamine 3000 reagent according to manufacturer instructions. Protein expression and morphological changes were monitored at different time points. Co-transfected cells show a morphological change in the cell body where one cell body displays two nuclei. Scale bars are 20  $\mu$ m for all images.

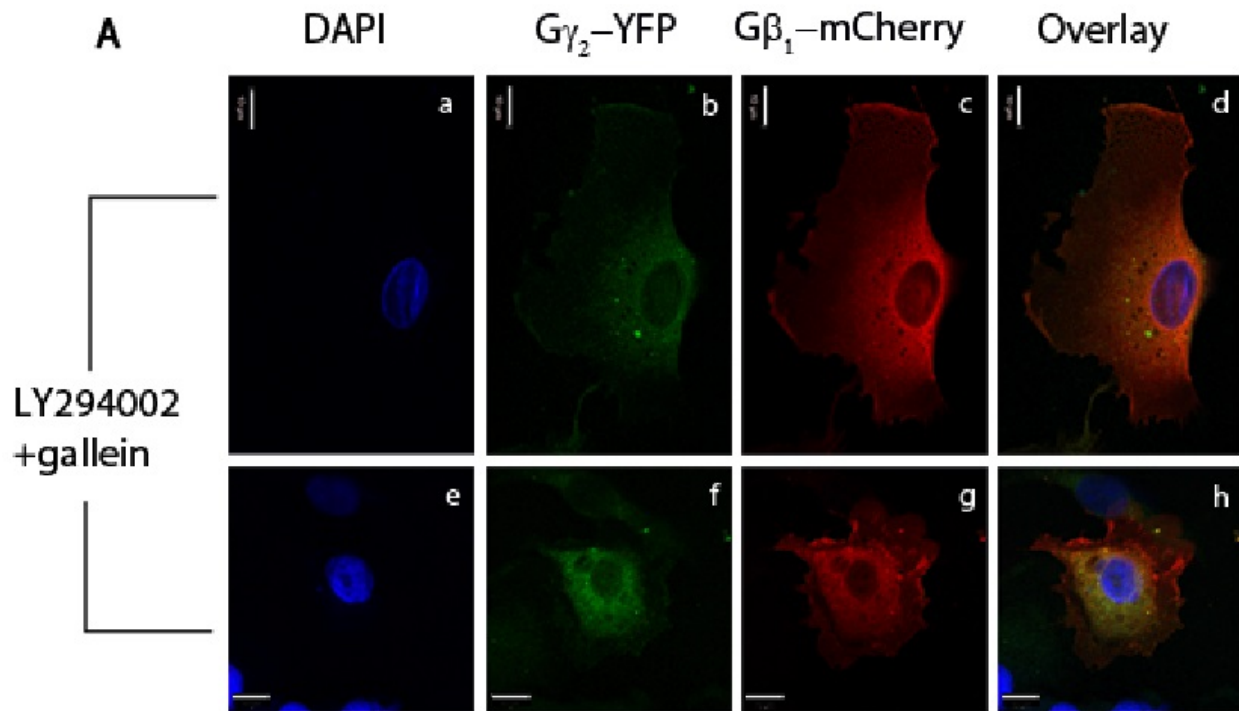


### **3.2.3 Gallein and LY294002 altered neuronal differentiation in overexpressed PC12 cells**

Both gallein and LY294002 affected neurite formation, cell morphology, and cell division. Gallein effect in  $G\beta\gamma$  overexpressed cells appears to be similar to that observed in NGF-differentiated cells, cells treated with gallein presented significant changes in morphology. Similarities in neurite outgrowth such as the ones present in  $G\beta\gamma$  overexpression were seen but neurites appeared distorted (Figure 12A, a-b). Also a distinguishing feature of cells treated with gallein was the elongated nuclei in the majority of the cells (Figure 12A, e-h). In the case of LY294002 a total inhibition of neurite outgrowth was noted on the majority of cells (Figure 12A, i-p), not only there were no neurites present but also the cells appeared to grow in size significantly when compared to untreated cells. Joint treatments with both gallein and LY294002 were also performed; the predominant morphology was that of LY294002 where neurites were absent in the majority of the cells, this could be due to the overexpression of  $G\beta\gamma$  causing an increase in size of the cell body inhibiting neurite growth (Figure 13A). The cell body was largely enlarged by the effect of the joint treatment, even more when compared to LY294002 alone (Figure 13A, a-d)



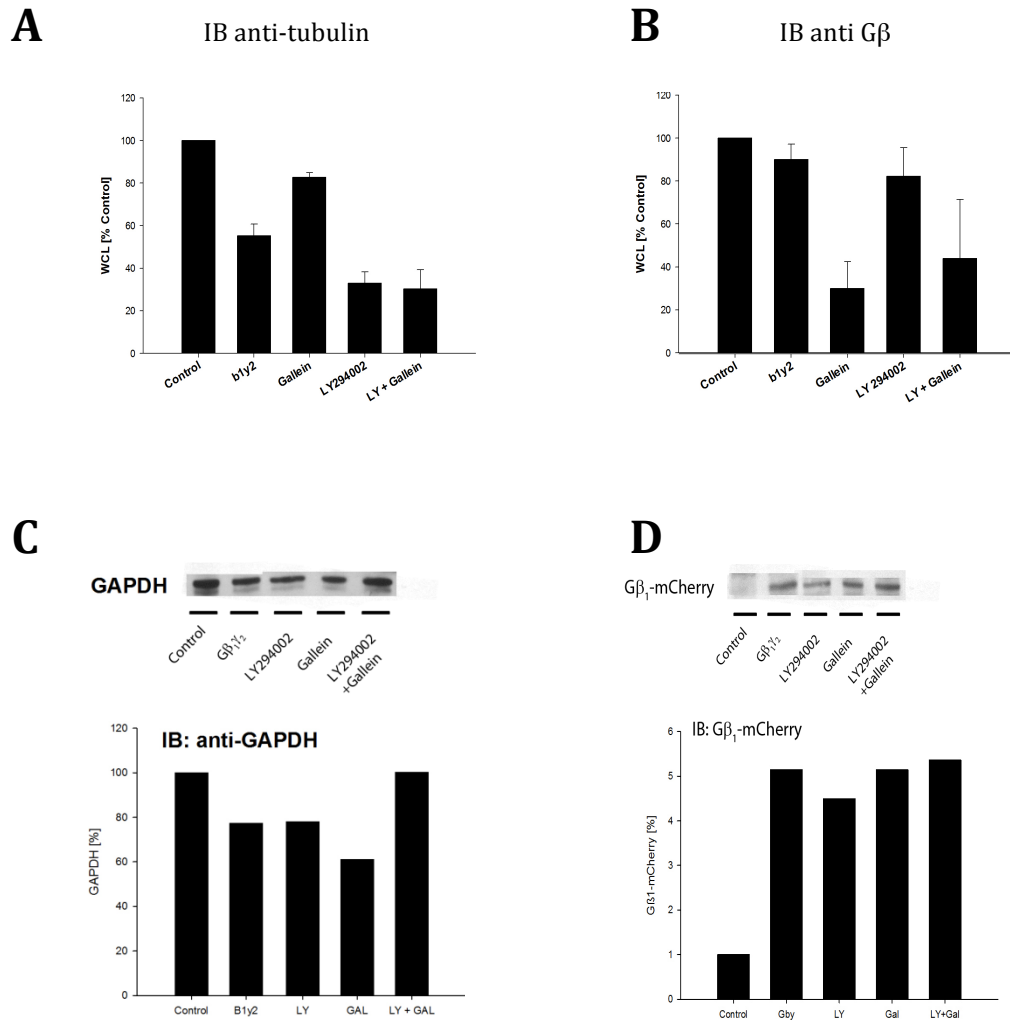
**[Figure 12. Gallein and LY294002 affected cell morphology as well as cell division by affecting cytokinesis in  $G\beta_1\gamma_2$  overexpressed cells. PC12 cells were co-transfected with YFP- and mCherry-tagged constructs encoding  $G\gamma_2$  and  $G\beta_1$  ( $\beta_1\gamma_2$ ), respectively, in the absence of NGF, using Lipofectamine 3000 reagent according to the manufacturer's instructions. Cells were then treated with gallein (10  $\mu$ M for 30 min), and LY294002 (100  $\mu$ M for 1 h). Scale bars: 20  $\mu$ m for all images.]**



**[Figure 13. PI3K inhibitor cause morphological changes in  $G\beta_1\gamma_2$  overexpressed cells.** PC12 cells were co-transfected with YFP- and mCherry-tagged constructs encoding  $G_{\gamma_2}$  and  $G\beta_1$  ( $\beta_1\gamma_2$ ), respectively, in the absence of NGF, using Lipofectamine 3000 reagent according to the manufacturer's instructions. Cells were then treated with gallein (10  $\mu$ M for 30 min), and LY294002 (100  $\mu$ M for 1 h). Scale bars: 20 $\mu$ m for all images]

### **3.2.4 Overexpression of $G\beta\gamma$ in PC12 cells promoted the activation of PI3k/AKT/GSK-3 $\beta$**

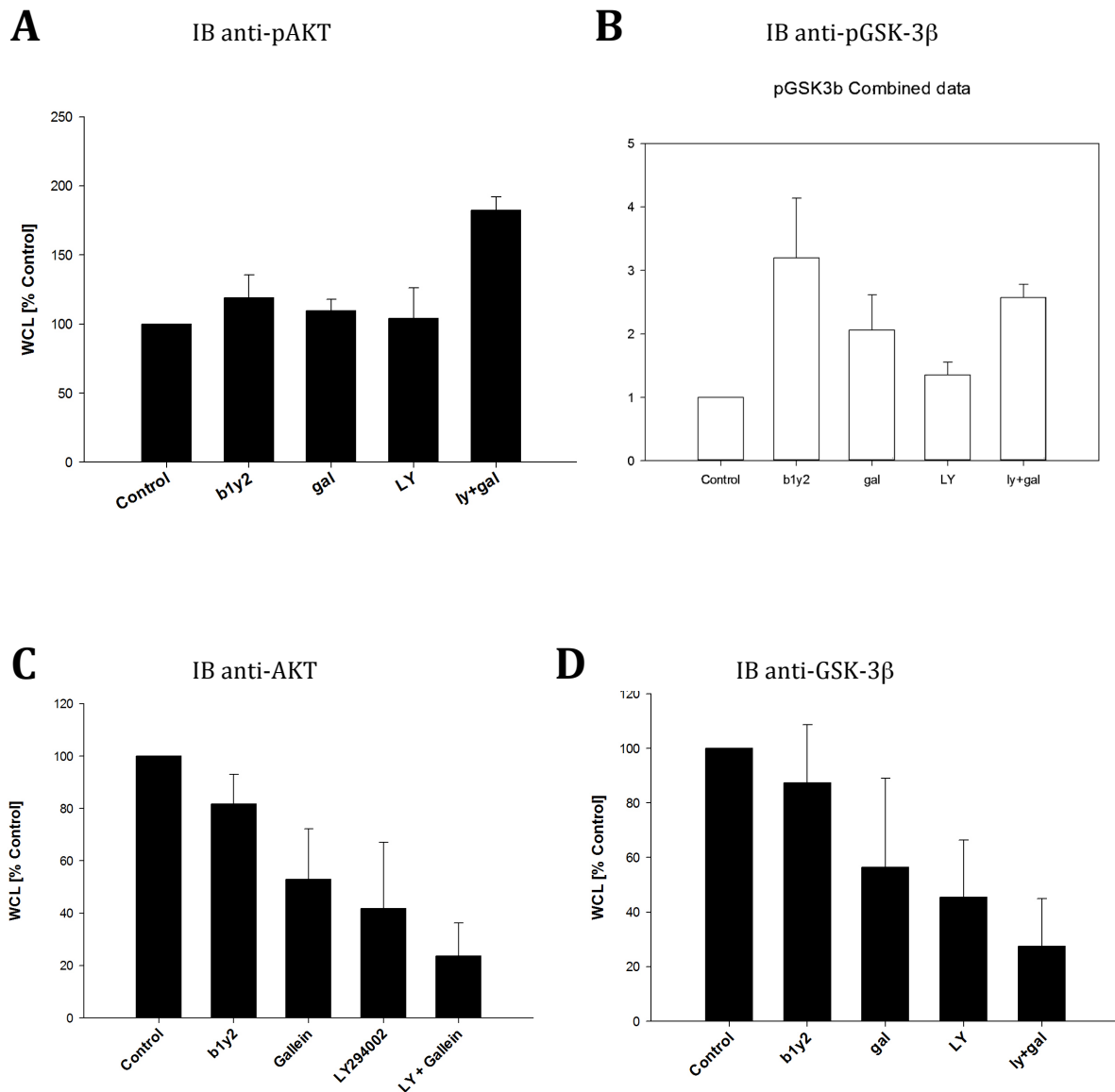
We found that overexpression of  $G\beta_1\gamma_2$  inhibited the expression of tubulin but the expression of  $G\beta$  was unaffected in overexpressed cells (Figure 14A and B), while expression levels of  $G\beta_1$  increased by a factor of 5, meaning that overexpression of  $G\beta_1\gamma_2$  was successful (Figure 14C). While  $G\beta\gamma$  inhibitor gallein significantly inhibited  $G\beta\gamma$ , tubulin expression in overexpressed cells was reverted to control levels. PI3K inhibitor LY294002 was very effective in enhancing the effect of overexpression of  $G\beta\gamma$  on tubulin expression, while reverting almost completely the effect of  $G\beta$  expression levels. The loading control GAPDH was also used to ensure that proper loading was performed efficiently (Figure 14C). Also in order to ensure that the  $G\beta\gamma$  overexpression was successful a blot using anti- $G\beta_1$  was done, and as expected all transfected subjects showed a five-fold increase in  $G\beta_1$  expression.



**[Figure 14. Effect of gallein and LY294002 in Gβ<sub>1</sub>γ<sub>2</sub> overexpressed cells.** PC12 cells were co-transfected with pcDNA 3.1 plasmids encoding for the β<sub>1</sub> and γ<sub>2</sub> genes, and then treated with gallein at a concentration of 10mM for 30 min., and LY294002 at a concentration of 100 mM for 1 hr. After incubation cell lysate extraction was performed and subjected to immunoblotting, Protein bands were detected using ECL-plus reagent and quantitated. A) WCLs were subjected to western blot analysis using anti-tubulin antibody as indicated in the methods to determine MT assembly. B) WCLs were subjected to western blot analysis using anti-Gβ antibody as

indicated in the methods to determine the expression levels of  $G\beta$  in the presence of gallein or LY294002. C) WCLs were subjected to western blot analysis using anti-GAPDH antibody as indicated in the methods to determine an even loading control of the samples. D) WCLs were subjected to western blot analysis using anti- $G\beta_1$  antibody as indicated in the methods to determine the expression levels of the  $G\beta_1$ -mCherry complex in order to ensure a successful transfection of the PC12 cells.

Overexpression of  $G\beta\gamma$  in PC12 cells promoted the activation of PI3k/AKT/GSK-3 $\beta$  pathway supporting the idea that  $G\beta\gamma$  and PI3K pathway works in concert. Both gallein and LY294002 decreases the total amount of protein of AKT, while the phosphorylation of this enzyme remained constant with either of the treatments. It was with the joint treatment that AKT phosphorylation increased by a factor of 74% (Figure 15A and C). However, stimulation of phosphorylation of GSK-3 $\beta$  was only partially inhibited both by LY294002 as well as gallein, it was the joint treatment that was ineffective in reversing the effects of  $G\beta\gamma$  by bringing its expression levels right below control levels (Figure 15B). The effect of the joint treatment on the phosphorylation of both AKT and GSK-3 $\beta$  isn't still fully understood, there is a possibility that these two compounds interact with one another. Both gallein and LY294002 have a substantial amount of aromatic rings in its chemical structure; because of this it is possible that there is a non covalent interactions between pi bonds found in its aromatic rings, cancelling the effect of these two compounds.



**[Figure 15. The effect of gallein and LY294002 in  $G\beta_1\gamma_2$  overexpressed cells on phosphorylation of PI3K downstream effectors.** PC12 cells were co-transfected with pcDNA 3.1 plasmids encoding for the  $\beta_1$  and  $\gamma_2$  genes, and then treated with gallein at a concentration of 10mM for 30 min., and LY294002 at a concentration of 100 mM for 1 hr. After incubation cell lysate extraction was performed and



subjected to immunoblotting, protein bands were detected using ECL-plus reagent and quantitated. A) WCLs were subjected to western blot analysis using anti-pAKT antibody as indicated in the methods to determine the phosphorylation levels of the protein kinase B in the presence or absence of gallein and LY294002. B) WCLs were subjected to western blot analysis using anti-pGSK-3 $\beta$  antibody as indicated in the methods to determine the phosphorylation levels of GSK-3 $\beta$  in the presence or absence of gallein and LY294002.

## Chapter 4: Discussion

Neuronal outgrowth is a complex process that requires the coordination of different cellular mechanisms. Since the microtubules are the major cytoskeletal filaments in axon, the control of microtubule assembly and stability is a key regulatory step during the growth of an axon. Previous studies demonstrated that  $G\beta\gamma$ , an important component of GPCR pathway promotes microtubule assembly and induces neurite outgrowth. The result presented here suggests that neurite outgrowth is dependent on both  $G\beta\gamma$ -MT dependent pathway as well as the activation of PI3K pathway.

Two downstream effectors of PI3K that have been shown to participate in the regulation of neurite outgrowth and are particularly associated with cytoskeletal remodeling, are AKT and the glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ). The PI3K/AKT pathway has been shown to be responsible for mediating neurotrophin responses, which are critical for neuronal outgrowth (Wu and Wong, 2005a and 2005b; Wu and Wong, 2006). Using inhibitors of  $G\beta\gamma$  and PI3K signaling, our results clearly suggest that PI3K signaling and  $G\beta\gamma$  coordinate to regulate NGF-induced neuronal differentiation of PC12 cells. As indicated in the figure, gallein, which is known to bind to  $G\beta\gamma$  and inhibit  $G\beta\gamma$ -dependent effector function including PI3K, also inhibited the expression of both tubulin and  $G\beta\gamma$  in NGF-differentiated PC12 cells. As expected, inhibitors of PI3K (LY294002) block the PI3K/AKT/pAKT pathway as

shown by inhibition of pAKT and pGSK-3 $\beta$  phosphorylation. Thus it appears that inhibitors affect both G $\beta\gamma$  and PI3k pathways.

Overexpression of G $\beta\gamma$  (in the absence of NGF) induced neurite outgrowth of PC12 cells similar to that observed in NGF-differentiated PC12 cells. Moreover, G $\beta\gamma$  overexpression stimulated phosphorylation of both AKT and GSK-3 $\beta$  (not significant results). The results suggest that overexpression of G $\beta\gamma$  mimics the effect of NGF in activating PI3K/AKT/GSK-3 $\beta$  pathway and promoting neurite outgrowth. Both gallein and LY294002 inhibited the phosphorylation of AKT. However, stimulation of phosphorylation of GSK-3 $\beta$  was only partially inhibited by LY294002 while gallein was ineffective in reversing the effect of G $\beta\gamma$  overexpression. This finding has very high implication. The result suggests once GSK-3 $\beta$  is phosphorylated by G $\beta\gamma$  overexpression (or receptor activation), pGSK-3 $\beta$  levels are unable to be reversed by blockage of G $\beta\gamma$  activation (by gallein) and subsequent inhibition of PI3K activity (as demonstrated by inhibition of pAKT activity by gallein). Since inactivation of GSK-3 $\beta$  by phosphorylation control MT polymerization/dynamics and stabilization via MT binding proteins (MBPs), the result may provide the mechanism by which G $\beta\gamma$ , PI3K and GSK-3 $\beta$  co-ordinate to regulate neuronal differentiation/MT assembly in neuronal cells.

We found that overexpression of G $\beta 1\gamma 2$  inhibited the expression of tubulin but the expression of all isoforms of G $\beta$  was unaffected in overexpressed cells

expression of  $G\beta_1$  on the other hand increased by 400%. While  $G\beta\gamma$  inhibitor gallein inhibited  $G\beta\gamma$  and tubulin expression in overexpressed cells when compared to overexpressed control cells, PI3K inhibitor LY294002 further inhibited the expression of tubulin. It appears that cytokinesis is affected in some of the  $G\beta\gamma$ -overexpressed cells inducing multinuclear formation. Thus, it appears that regulated expression of  $G\beta\gamma$  is important for normal neurite outgrowth and altered expression of  $G\beta\gamma$  may damage neuronal cells by altering cytoskeleton.

Since GSK-3 $\beta$  is thought to play significant roles in microtubule assembly in axon growth, morphology and guidance (reviewed in Zhou and Snider 2005), It is the best course of action to focus on understanding the mechanism of how  $G\beta\gamma$  and GSK-3 $\beta$  work together to achieve this goal. pGSK-3 $\beta$  is a unique enzyme that is usually active in resting cells, and inactivated through phosphorylation of the ser-9 position at the amino terminal by activation of PI3K. Results indicate that  $G\beta\gamma$  inhibitor gallein affects both pGSK-3 $\beta$  phosphorylation and axonal morphology; therefore it is possible that  $G\beta\gamma$  modulates GSK-3 $\beta$  mediated regulation of neurite outgrowth. Similarly, both LY294002 and gallein affects both  $G\beta\gamma$  and tubulin expression. However, to understand the relationship between  $G\beta\gamma$  and PI3K pathways in inducing neuronal differentiation, it is important to determine if MT assembly as well as MT-  $G\beta\gamma$  interaction is affected by gallein and LY2940029. Future experiments will address this issue. Quantitative analyses of confocal

microscopic studies are required to better understand the effect of gallein and LY294002 on neurite outgrowth and morphology.

G $\beta$  $\gamma$  overexpression stimulated phosphorylation of both AKT and GSK-3 $\beta$  in the absence of NGF. Similarly, we found overexpression of G $\beta$  $\gamma$  induced neuronal differentiation in the absence of NGF. The results suggest that G $\beta$  $\gamma$  mimics the effect of NGF in activating PI3K/AKT/GSK-3 $\beta$  pathway and promoting neurite outgrowth. Although G $\beta$  $\gamma$  overexpression induced neurite outgrowth, many of these overexpressed cells produced altered morphology. It was found that the stimulation of phosphorylation of GSK-3 $\beta$  in G $\beta$  $\gamma$ -overexpressed cells was only partially inhibited by LY294002, as well as gallein. This suggests that phosphorylation pattern of GSK-3 $\beta$  by G $\beta$  $\gamma$  overexpression could be similar to that found in NGF-induced phosphorylation.

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## Vitae

Jose L. Varela was born in El Paso Texas, US. The first son of Jose Luis Varela and Lucia Margarita Baca de Varela, he graduated from Franklin High School, El Paso Texas, in 2008, after which he entered the University of Texas at El Paso earning a Bachelor of science degree in Biomedical Science in 2012. While pursuing his bachelor's degree, he joined a neuroscience reserach laboratory, where he conducted research in the areas of molecular biology and neuroscience for three years under the guidance of Dr. Manuel Miranda. Meanwhile he entered the master's program in Biological Sciences at the University of Texas at El Paso in 2013, joining the Cytoskelteon and Cell Signaling Laboratory. Here, he developed a research project under the supervision of Dr. Sukla Roychowdhury, studying the intracellular signals that regulate the cytoskeleton during neuronal differentiation and neurodegeneration. He presented the results of his research in local scientific meetings such as the annual Border Biomedical Research Symposium, and the first ever Medical Center of the America's Cardwell Collaborative poster presentation. He concurrently trained several undergraduate and graduate students in the laboratory, and worked as a Teaching Assistant at the University, teaching undergraduate-level courses for three years.

Permanent address

7040 Luz de espejo Dr.

El Paso Tx. 79912

This thesis was typed by Jose L. Varela