University of Texas at El Paso
ScholarWorks@UTEP

Open Access Theses & Dissertations

2019-01-01

Molecular Mechanism of Neurodegeneration Induced by 4-Nonylphenol

Michelle Alejandra Aranda
University of Texas at El Paso

Follow this and additional works at: https://digitalcommons.utep.edu/open_etd

 Part of the Biology Commons, Cell Biology Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation

Aranda, Michelle Alejandra, "Molecular Mechanism of Neurodegeneration Induced by 4-Nonylphenol" (2019). *Open Access Theses & Dissertations*. 2827.
https://digitalcommons.utep.edu/open_etd/2827

This is brought to you for free and open access by ScholarWorks@UTEP. It has been accepted for inclusion in Open Access Theses & Dissertations by an authorized administrator of ScholarWorks@UTEP. For more information, please contact lweber@utep.edu.

MOLECULAR MECHANISM OF NEURODEGERATION INDUCED BY 4-NONYLPHENOL

MICHELLE ALEJANDRA ARANDA BARROSO

Master's Program in Biology

APPROVED:

Sukla Roychowdhury, Ph.D., Chair

Igor Almeida, Ph.D.

Anita Quintana, Ph.D.

Binata Joddar, Ph.D.

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

Copyright ©

by

Michelle A. Aranda Barroso

2019

Dedication

Para mi mamá, sin su apoyo habría sido imposible estar aquí.

MOLECULAR MECHANISM OF NEURODEGERATION INDUCED BY 4-NONYLPHENOL

by

MICHELLE ALEJANDRA ARANDA BARROSO, B.S.

THESIS

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

December 2019

Acknowledgments

An accomplished goal is always the result of a team effort. This thesis is the result of the support and guidance I got from the amazing people around me.

First of all, I want to thank Dr. Sukla Roychowdhury, my principal investigator, and dearest professor, without her guidance, support, and encouragement none of this would have been possible. Thank you, Dr., for the opportunity to join your laboratory, and for all the talks, the advice, and the laughs during these past years.

My sincere gratitude to the members of my committee, Dr. Igor Almeida, Dr. Anita Quintana, and Dr. Binata Joddar, for their guidance and advice towards this project which made possible this accomplishment.

I want to acknowledge the staff of Cytometry, Screening, and Imaging, and Cell Core facility, Dr. Varela, Ms. Gladys Almodovar and Denisse Gutierrez for their constant advice and support during the different experiments and analysis performed for this thesis.

I will always be grateful to the members of Dr. Roychowdhury's and Dr. Das's laboratories as well as teaching colleges, Roberto Castro, Clarissa Palacios, Luisa Sanchez, Vanessa Enriquez, Alejandra Gomez, Diana Olivas, Chelsea George, Melissa Lerma, and Sara Bustamante. Thank you for all the knowledge shared, as well as the lunchtime stories, laughs, fights and support. Your friendship is one of the most valuable things I am taking with me from this program.

I also need to thank my friends and family who were always willing to share my frustration, and my happiness even without having an idea of what I was talking about. My parents, who were the main source of encouragement and support during both my undergraduate and graduate studies; Ricardo, the pillar of my sanity and productivity during this last year, thank

you for all the love, games, dinners, jokes, and for your infinite patience towards me; my siblings, cousins, nephews, nieces, and all my friends, especially those who have been there since the beginning, I love you all.

Lastly, I want to thank God, my personal source of peace.

Abstract

Neurodegeneration, a progressive loss of nerve cells, occurs in many neurological disorders, including Alzheimer's disease (AD) and Parkinson's diseases (PD), as well as in dementia. The pathogenesis of these diseases is unknown, and recent evidence suggests that environmental factors, which act as endocrine-disrupting compounds (EDCs) could play a significant role in developing the disease process. 4-Nonylphenol (4-NP), an EDC, and a ubiquitous environmental toxin has been shown to affect brain development and may cause neurodegeneration. 4-NP is produced in large quantities in the U.S. and used as raw materials for making detergents, pesticides, plastics, paints, cosmetics, rubber, and other industrial/household products that lead to its widespread release to the environment. However, the underlying molecular mechanism of 4-NP-induced neurodegeneration is not understood. Recent results from our laboratory indicate that 4-NP inhibits nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells, causes cellular aggregation and interferes with microtubule (an important component of the neuronal cytoskeleton) organization. The expression of tau, a microtubule-associated protein (MAP) known to play a key role in neurodegeneration was increased significantly in the presence of 4-NP. High-Resolution proteomic analysis of a cytoskeletal fraction (CSKF) reveals that 4-NP altered the proteomic landscape of CSK and increased the association of several proteins, including proteins of Alzheimer's (AD) and Parkinson's disease (PD) pathways, with the cytoskeleton (CSK). 4-NP has been shown to inhibit tubulin-Gβγ interactions. Gβγ is an important component of the GPCR (G protein-coupled receptor) signaling pathway and its interaction with tubulin/MTs has been shown to be important for MT assembly and neurite outgrowth. Based on these results I hypothesize that *4-NP induces neurodegeneration by disrupting cytoskeleton and altering expression/localization of*

Tau protein. We further hypothesize that Gβγ and downstream PI3K/pAkt/pGSK3β signaling pathway is involved in this process. Because PC12 cells are not of neuronal origin, in the current study I also used human neuronal cells SHSY5Y. In Specific Aim 1, using biochemical, and immunoconfocal methodologies, I have demonstrated that 4-NP inhibited neurite formation and disrupted MT assembly and organization in both SHSY5Y and PC12 cells. Tau and tubulin localization was dramatically altered in the presence of 4-NP and no colocalization was observed as seen in control cells. In Specific Aim-2, I have conducted co-immunoprecipitation analysis and demonstrated the 4-NP significantly inhibited tubulin-Gβγ interactions in SHSY5Y cells. In addition, the PI3K pathway was also affected by 4-NP as shown by inhibition of phosphorylation of Akt and GSK3b in the presence of 4-NP.

Our study clearly demonstrates the potential risk of 4-NP in disrupting cytoskeleton and inducing neurodegeneration and should provide essential information in accessing the environment risk of 4-NP.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 4-Nonylphenol.

Endocrine-disrupting compounds (EDCs) have been the focus of research in recent years because of the growing concerns of the health risks they pose. They are widespread in our environments. It has been shown that EDCs may disturb homeostatic control and cause psychological disorders. It has toxic effects on the reproductive, digestive, and immune systems (Jie, 2013; Matsunaga, 2010). The potential effects of EDCs on the central nervous system (CNS) have prompted concern in recent years (Jie, et al., 2013).

4-Nolylphenol (4-NP), one of the most prevalent EDC, is a synthetic organic chemical produced in relatively large quantities in the U.S. to synthesize Nonylphenol polyethoxylate (NPE). NP is a compound that has numerous isomers. The side chain has nine carbons and can be attached to phenol at different points on the ring, thus producing different isomers. 4-NP is the most common commercial form of NP (Mao, et al., 2012) and has been reported to have deleterious effects on CNS altering cognitive function and neurotoxicity of tissues (Jie et al., 2013). 4-NP is usually used to produce nonylphenol polyethoxylates (NPEs). NPEs are mainly used in a number of industrial processes and products, including cleaners, pesticides, paint, detergents, and plastics. (Mao, et al., 2012) (**Fig.1**). 4-NP is widely released in the environment as a degradation product of NPE (US Environmental Agency, 2010; IRA Office of Environmental Health Hazard Assesment, 2009). It has been detected in surface and groundwater, sediment, wastewater effluent, and air (Knepper, 2003; Gautam et al, 2015; Toxnet). It is highly toxic to aquatic organisms. Human exposure to 4-NP may occur through contact with detergents and products containing this chemical compound, as well as through the ingestion of contaminated water or food. The molecule has been detected in human breast milk,

blood, and urine, raising a concern of toxic effects of 4-NP in human health (Sise, 2017; Li X, et al., 2013). NP easily accumulates in animal and human tissues due to its hydrophobicity (Zhang, et al., 2016). Because of its lipophilic nature, 4-NP can cross the blood-brain barrier (BBB), exerting its diverse effects on the central nervous system (CNS) (Zhang et al., 2008). More recently, oral administration of low doses of 4-NP in adult rats resulted in learning and memory impairment, which correlated with its concentration in specific brain regions (Kazemi, 2018), and further supports the ability of the molecule to concentrate in the brain tissue and affect the functional dysfunction of the nervous system. Due to the potential toxicity issues of NP, the "Oslo and Paris Commission for the Protection of the Marine Environment of the north-east Atlantic" called for phasing out the use of NPEs in domestic cleaning agents by 1995 and in industrial cleaning agents by 2000 (Ferra, et al. 2001). Following these recommendations, European countries have restricted or banned the use of NPEs. In the United States, the use of NPs is still permitted, with certain limitations. In 2006, the U.S. Environmental Protection Agency (EPA) released final aquatic life ambient water quality criteria for NP, which recommends NP concentrations in both freshwater (28 μg/L, acute; 6.6 μg/L, chronic) and saltwater (7.0 μg/L, acute; 1.7 μg/L chronic). However, a debate concerning the level of risk of 4-NP is still ongoing by researchers, regulators, and manufacturers.

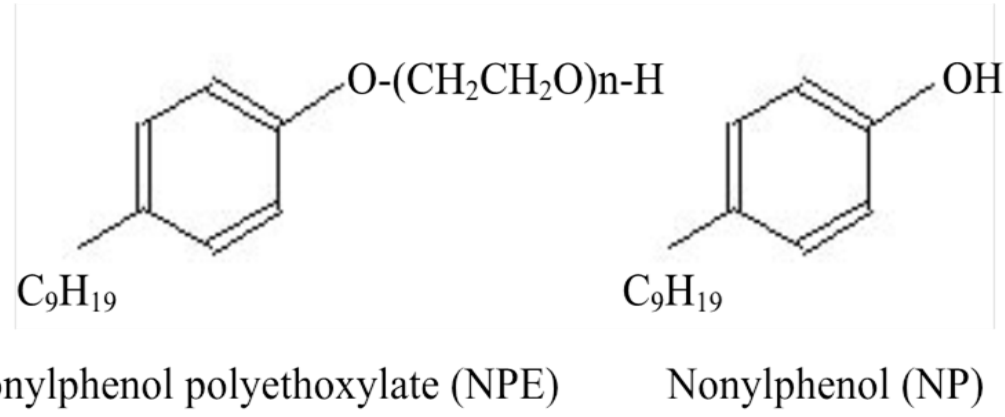

Figure 1. Chemical Structures of nonylphenol polyethoxylate and nonylphenol

1.2 Neurodegeneration and 4-NP.

Neurodegeneration refers to a pathological condition associated with the progressive atrophy, loss of function, and ultimately death of nerve cells in the brain and peripheral nervous system, and occurs in diseases or disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), dementia, and others. Cytoskeletal disruption and aggregation of proteins in neurons is the hallmark of neurodegeneration. (Alonzo, 2008; Amniai, 2008; Gustav-rothenbergg, 2010). The most consistent risk factor for developing a neurodegenerative disorder is aging (Tanner. 1992) and recent evidence suggest a role of genetic and environmental factors in the initiation of these diseases. However, the causes of neurodegenerative diseases are essentially unknown, and the mechanisms by which they initiate the disease remain, at best, speculative. (Przedborski, et al., 2003).

4-NP has been shown to induce behavioral as well as learning and memory capacity alterations in the male offspring of Sprague Dawley rats. (Xu, et al., 2010). Alongside, 4-NP treatments administrated to mothers during gestation, resulted in deterioration of neuronal development, decrease in memory and learning capacity and spatial learning in monkey and rat offsprings. (Yoshikawa, 2005) These data suggest that 4-NP might have a potentially damaging effect during early neurodevelopment. Other studies indicate that 4-NP treatment causes the death of neuronal stem cells suggesting that 4-NP could directly cause neurodegeneration (Kudo et al., 2004). 4-NP also was able to inhibit the MAP2-mediated neurite outgrowth. (Matsunaga et al, 2010).

More recently, 4-NP has been shown to inhibit neurotrophin-dependent neurite outgrowth in cultured embryonic Xenopus spinal-cord neurons and PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla that can show neurite like morphologies after

NGF treatment). Surprisingly, the effect of 4-NP was not inhibited by nuclear estrogen receptor antagonist, ICI182-780, but was inhibited by the G-protein antagonist, pertussis toxin (Bevan, et al, 2006) suggesting a role of G-protein signaling in this process.

1.3 Neuronal cytoskeleton and neurodegeneration.

The cytoskeleton provides structural organization for the cell interior, serve as tracks for intracellular transport, and comprises the core framework of cellular morphologies. The shape of the cells in the nervous system is closely connected to their functions. (Kirkpatrick and Brady, 1999). In neurons, the ability to achieve proper connections between the correct set of cells depends on the polarized organization into axons and dendrites. Microtubules (MTs) and actin filaments are core components of the cytoskeleton and are assembled through head-to-tail polymerization of α- and β-tubulin heterodimers and actin monomers, respectively, resulting in asymmetric, polarized polymers with two different ends. (Tas, et al., 2018). Both MTs and actin filaments are dynamic structures with the ability to continuously grow and shrink, which facilitates the continuous remodeling of the cytoskeleton. (Kevenaar and Hoogenraad, 2015). Microtubules (MTs) form dense, parallel arrays in axons and dendrites that are required for the growth and maintenance of these neurites (Witte and Bradke; Geraldo and Gordon-Weeks, 2009). 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) (Witte, 2008; Geraldo, 2009) (**Fig.2**). The dynamics of MTs are regulated by a large number of factors, including microtubule-associated proteins (MAPs). (Dehmelt and Halpain, 2005).

During neurodegeneration, the proper association and arrangement of cytoskeleton components are severely compromised. When neurodegeneration occurs several hallmark conditions are observed, such as altered protein folding, amyloid plaques, neurofibrillary tangles, Lewy bodies, impaired cellular transport, increased oxidative stress among many others

(Jellinger, 2010). However, as mentioned before, the mechanism by which diseases are triggered and then progress is poorly understood.

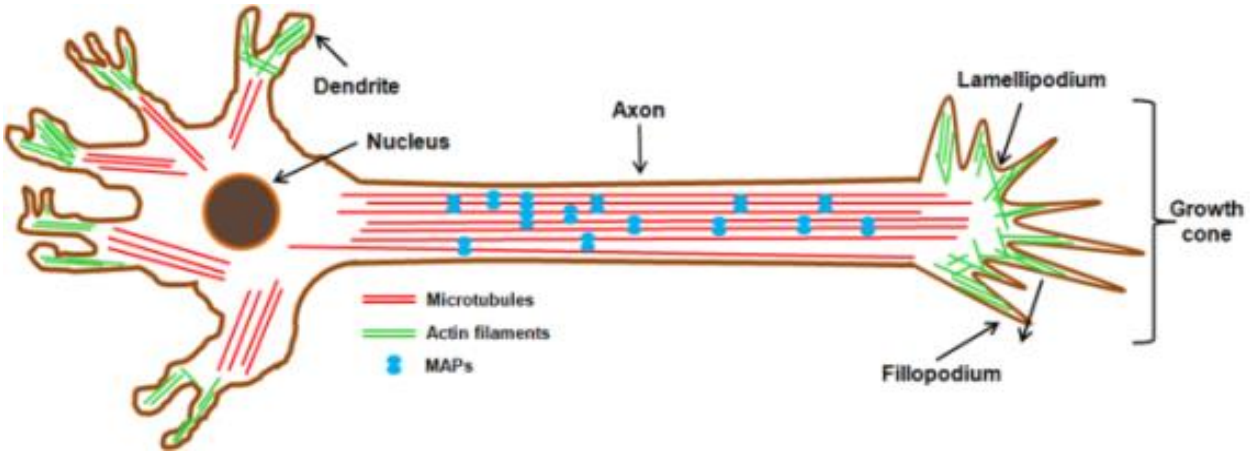

Fig. 2 Neuronal Cytoskeleton. Representation of the cytoskeletal organization in neuronal cells. Microtubules form dense arrays mainly along the axon and dendrites of the cell (shown in red). The actin filaments, represented in green, localized primarily on the tips of the processes, forming lamellipodium and filopodium. Lastly, shown in blue are the microtubule-associated proteins, which help in the dynamic stability of the cell.

1.4 Microtubule associated proteins, Tau and Neurodegeneration.

Microtubule dynamics in neurons is modulated by several accessory proteins termed microtubule-associated proteins (MAPs). Tau is an important microtubule-associated protein found in axons and is known to regulate MT assembly and neurite outgrowth. Tau functions in the stabilization and regulation of microtubule assembly are regulated by its phosphorylation state. (Herbert, 2013). Tau protein is a family of six isoforms, each with either three or four MT-binding repeats located in the C-terminal half of the protein and zero to two inserts located in the N-terminal portion. (Goedert and Jakes, 1990). Tau isoforms are developmentally regulated and have similar levels in the adult human brain. (Kosik, 1989). When detached from microtubules, Tau can self-aggregate through its hexapeptide motifs in the repeat domain. (Bergen, et al., 2000). In several neurodegenerative diseases, tau is hyperphosphorylated and aggregated to neurofibrillary tangles (NFT) which is likely to play a role in the pathogenesis of diseases. (Johnson, and Stoothoff, 2004).

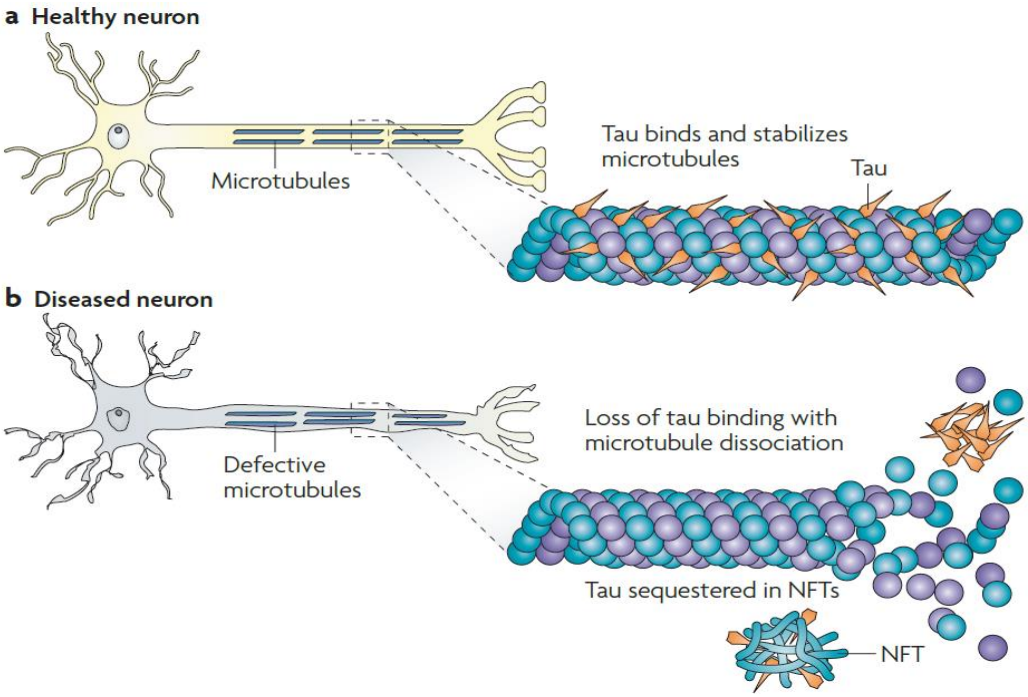

Figure 3. Tau in healthy neuronal cells and formation of NFTs in diseased cells. In normal

neurons, tau participates in MT stabilization, helping to preserve neuronal integrity and facilitating neuronal function. During neurodegeneration, tau becomes hyperphosphorylated and does not bind to MTs, forming abnormal aggregates that are deposited in the cells. This leads to impaired MT stability, axonal damage, and overall loss of neuronal integrity. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Brunden et al., 2009).

1.5 G-protein signaling pathway, Microtubule assembly and Neurite outgrowth.

Microtubule assembly, as mentioned above, is a dynamic process that must be tightly regulated in order to accomplish the proper morphology and therefore connectivity between neuronal cells. There are different pathways that can regulate microtubule assembly and disassembly in neurons. Previous studies from our laboratory have indicated that MT assembly could be regulated by G protein-mediated signaling (Roychowdhury and Rasenick, 1997; Roychowdhury et al., 1999; Roychowdhury et al., 2006; Montoya et al., 2007). G-protein-mediated signaling, a major signaling pathway, consists of three major components: G-protein coupled receptors (GPCRs), G protein, and effector molecules. GPCRs are transmembrane proteins and mediate different extracellular signals, like light, odorants, peptide hormones, and neurotransmitters. G proteins serve as signal transducers and are composed of three subunits: Gβ, Gγ, and Gα. When an agonist binds to GPCR, the G protein goes into an active state, disassociating the Gα subunit from the Gβγ allowing both to participate in intracellular signaling process (Gilman, 1987; Dohlman et al., 1991; Neves et al., 2002; McCudden et al., 2005). It is the Gβγ complex that has been shown to promote microtubule assembly and induce neuronal differentiation. (**Fig. 4**) (Roychowdhury and Rasenick, 1997; Montoya et al., 2007; Sierra-Fonseca et. al 2014). 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 Gβγ dependent MT assembly (Roychowdhury et.al 2006) (**Fig. 4**). Using the anti-mitotic agent nocodazole, it has been demonstrated that the tubulin-Gβγ interaction is important for MT assembly in cultured PC12 and NIH3T3 cells (Montoya et al., 2007). More recently, it was found that Gβγ–MT interaction is critical for NGF-induced neuronal differentiation of PC12 cells (Sierra-Fonseca et. al 2014). Overexpression of Gβγ in PC12 cells (in the absence of NGF) induced neurite formation similar to that seen in NGF-differentiated cells. Altogether, results from our laboratory suggested that the Gβγ, initiated by the activation of the G protein-coupled receptor (GPCR), is a signal-transducing protein complex, and its

interaction with microtubules (MTs) is important to neuronal development, differentiation, structure, and neurodegeneration (**Fig. 4**).

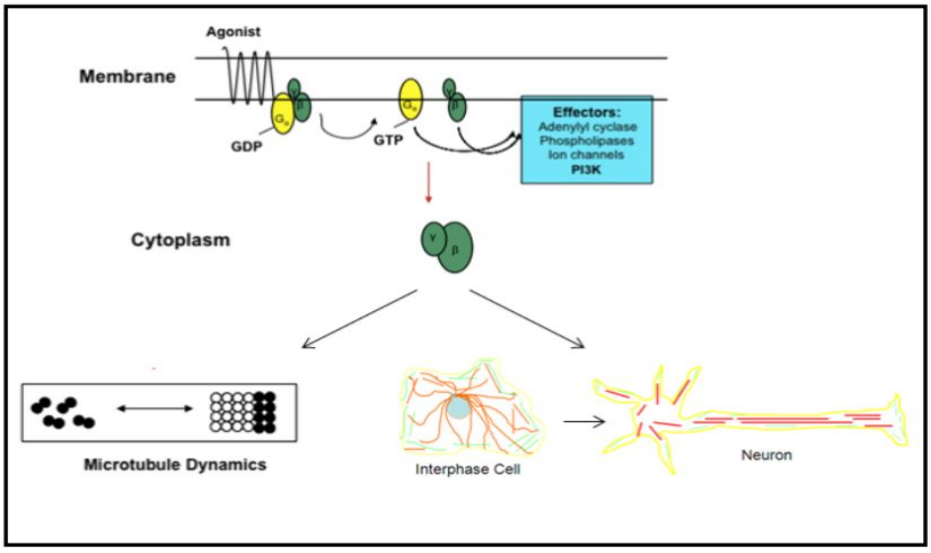

Fig 4. Gβγ signaling, MT assembly and Neurite outgrowth. Activation of GPCR triggers the disassociation of the Gαβγ heterotrimer into Gα and Gβγ subunits. These subunits play a role in the activation of several signaling pathways such as adenylyl cyclase, phospholipases, and PI3-Kinases. It has been previously found from our laboratory that Gβγ subunit interacts with αβ tubulin heterodimers to stimulate microtubule assembly and to induce neurite outgrowth in pheochromocytoma (PC12) cells (Roychowdhury and Rasenick, 1997; Montoya et al., 2007; Sierra-Fonseca et. al 2014)

1.5.1 Receptor Tyrosine kinase-A (TrkA), PI3K, MT assembly and Neurite outgrowth.

A different signaling pathway that can influence microtubule assembly, involves the phosphatidyl inositol-3-kinase (PI3K) enzyme. Being part of the downstream signaling for the receptor Tyrosine kinase-A (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 cytoskeletal remodeling, are Akt (a

serine/threonine-specific protein kinase) and the glycogen synthase kinase 3β (GSK3β). The regulation is neurite outgrowth by PI3K is dependent on the ability of the PI3-kinases to phosphorylate and activate the AKT that is followed by the inhibition of the GSK-3β by the phosphorylation of this enzyme (**Fig.5**). The role of PI3K in neuronal differentiation has been demonstrated using PC12 cells (endocrine chromaffin cells), a model cell line to study neuronal differentiation. PC12 cells respond to nerve growth factor (NGF) with growth arrest and exhibit a typical neuronal phenotype of neurite extensions. NGF has been shown to activate PI3K/Akt/GSK3β (Trk-A receptor pathway) to induce neurite outgrowth (**Fig.5**).

1.5.2 βγ signaling and PI3K/Akt/GSP3β pathway: Regulation of MT assembly and neurite outgrowth.

The connection between the PI3K pathway and Gβγ has been demonstrated previously. PI3K-γ was found to be a downstream effector of Gβγ-in neutrophil migration, inflammation and chemotaxis (Stephens et al., 1997) and recent results suggest that the activation of PI3K/Akt pathway by NGF is, in part, mediated through the βγ subunit (Wu and Wong, 2005a and 2005b; Wang and Wong, 2009). This correlates with previous studies from our laboratory that have shown that Gβ overexpression in PC12 cells was sufficient in the promotion of neurite formation (Sierra-Fonseca et. al 2014) as well as in increasing levels of pAkt and pGsk3β (Jose Varela, MS thesis UTEP 2016). These results suggest that Gβγ co-ordinates with PI3K/AKT/GSK3β to regulate neurite outgrowth and morphogenesis (**Fig.5**), and my research focuses to determine if 4-NP interferes with this pathway to disrupt MT assembly/neurite outgrowth, and induce neurodegeneration.

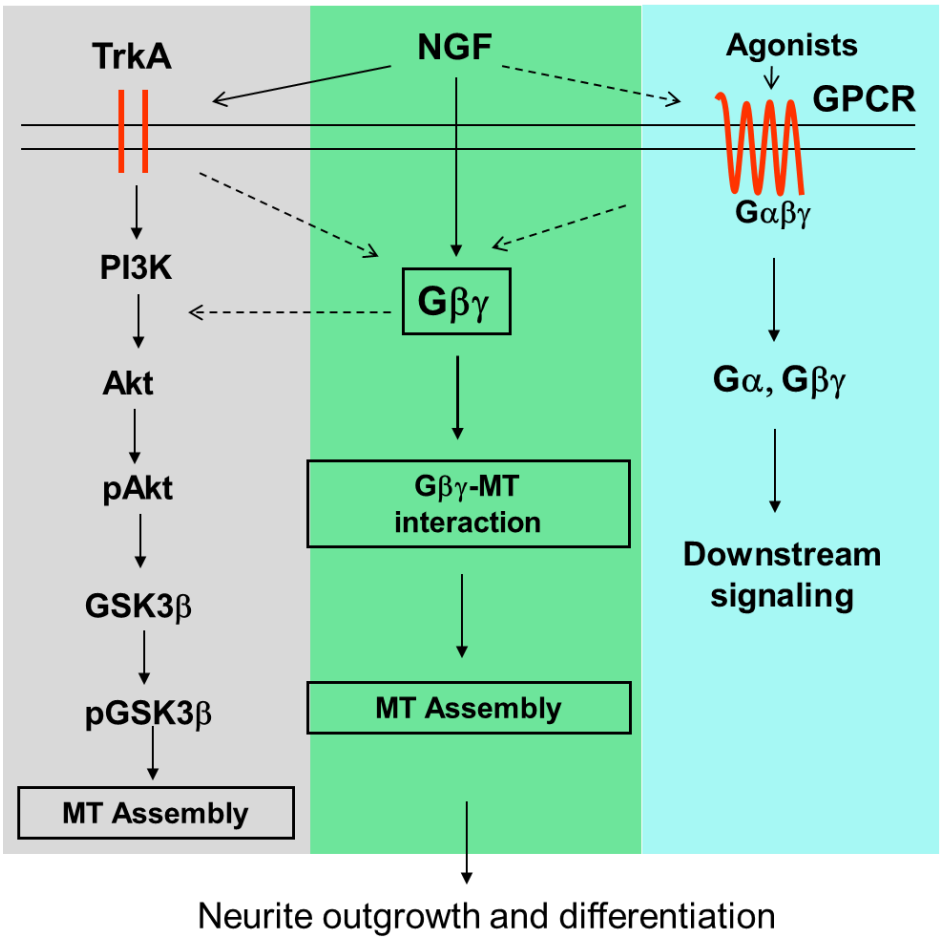

Fig. 5. Gβγ-MT mediated pathway and PI3K signaling coordinate to regulate MT assembly and neurite outgrowth. NGF is known to acts through TrkA receptors to activate PI3K/AKT/GSK3β to induce neurite outgrowth (left panel). The results from our laboratory demonstrate that NGF utilizes Gβγ, a key component of the GPCR pathway (right panel), to alter MT assembly by means of its, interaction with MTs, ultimately leading to neurite outgrowth and differentiation (central panel).

Hypothesis and specific aims.

The goal of the proposed study is to examine how 4-Nonylphenol (4-NP), a prevalent endocrine-disrupting compound (EDC) causes neurodegeneration. Although 4-NP has been shown to affect brain development and cause neurodegeneration, the mechanism by which 4-NP exerts its effect in neurons is not understood. Recent results from our laboratory indicate that 4-NP inhibits nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells and causes disruption of microtubule (an important component of the neuronal cytoskeleton). The expression of the tau protein was increased significantly in the presence of 4-NP. Tau is a microtubule-associated protein found in axons and it is known to regulate MT assembly and neurite outgrowth. High-Resolution proteomic analysis of a cytoskeletal fraction (CSKF) reveals that 4-NP altered the proteomic landscape of CSK and increased the association of several proteins, including proteins of Alzheimer's (AD) and Parkinson's Disease (PD) pathways, with the cytoskeleton (CSK). 4-NP has been shown to inhibit tubulin-Gβγ interactions. Gβγ is an important component of the GPCR (G protein-coupled receptor) signaling pathway and its interaction with tubulin/MTs has been shown to be important for MT assembly and neurite outgrowth. Based on these results, in the current investigation, I hypothesize that 4-NP induces neurodegeneration by disrupting cytoskeleton and altering expression/localization of Tau protein. I further hypothesize that Gβγ and downstream PI3K/pAkt/pGSK3b signaling pathways is involved in this process. PC12 cells and human neuroblastoma SHSY5Y cells are used for this study. We propose to test our hypotheses by addressing the following two Specific Aims.

Specific Aim-1: Elucidate the cytoskeletal changes that occur in response to 4-NP leading to neurodegeneration

Specific Aim-2: Determine if 4-NP interferes with Gβγ/PI3K dependent signaling pathway to promote cytoskeletal disruption and neurodegeneration.

Chapter 2: Materials and Methods

2.1 Cell culture, NGF, and 4-NP treatment.

PC12 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (4.5 g/L glucose, L-glutamine, without pyruvate), supplemented with 10% bovine calf serum (BCS), and penicillin (100U/mL)-streptomycin (100um/mL) at 37°C in 5% CO_2 in 75 cm^2 culture flasks. For NGF treatment, PC12 cells were grown in 100 mm plates to 75% confluence over 1-2 days, then treated with 100ng/mL of nerve growth factor (NGF) (Sigma-Aldrich, St. Louis, MO) dissolved in complete media for 2-3 consecutive days. Control cells were grown in the same conditions without NGF. For 4-NP treatment, a 5μM or 10μM solution of 4-NP dissolved in complete media was added to the cells overnight. A stock solution of 10mM 4-NP was prepared by dissolving the compound in DMSO to perform the treatments.

SHSY5Y cells were grown in Dulbecco's modified medium/Ham's F-12 50/50 mix (DMEM/F-12 50/50) supplemented with 10% fetal bovine serum (FBS), and penicillin (100U/mL)-streptomycin (100um/mL) at 37°C in 5% CO_2 in 75 cm^2 culture flasks. For 4-NP treatment, SHSY5Y the method described above for PC12 cells was followed. A stock solution of 10mM 4-NP was prepared by dissolving the compound in DMSO to perform the treatments.

2.2 Preparation of whole-cell lysate.

After treatments with NGF, and/or 4-NP, culture media was removed and the cells were washed with PBS and lysed by incubation in 500uL of lysis buffer (10mM Tris-HCl, pH 7.9, 1.5mM $MgCl_2$, 0.3M sucrose, 0.1% Triton X-100, 1mM DTT, 10 uM GTP) supplemented with protease and phosphatase inhibitor cocktail for 10 min over ice. Cells where then scraped and sonicated for 1 min. (3 sonications of 20 seconds each, on ice to prevent heating of samples), followed by centrifugation at 12,000 rpm for 10 min. at room temperature. Proteins

concentrations were determinate by the Bradford assay and all samples were leveled to obtain the same protein concentration in all the experimental vials. Loading dye was added as required for the immunoblotting protocol.

2.3 Electrophoresis and immunoblotting.

Samples for immunoblotting were run through a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a nitrocellulose membrane (Leammil 1970; Towbin et al., 1970) which were then incubated for 1 hour at room temperature in blocking solution (5% dry milk in 10%Tris-Sulfate Buffer). Subsequently, the membranes were incubated overnight at 4^{o}C in with mouse monoclonal anti-alpha-tubulin (DMIA, Sigma Aldrich, 1:100), Rabbit polyclonal anti Gβ (Santa Cruz Biotechnology, 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; 1:1000) in TBST for one hour. Chemiluminescence (ECL) technique (SuperSignal West Pico Chemiluminescence Substrate) was used according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Quantitative analysis of the protein bands was performed with the ThermoFisher, iBright image acquisition and analysis software.

2.4 Co-immunoprecipitation.

Whole-cell lysate from SHSY5Y cells sample was incubated in anti-alpha-Tubulin-protein specific antibody (mouse monoclonal anti-alpha-Tubulin, DMIA (or mouse monoclonal antibody derives from hybridoma in Dr. Francia's laboratory at UTEP) overnight at 4^{o}C. 100uL of 50% protein A-sepharose (Amersham Biochemical, Piscataway, NJ), pre-equilibrated in 10mM Tris-HCl, pH 8.0 was added to the samples to allow for a 2 hours incubation. Samples

were then centrifuged at 12,000rpm for 8 min, and the pellet was washed with wash buffer (25mM Tris-HCl, pH 7.2, 150mM NaCl). The pellets were washed 6 times in total, followed by immunoblotting sample preparation.

2.5 Immunofluorescence and confocal microscopy.

Cells were allowed to attach to glass coverslips placed in 12-wells plates and were then treated as described above. Media was then removed, cells were fixed by methanol fixation method [Montoya et al., 2007], then washed three times with PBS, blocked for 1 hour at room temperature in 500 μL of 5% normal goat serum (NGS) (Sigma-Aldrich) in PBS. Primary antibody incubation was performed for 1 hour at room temperature in the dark (anti-alpha-tubulin-Alexa Fluor 488-conjugated, and anti-Gβ-Alexa Fluor 647-conjugated). In 1% NGS in PBS. Coverslips were then mounted into slides using a drop (~15uL) of ProLong Gold anti-fade reagent with DAPI. High-resolution, digital, fluorescent images were captured by using an inverted, confocal laser-scanning microscopy (LSM 700; Zeiss, Thornwood, NY) employing a Plan-Apochromat 40x/1.4 and 60x/1.6 immersion oil DIC objectives and assisted with 2009 ZEN software (Zeiss, Thornwood, NY). DAPI (blue), Alexa Fluor 488 (green), and Alexa Fluor 647 (red) were excited with laser emissions of 405-, 488-, and 555-nm wavelengths, respectively.

2.6 Polymerization Assay.

In vitro, MT polymerization assay was performed using the Tubulin Polymerization Assay Kit by Cytoskeleton Inc. The kit provides neuronal tubulin subunits attached to a fluorescent reporter. Tubulin samples were prepared according to the kit manual provided to obtain the tubulin control samples, positive control samples (using Taxol as a polymerization enhancer), negative control samples (containing Nocodazol as polymerization inhibitor), and 4-NP samples to assess the effects of the toxin in tubulin polymerization. Besides the treatment

mentioned above, all samples contained PEM buffer along with GTP to allow for normal polymerization conditions. After the samples were placed in a 96 wells plate, they were analyzed using a fluorimeter for a 1 hour period.

2.7 Cytotoxicity Assay.

To determine the levels of cytotoxicity caused by the 4-NP, a previously described DNS 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, PC12 cells were seeded in a 96-well plate format and incubated with 4-NP at different concentrations overnight (1uM, 5uM, 10uM, 12uM, 15uM, 18uM, 20uM, and 25uM), as well as H_2O_2 (positive controls). Two hours before image capturing, cells were added with a staining mixture of Hoechst and PI at a final concentration of 1 μg/mL for each dye. 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 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 v1.6.2 software (BD Biosciences), and each experimental condition was assessed with 8 replicates.

2.8 Statistical analysis.

All statistical analyses were performed using Sigma Plot 11 software (Systat Software, Chicago, IL, USA). In the 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: Establish the cytoskeletal changes that occur in response to 4-NP leading to neurodegeneration.

3.1.1Objective and overview

During the process of neurodegeneration, MT assembly and proteins associated with MTs are severely altered. Under normal conditions, microtubule-associated protein Tau binds to MTs, stabilizing neuron structure and integrity. In Alzheimer's disease (AD), tau is hyper-phosphorylated and does not bind to the MTs. Lewy bodies, which are considered cytopathological markers of Parkinson's disease (PD), are comprised of tubulins, MAP1, and MAP2 (17-19). Our preliminary results indicate that the integrity of the MT network is severely altered by 4-NP treatment in PC12 cells. In addition, Tau protein expression was increased in the presence of 4-NP. Proteomic analysis revealed that the association of several proteins of AD/PD pathways with the CSK fraction was increased after 4-NP treatment. Therefore, in **Aim-1**, cytoskeletal alterations in the presence of 4-NP were investigated using neuronal cells SHSY5Y. In addition, PC12 cells were also used to accomplish this aim. Following two sub-aims were addressed.

(1) Establish 4-NP-induced disruption of neurite outgrowth and MT assembly/organization.

(2) Does 4-NP alter the expression/localization of Tau proteins?

3.1.2 Results

3.1.2.1 4-NP inhibits neurite formation and disrupts MT organization in PC12 cells

To determine the effects of 4-NP in MT organization, and neurite formation in NGF differentiated PC12 cells, PC12 cells treated with 100 ng/mL NGF over the curse of two days to allow for neuronal differentiation followed by 4-NP (1 µM and 5 µM) treatment overnight, as indicated in the figure (**Fig. 6**). Cells were fixed by methanol and label with monoclonal anti-tubulin (red) primary antibody and processed for confocal microscopy. In control cells, tubulin is localized evenly throughout the cell body. After NGF treatment, most of the cells showed neurite formation and tubulin can be seen localized along the neurites of the cells as well as in the cell bodies. In the presence of 4-NP, neurite formation is inhibited (1 and 5 µM treatments). As the concentration of 4-NP increases, cell aggregation, and neurite loss were more persistent. A quantitative assessment of neurite measurement indicated that the percentage of cells bearing neurites was inhibited by 4-NP in a dose-dependent manner (**Fig. 6 B**). A cell was considered neurite-bearing if it contained at least one neuronal process that was longer than the cell body (~14mm in diameter). However, the average neurite length of the surviving neurites was increased by 5mM 4-NP treatment (**Fig. 6 C**). This outcome was expected because we found that only a few longer neurites survived in the presence of 4-NP. Shorter neurites (developing neurites) were not found after 4-NP treatment. These results clearly suggest that 4-NP disrupts MTs, inhibits neurite formation and promotes cellular aggregation in NGF-differentiated PC12 cells.

Using cytotoxicity assay it was determined earlier that the viability of NGF differentiated PC12 cells was not altered after treatment with 4-NP (Jessica Martinez) indicating that 4-NP effect on neuronal differentiation is not due to cells death and could be due MT disruption.

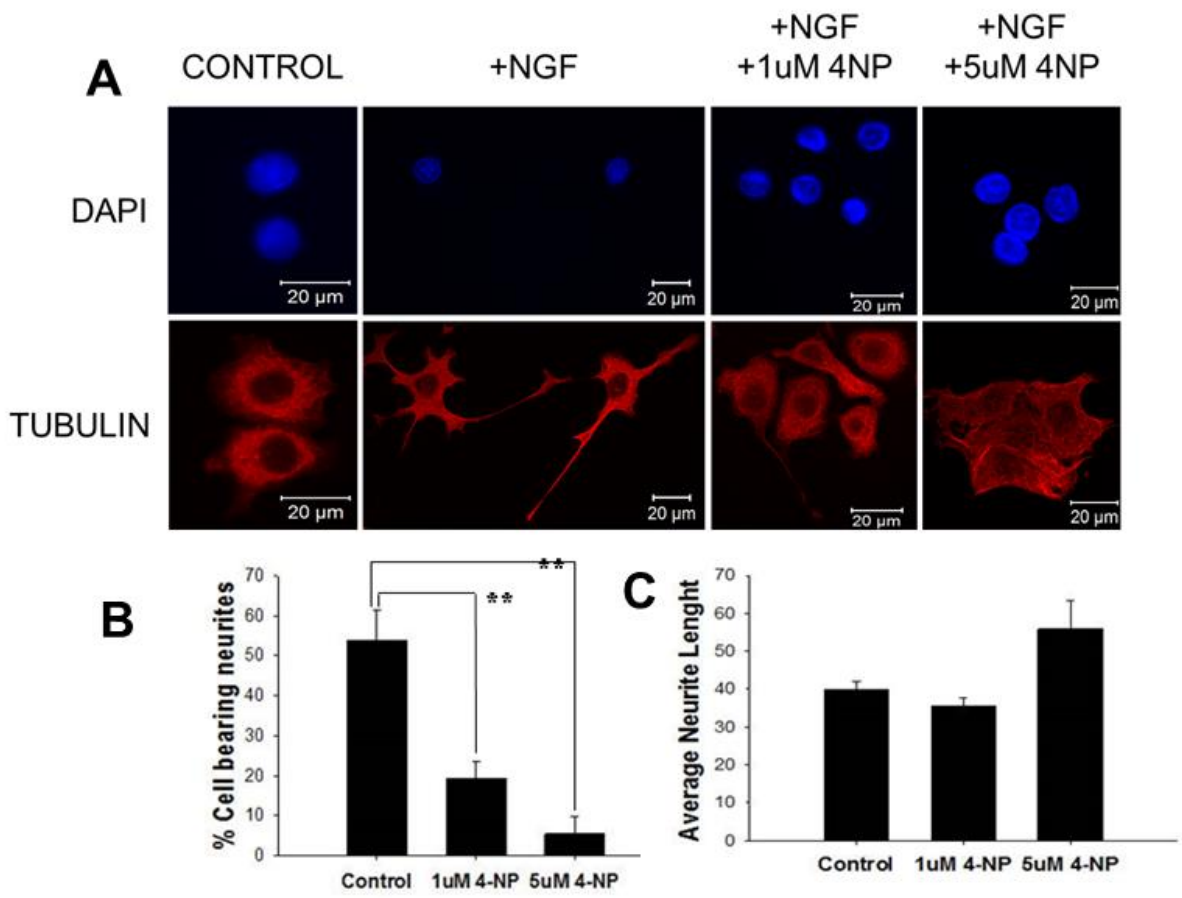

Fig. 6. Effect of 4-NP on (A) MT organization and cellular morphology in PC12 cells; (B) Quantitative assessment neurite formation; (C) average neurite length. PC12 cells were allowed to differentiate in the presence of NGF followed by treatment with 4-NP overnight. Samples were processed for confocal microscopy using anti-DAPI (blue), and anti-tubulin (red). In NGF treated cells, neurite outgrowth was observed. After 4-NP treatment, most of the cells had lost their neurites, and aggregation of cells was observed. Increases in the treatment concentration showed a more dramatic change in morphology and increased aggregation.

3.1.2.2 4-NP inhibits neurite formation and disrupts MTs in SHSY5Y cells.

Although PC12 cells are used as model cell lines to study neuronal differentiation, they are derived from the adrenal glands and are not of neuronal origin. Therefore, we tested the effect of 4-NP (10 μM) on neuronal cells SHSY5Y (human-derived). To determine the effects of 4-NP in MT organization, and neurite formation in SHSY5Y cells, the cells were seeded in 12-well plates with glass coverslips overnight to allow for cell attachment, followed by 4-NP (10 μM) treatment overnight, as indicated in the figure (**Fig. 7**). Cells were fixed by methanol protocol and label with monoclonal anti-tubulin (red) primary antibody and processed for confocal microscopy. In control cells incubated in the absence of 4-NP, tubulin is localized evenly throughout the cell body of the cell. In these cells, tubulin can be seen localized also along the neurites and processes they exhibit. In 4-NP presence, neurite formation is inhibited, aggregation of cells was also observed. These results clearly suggest that 4-NP disrupts MTs, inhibits neurite formation and disrupts the MT organization in SHSY5Y cells.

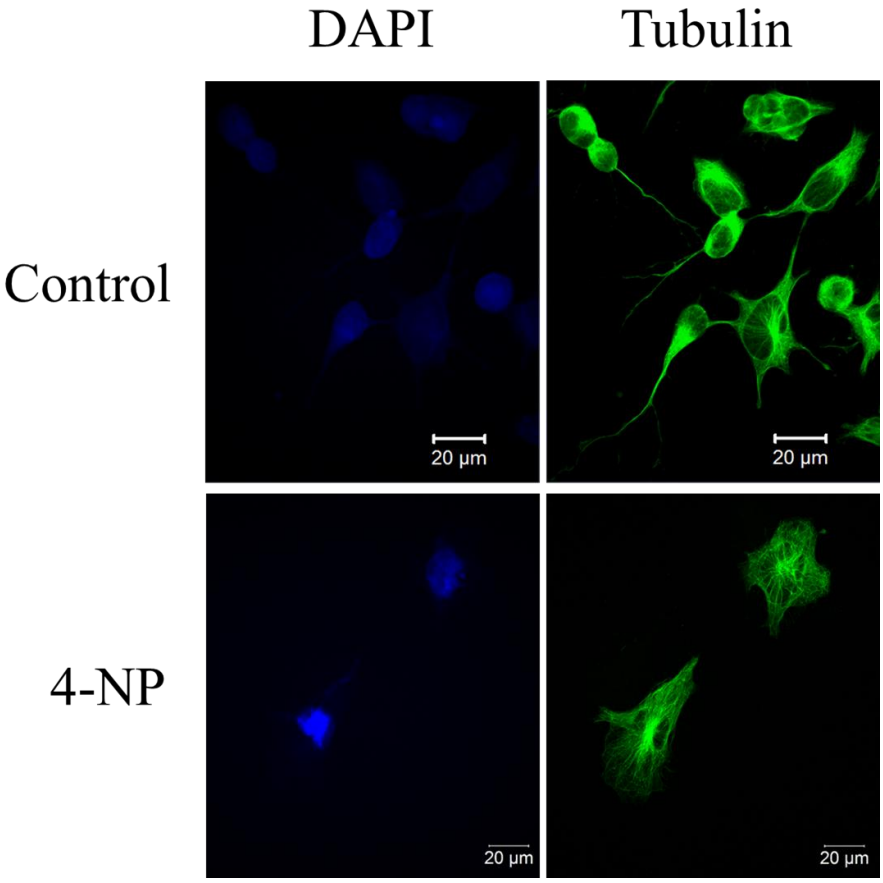

Fig. 7. 4-NP effect on tubulin expression in SHSY5Y cells. SHSY5Y cells were allowed to attach to glass coverslips followed by treatment with 4-NP overnight. Samples were processed for confocal microscopy using anti-DAPI (blue), and anti-tubulin (red). In control SHSY5Y cells, neurite formation is observed and tubulin is expressed along the neurites and cell body. In 4-NP treated cells, neurite formation is loss and an increase in the cell body is observed.

3.2.1. 3. 4-NP does not have a significant effect in SHSY5Y cells viability.

To determine if 4-NP induced neuronal cell death at the concentration used in this study (10μM), a Cytotoxicity assay was performed using the Dual Nuclear Staining DNS) assay as described in the method. SHSY5Y cells were treated with different concentrations of 4NP overnight. Cells were prepared for cytotoxicity analysis using Hoechst and PI at a final concentration of 1μg/mL Samples were then analyzed using IN Cell Analysis 1000. Hydrogen peroxide were used as positive controls. 4-NP at 10uM shows the same level of cell death (10%) as control cells (in the absence of 4-NP) (**Fig. 7**). These results suggest that 4-NP at 10μM concentration is not toxic to cause cell death. Therefore, the results observed on the SHSY5Y cells by the exposure of 4-NP are not due to cell death.

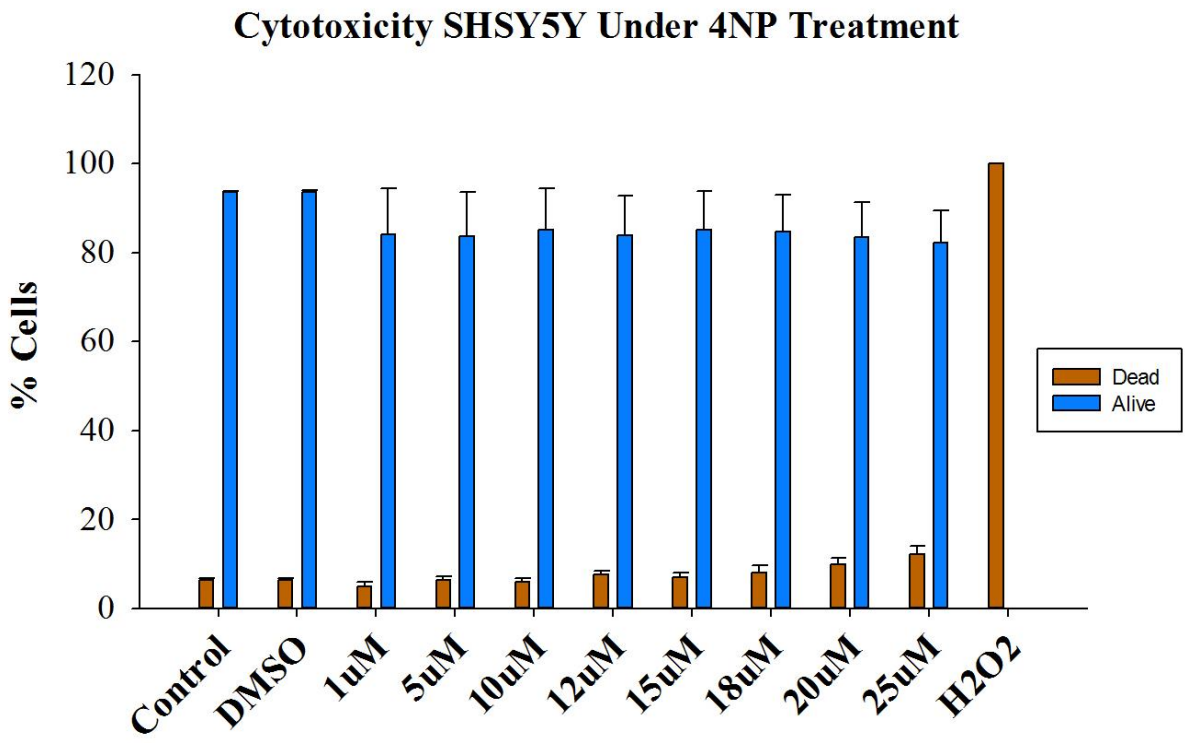

Fig. 8. Effect of 4-NP on SHSY5Y cells viability. SHSY5Y cells were treated with different concentrations of 4NP overnight (1uM, 5uM, 10uM, 12uM, 15uM, 18uM, 20uM, and 25uM), as well as DMSO and H2O2 at 1uM (positive and negative controls). Cells were prepared for cytotoxicity analysis using Hoechst and PI at 1mg/mL Samples were then analyzed using IN Cell Analysis 1000. 4-NP at 10uM shows the same level of cell death (10%) than cells under normal conditions and cells under DMSO treatment. Cell death percentage starts to increase at 4-NP 20uM concentration.

Proteomic analysis of cytoskeleton

Proteomic analysis reveals that 4-NP alters the profile of cytoskeletal proteins, and G protein subunits in NGF-differentiated cells (Carreon 2017). Many proteins in AD, PD, and HD pathways were significantly up-/down-regulated in CSK after treatment with 4-NP. Reviewing the proteomic results we identified the following potential targets by 4-NP for inducing neurodegeneration (**Table-I**). Future studies will involve understanding the role of these proteins in 4-NP-induced neurodegeneration.

TABLE-I: Potential targets by 4-NP for inducing neurodegeneration

Protein ID	4-NP Effect	Cellular function
Tau	protein expression increases (Immunoblot analysis)	Microtubule-associated protein known to be involved in neurodegeneration, Forms NFT, Taupathies
Protein DJ1(Perk7)	CSK association upregulated	involved in Parkinson disease, DJ1 immunoreactivity in Taupathies
Carbonyl reductase 1-Cbr1	NGF-downregulated NGF+4-NP-upregulated	Essential for neuronal cell survival
MAP1b	Upregulated in NGF treated cells	Involved in MT assembly, associated with Aβ-mediated synaptic dysfunction
Huntington interacting protein 1	Downregulated in the presence of 4-NP	involved in clathrin-mediated endocytic trafficking Abnormal endocytic trafficking found in AD and PD

3.1.2.3 4-NP increases Tau protein expression and decreases the interaction between Tau and Tubulin protein in SHSY5Y cells.

To determine if 4-NP affects tau expression and its interaction with MTs in SHSY5Y cells, cells were seeded in 100mm plates, followed by treatment with 4-NP as indicated in the figure (**Fig. 9 a, b**). Whole-cell lysates (WCL) were prepared as indicated in the methods to determine if tau expression is altered in the presence of 4-NP. The co-immunoprecipitation (CO-IP) analysis was used to determine the interactions between tubulin and tau as described in the method. Briefly, the monoclonal anti-tubulin antibody was used for immunoprecipitation. Immunocomplex was then subjected to western blotting using anti-tau antibody (**Fig. 9 a, b**). This experiment was repeated two times with duplicates and statistical analysis was carried out using one-way ANOVA. Controls represent 100%. Tau expression was increased after 4-NP. The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = 0.031$). Tau interaction with tubulin was decreased after 4-NP exposure. The differences in the mean values among the sample groups are greater than would be expected by chance; there is a statistically significant difference ($P=0.044$).

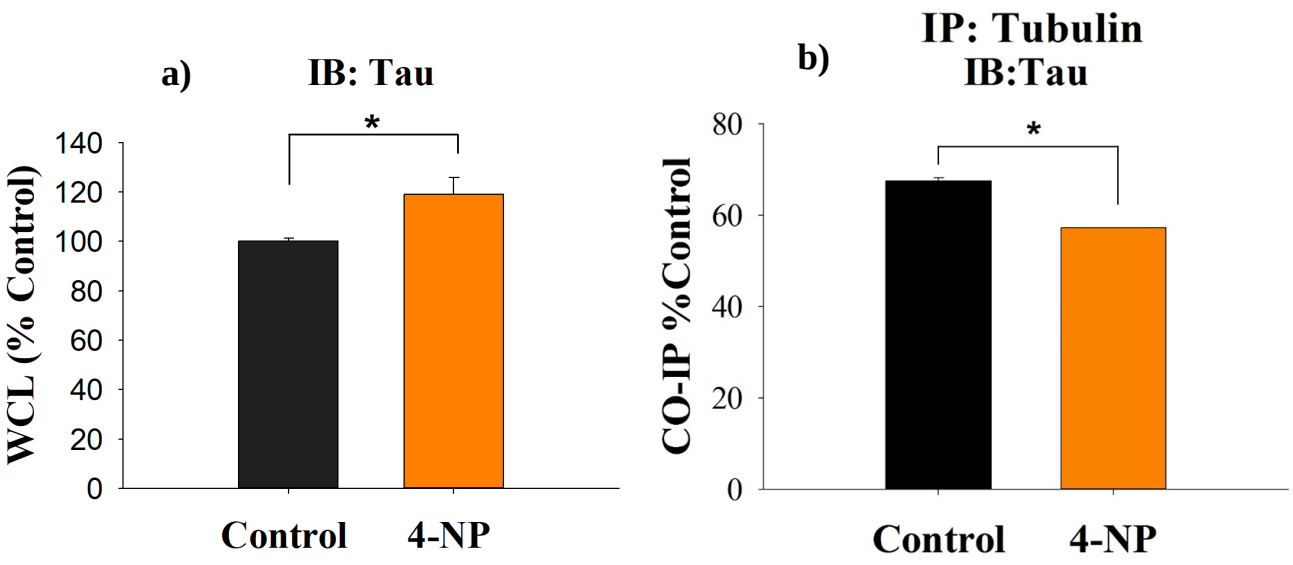

Fig. 9 Tau protein expression is increased and its interaction with MT decreased in SHSY5Y after 4-NP treatment. Effect of 4-NP on the expression of Tau and the interaction between Tau and Tubulin protein in SHSY5Y cells. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with or without 4-NP overnight. Whole-cell lysates and Co-IP samples were prepared as described in the methods. 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.

3.1.2.4 4-NP disrupts MT and Tau organization on SHSY5Y cells.

To determine the effects of 4-NP in MT and Tau organization, as well as neurite formation in SHSY5Y cells, SHSY5Y cells were seeded in 12-well plates with glass coverslips overnight to allow for cell attachment, followed by 4-NP (10 µM) treatment overnight, as indicated in the figure (**Fig. 10**). Cells were then fixed by methanol protocol and labeled with monoclonal anti-tubulin (green), and anti-tau (red) primary antibodies, and processed for confocal microscopy. In control cells, tubulin and tau are colocalized throughout the cell body and the neurites of the cell. After 4-NP treatment, neurite formation is inhibited, aggregation of cells is observed and the colocalization and general organization of the MT and tau proteins are altered. Both tubulin and tau proteins are being localized mainly in the cell membrane, instead of thought the cell. These results clearly suggest that 4-NP disrupts MTs and tau organization, inhibits neurite formation and disrupts the MT organization in SHSY5Y cells.

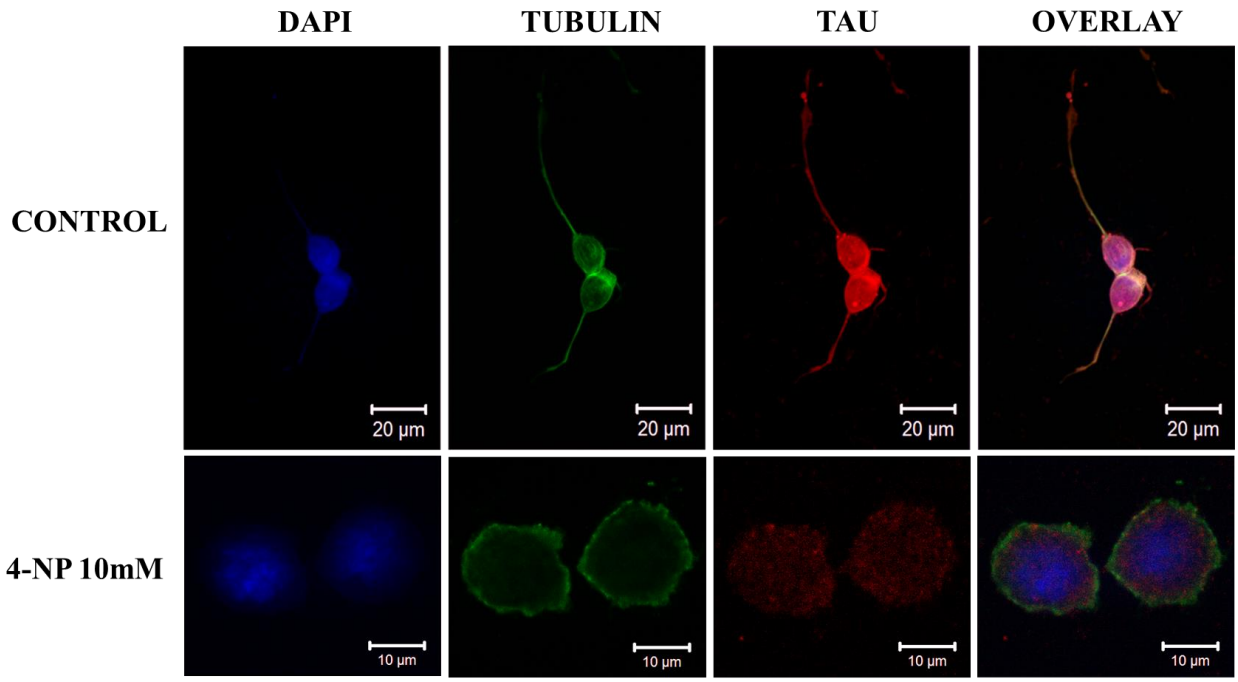

Fig. 10. 4-NP disrupts MT and Tau organization in SHSY5Y cells. Effect of 4-NP on the organization of Tubulin and Tau in SHSY5Y cells. Samples were processed for confocal microscopy using anti-DAPI (blue), anti-tubulin (green), and anti-tau (red). Colocalization between Tau and tubulin was observed mainly in the neuronal processes in the untreated cells. In 4-NP treated cells suffered a change in morphology, a loss of neurites is observed, and even when colocalization of Tubulin and Tau is still observed, it is more prevalent on the cell membrane of the cells.

3.1.2.5 4-NP does not have a significant direct effect in tubulin polymerization in vitro.

To determine if the effects of 4-NP in MTs was caused directly by 4-NP, an in vitro MT polymerization assay was performed using the Tubulin Polymerization Assay Kit by Cytoskeleton Inc. Tubulin molecules were mixed along with PEMP buffer, and 4NP. For this experiment nocodazole was used as a negative control, and Taxol was used as a positive control. The results were observed using a fluorometer. Even when some inhibition of MT assembly can be observed in the graph, it is not significant compared with the control sample (**Fig. 11**). The result indicates that 4-NP does not have any direct effect on MT assembly in vitro. Therefore, the effects of 4-NP in MT disruption as observed in PC12 and SHSY5Y cells, are more likely to be mediated by tau, Gβγ and downstream signaling pathways as described in this study.

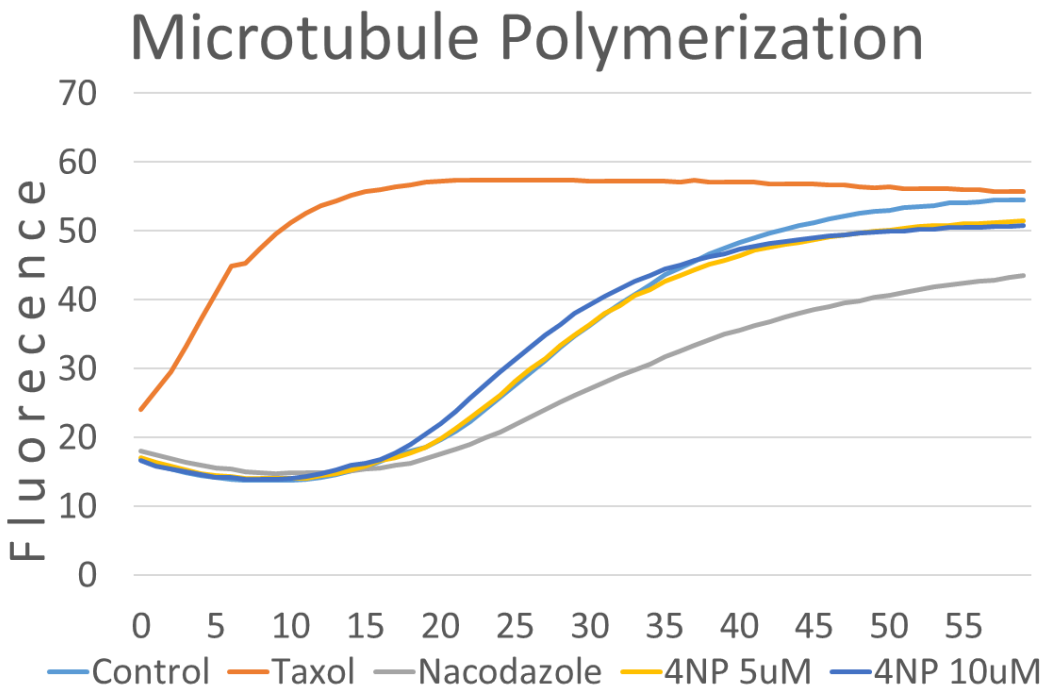

Fig. 11. Polymerization of MT in vitro is not significantly altered by 4-NP exposure. A representative graph of the effect of 4-NP on the assembly of tubulin subunits in vitro. Using the Tubulin Polymerization Assay Kit by Cytoskeleton Inc. the samples were treated with taxol, (orange) nocodazole, (gray) buffer (light blue) or 4-NP at 5uM (yellow) or 10uM (dark blue). Samples treated with 4-NP show a partial assembly inhibition of tubulin compared with the control samples.

3.2 Specific aim 2: Determine if 4-NP interferes with Gβγ/PI3K dependent signaling pathway to promote cytoskeletal disruption and neurodegeneration.

3.2.1 Objectives and Overview:

The goal of this aim is to determine the signaling pathway by which 4-NP interferes with MT assembly and neurite outgrowth and induces neurodegeneration. Recently, it was found that the interaction of Gβγ with MTs is important for nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells and that the blocking of the interaction between Gβγ and tubulin/MT disrupted MTs, inhibited neurite outgrowth and induced axonal damage indicating the involvement of Gβγ in these processes. NGF-induced neuronal differentiation of PC12 cells also activates receptor tyrosine kinase (TrkA) and its downstream effector Phosphatidyl inositol-3-kinase (PI3K). Activation of PI3K promotes the phosphorylation of downstream AKT and GSK-3β, which subsequently participate in the regulation of neurite outgrowth, and are particularly associated with microtubule remodeling. Overexpression of Gβγ in PC12 cells has been shown to induce neurite outgrowth (Sierra-Fonseca et al.,2014) and phosphorylate Akt and GSK3β supporting the notion that Gβγ and PI3K pathway co-ordinate to regulate MT assembly and neurite outgrowth. 4-NP has been shown to inhibit tubulin-Gβγ interactions, disrupts MTs/Gβγ organization in NGF-differentiated PC12 cells (MS thesis, Cynthia Carreon). The goal of the aim-2 is to determine if 4-NP inhibits Gβγ-MT interactions in SHSY5Y cells, and interferes with the PI3K pathway both in PC12 and SHSY5Y cells. **PI3K signaling pathway in both PC12 and SHSY5Y cells (erase)** Both PC12 cells and neuronal cells SHSY5Y were used to accomplish this aim using the following sub-aims.

(1) *Determine whether 4-NP inhibits the interaction of Gβγ and tubulin/MT interaction in SHSY5Y cells.*

(2) *Determine if 4-NP interferes with the phosphorylation of Akt and GSK3β in SHSY5Y cells.*

3.2.2 Results.

3.2.2.1 4-NP disrupts tubulin/MT and Gβγ organization on SHSY5Y cells.

To analyze the effect 4-NP has on the organization and localization of Gβ in SHSY5Y cells, SHSY5Y cells subjected to 4-NP (10uL) treatment overnight were subjected to confocal microscopy as described above. Methanol fixation protocol was employed. Cells were incubated in Alexa Fluor 546 conjugated-Gβ primary antibody, and Alexa Fluor 488 conjugated-tubulin primary antibody. After mounting the slides with mounting media with DAPI. In control cells incubated in the absence of 4-NP, tubulin and Gβ are colocalized throughout the cell body and the neurites of the cell. After 4-NP treatment, neurite formation of cells is inhibited, and the colocalization and general organization of the MT and Gβ/tubulin proteins are altered. Both tubulin and Gβ proteins are being localized mainly in the cell membrane, instead of thought the cell (**Fig. 12)**. These results clearly suggest that 4-NP might inhibit the interaction of Gβ and tubulin/MT organization, inhibits neurite formation and disrupts the general MT organization in SHSY5Y cells.

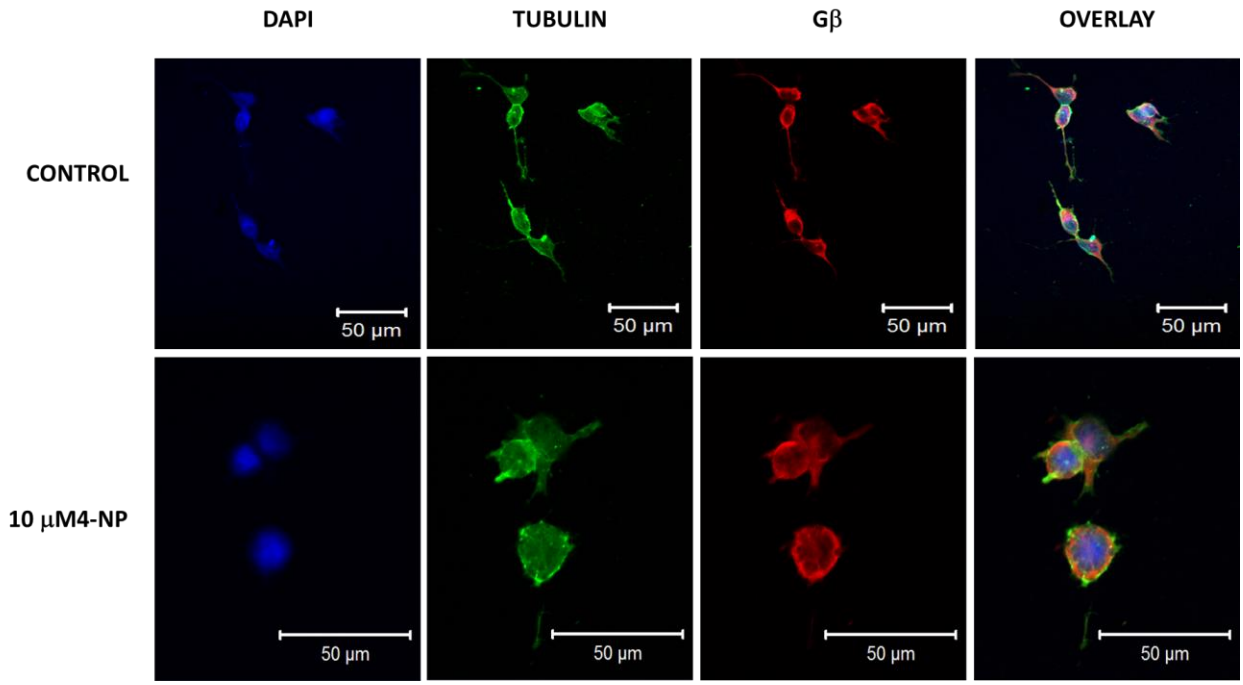

Fig. 12. Disruption of tubulin/MT and Gβγ organization on SHSY5Y cells after 4-NP treatment. Effect of 4-NP on the organization of Tubulin/MT, and Gβ in SHSY5Y cells. Samples were processed for confocal microscopy using anti-DAPI (blue), anti-tubulin (green), and anti-Gβ (red). Colocalization between Gβ and tubulin was observed mainly in the neuronal processes in the control (untreated) cells. In 4-NP treated cells suffered a change in morphology, a loss of neurites is observed, and even when colocalization of Tubulin and Gβ is still observed, it is more prevalent on the cell membrane of the cells.

3.2.2.2 4-NP increases Gβ expression and decreases its interaction with MT in SHSY5Y cells.

To determine if 4-NP affects the interaction of Gβ with tubulin and its expression levels in SHSY5Y, SHSY5Y cells were plated in 100 mm plates, following the methodology previously described, followed by overnight treatment of 4-NP at a concentration of 10μM as indicated in the figure (**Fig. 13**). Whole-cell lysate (WCL) and co-immunoprecipitation (CO-IP) samples were prepared as indicated in the methods. For CO-IP samples, the monoclonal anti-tubulin antibody was used. Samples were then processed for western blotting using anti-Gβ. This experiment was repeated two times with duplicates and statistical analysis was carried out using paired t-Test. Controls represent 100%. Gβ expression was increased after 4-NP. Gβ interaction with tubulin protein was decreased after 4-NP exposure. However, the differences in the mean values among the sample groups are not greater than would be expected by chance; there is not a statistically significant difference. These results suggest that 4-NP might inhibit the interaction of Gβ and tubulin, even when the levels of Gβ expressed by SHSY5Y cells is increased.

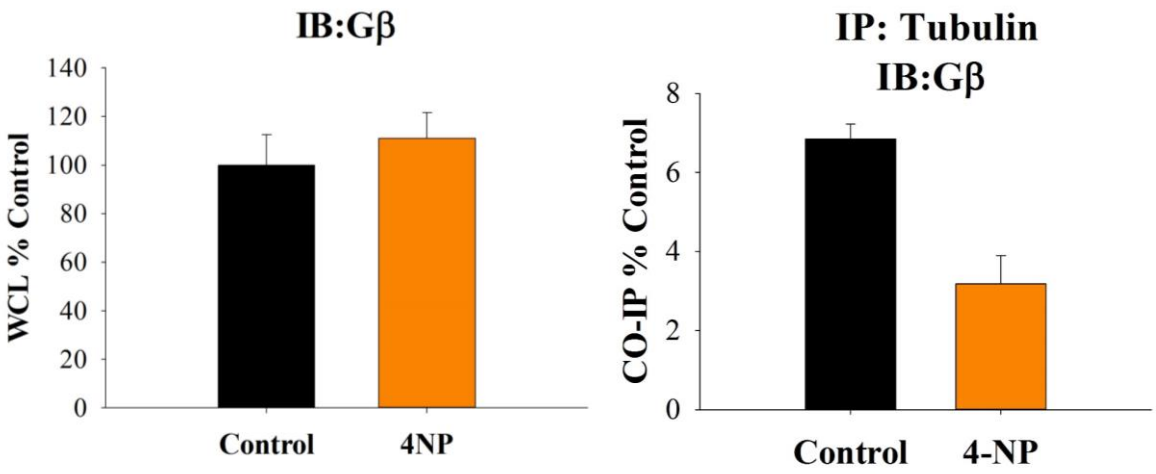

Fig. 13. Gβ expression increased while its interaction with tubulin decreases after 4-NP treatment. Effect of 4-NP on the expression of Gβ and the interaction between Gβ and Tubulin protein in SHSY5Y cells. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with or without 4-NP overnight. Whole-cell lysates and Co-IP samples 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- Gβ antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure.

3.2.2.3 4-NP decreases pAkt expression in SHSY5Y cells.

To examine the effect of 4-NP on the phosphorylation of Akt molecules in SHSY5Y cells, SHSY5Y cells were seeded in 100 cm^2 plates. Afterward, the cells were treated or not overnight with 4-NP at 10mM. Whole-cell lysates were then processed as indicated in the methods and prepared for western blotting, followed by immunoblotting with the aid if anti-Gβ antibodies. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure. This experiment was repeated two times with duplicates and statistical analysis was carried out using paired t-Test. Controls represent 100%. pAkt expression was decreased after 4-NP (**Fig.14**). However, the differences in the mean values among the sample groups are not greater than would be expected by chance; there is not a statistically significant difference. These results suggest that 4-NP might inhibit the expression of pAkt, which could suggest that it affects the PI3K pathway in SHSY5Y cells.

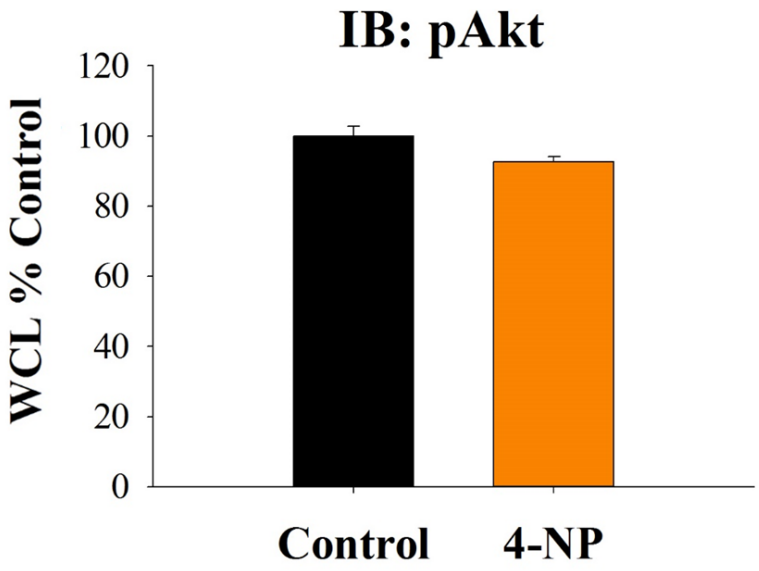

Fig.14 pAkt expression decreases after 4-NP exposure in SHSY5Y. Effect of 4-NP on the expression of pAkt in SHSY5Y cells. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with or without 4-NP at 10μM overnight. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti- Gβ antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure.

3.2.2.4 4-NP decreases pGSK3β protein expression after 4-NP treatment in SHSY5Y cells.

To determine the effect of 4-NP on the expression of phosphorylated GSK3β molecules in SHSY5Y cells, SHSY5Y cells were seeded in 100 cm^2 plates. Afterward, the cells were treated or not overnight with 4-NP at 10mM. Whole-cell lysates were then processed as indicated in the methods and prepared for western blotting, followed by immunoblotting with the aid if anti-pGSK3β antibodies. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure. This experiment was repeated two times with duplicates and statistical analysis was carried out using paired t-Test. Controls represent 100%. pGSK3β expression was decreased after 4-NP (**Fig. 15**). The differences in the mean values among the sample groups are greater than would be expected by chance; there is a statistically significant difference (P=0.046)

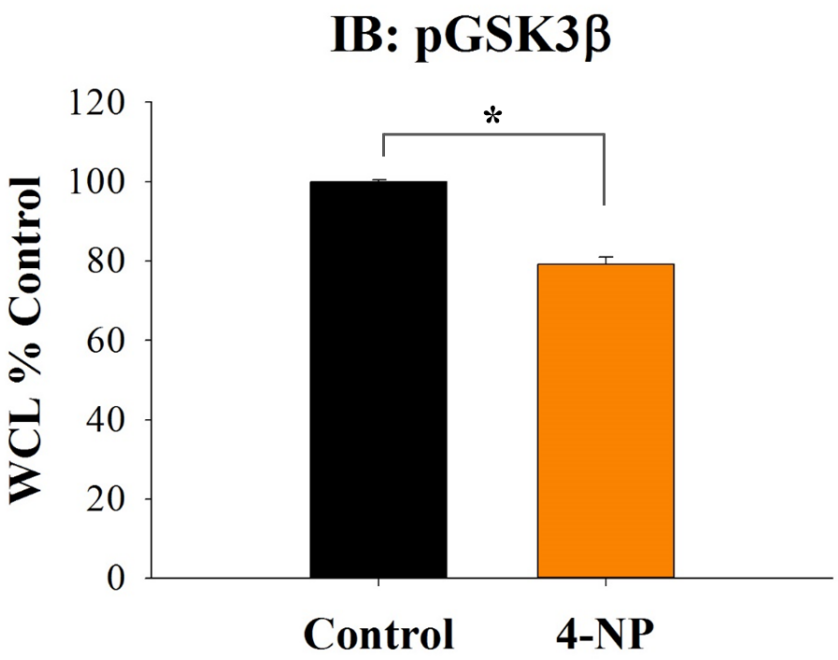

Fig. 15. pGSK3b expression decreases significantly after 4-NP treatment. SHSY5Y cells were plated in 100 mm. Subsequently, cells were treated with or without 4-NP at 10μM overnight. Whole-cell lysates were prepared for western blot as described in the methods. Equal amounts of proteins from each sample were subjected to immunoblot analysis using an anti- Gβ antibody as indicated. Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as indicated in the figure.

Chapter 4: Discussion

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 ALS. It is the most common cause of dementia among people 65 and older. Because of increasing life expectancy, the prevalence of neurodegenerative diseases is increasing rapidly. Although the speed of research has also been increased significantly, clinical trials have yielded mostly disappointing results, suggesting that focus on identifying new strategies and target(s) for therapeutic interventions should be re-evaluated. Recent evidence suggests that environmental chemicals that act as endocrine disruptors (EDC) may adversely affect brain development and induce neurodegeneration. 4-NP is an endocrine disruptor that can induce neural stem cell death and affect neurite outgrowth in Xenopus spinal cord neurons. Since cytoskeletal defect and dysfunction is the hallmark of neurodegeneration, we proposed to elucidate the cytoskeletal changes that occur in response to 4-NP and to investigate the signaling pathway by which 4-NP alters the cytoskeleton and induces neurodegeneration.

It was found that 4-NP inhibited NGF-induced neuronal differentiation of PC12 cells and disrupted MTs (**Fig.6**). Since PC12 cells are not of neuronal origin, neuronal cells SHSY5Y were used to test the effect of 4-NP and found that 4-NP has a similar effect on SHSY5Y cells (**Fig.7**). During the process of neurodegeneration, MT associated protein Tau is severely altered. Our results indicate that tau expression increases significantly when exposed to 4-NP in SHSY5Y cells and the interaction between tau and MT is significantly decreased (**Fig. 9**). These results are similar to those obtained by confocal microscopy, where it can be appreciated that the morphology of SHSY5Y cells, as well as the location/organization of the tau and tubulin

proteins, is disrupted and changes dramatically after 4-NP exposure (**Fig.7 and Fig.10**). These support the initial hypothesis that 4-NP induces neurodegeneration by disrupting the expression/localization of Tau protein and its interaction with MTs.

Dynamic rearrangement of microtubules (MTs) is critical for growth-cone motility and neurite outgrowth. Previously, Gβγ has been shown to play a key role in this process by stimulating MT assembly required for neurite outgrowth. This was further supported by the fact that blocking Gβγ-MT interaction by a Gβγ-sequestering peptide, GRK2i, inhibited neurite outgrowth and induced neurodegeneration (Sierra Fonseca et. al., 2014). The observation that 4-NP alters MT organization and its association with Gβγ suggests that 4-NP induces neurodegeneration by affecting the Gβγ-MT mediated pathway. Our results indicate that after 4-NP exposure, not only is Gβ expression decrease, but also the expression of downstream effectors of the Gβγ modulated pathway, PI3K, show a significant decrease, specifically pGSK3β. These results support the proposed hypothesis that 4-NP dependent MT disruption and neurodegeneration involves the interaction with Gβγ and the effectors of the PI3K pathway in order to induce neurodegeneration.

In summary, I have demonstrated that 4-NP at a concentration of 10μM was potent enough to induce neurodegeneration by the PI3K/pAkt/pGSK3β pathway. Using the neuronal SHSY5Y cell line as a model system, this study clearly demonstrates the potential risk of 4-NP in inducing neurodegeneration and should provide essential information to assess the environmental risk of 4-NP and regulate the production of it.

References

Amniai L., Barbier, P., Sillen, A., Wieruszeski, J.M., Peyrot, V., Lippens, G., and Landrieu, I. (2008). Alzheimer disease specific phosphoepitopes of Tau interfere with assembly of tubulin but not binding to microtubules. *FASEB J.* 23, 1146–11452.

Bevan, C.L., Porter, D.M., Schumann, C.R., Bryleva, E.Y., Hendershot, T.J., Liu, H., Howard, M.J., Henderson, L.P. (2006). The endocrine-disrupting compound, nonylphenol, inhibits neurotrophin-dependent neurite outgrowth. Endocrinology. 147, 4192-204.

California Environmental Protection Agency. Office of Environmental Health Hazard Assessment. 2009. Toxicological profile for nolylphenol

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science. 296, 1655-1657.

Carreon, C. 4-Nonylphenol Induces Neurodegeneration by Altering Cytoskeleton. Master thesis. Biological sciences, UTEP 2017

Dehmelt L., Halpain S. The MAP2/Tau family of microtubule-associated proteins. *Genome Biology*. 2005;6(1):p. 204. doi: 10.1186/gb-2004-6-1-204.

Ferrara F, Fabietti F, Delise M, Bocca AP, Funari E. (2001) Alkylphenolic compounds in edible molluscs of the Adriatic Sea (Italy). Environ Sci Technol. 35153109-3112

Gilles Breuzard, Pierre Hubert, Roqiya Nouar, Tiphany De Bessa, François Devred, Pascale Barbier, James N. Sturgis, Vincent Peyrot (2013) Molecular mechanisms of Tau binding to microtubules and its role in microtubule dynamics in live cells. Journal of Cell Science 2013 126: 2810-2819; doi: 10.1242/jcs.120832

Geeta J. Gautam, Radha Chaube & Keerikkattil Joy (2015) Toxicity and tissue accumulation of 4-nonylphenol in the catfish Heteropneustes fossilis with a note on prevalence of 4- NP in

water samples, Endocrine Disruptors, 3:1, e981442, DOI: 10.4161/23273747.2014.981442

George J. Siegel, Bernard W. Agranoff, Stephen K. Fisher, Michael D. Uhler (6th edition) Basic Neurochemistry: Molecular, Cellular, and Medical Aspects.

Geraldo, S., Gordon-Weeks, P. R. 2009. Cytoskeletal dynamics in growth-cone steering. *J. Cell. Sci.* 122, 3595–3604.

Gustav-Rothenberg, K., Lerner, A., Bonda, D.J., Lee, H.G., Zhu, X., Perry, G., Smith, M.A. (2010). Biomarkers in Alzheimer's disease: past, present and future. Biomark Med. 4, 15–26.

Herbert MK, Eeftens JM, Aerts MB, Esselink RA, Bloem BR, Kuiperij HB, et al. (2014) CSF levels of DJ-1 and tau distinguish MSA patients from PD patients and controls. Parkinsonism Relat Disorder; 20(1):112–5. 10.1016/j.parkreldis.2013.09.003.

Jie X, Jianmei L, Zheng F, Lei G, Biao Z, Jie Y. (2013)Neurotoxic effects of nonylphenol: A Review. J Wien Klin Wochenschr. 125(3-4):61–70. doi:10.1007/s00508-012-0221-2. PMID:23334477

Johnson, G.V.W., Stoothoff, W.H. Tau phosphorylation in neuronal cell function and dysfunction.*J Cell Sci*. 2004;117:5721–5729

Kadavath, H., R.V. Hofele, J. Biernat, S. Kumar, K. Tepper, H. Urlaub, (2015). Tau stabilizes microtubules by binding at the interface between tubulin heterodimers Proc. Natl. Acad. Sci. U. S. A., 112 , pp. 7501-7506

Kazemi S (2018) The correlation between nonylphenol concentration in brain regions and resulting behavioral impairments. Brain Research Bulletin. 139, 190-196

Kevenaar, J. T., & Hoogenraad, C. C. (2015). The axonal cytoskeleton: from organization to function. *Frontiers in molecular neuroscience*, *8*, 44. doi:10.3389/fnmol.2015.00044

Knepper, TP (2003) Analysis and fate of surfactants in the aquatic environment

Kudo, C., Wada, K., Masuda, T., Yonemura, T., Shibuya, A., Fujimoto, Y., et al. 2004. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J Neurochemistry 88,1416–23.

Li Xu, Ying G-G, Zhao J-L, Chen Z-F, Lai H-J, and Su H-C. 4-Nonylphenol, bisphenol A and triclosan levels in human urine of children and students in China, and the effects of drinking these bottled materials on the level. (2013) Environment International 52, 81-86

Lei, P., Ayton, S., Finkelstein, D.I., Adlard, P.A., Masters, C.L., Bush, A.I. Tau protein: relevance to Parkinson's disease. *Int J Biochem Cell Biol*. 2010;42:1775–1778

Mao Z, Zheng XF, Zhang YQ, Tao XX, Li Y, Wang W. (2012) Occurrence and biodegradation of nonylphenol in the environment. Int J Mol Sci. 13:491–505. 10.3390/ijms13010491.

Martin, E.R., Scott, W.K., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C. et al, Association of single-nucleotide polymorphisms of the tau gene with late-onset Parkinson disease. *JAMA*. 2001;286:2245–2250

Martinez Jurado, Jessica. Endocrine disrupting compound 4-Nolylphenol and neurodegeneration. Master thesis. Biological sciences, UTEP 2013.

Matsunaga H., Mizota, K., Uchida, T., Ueda, H., (2010) Endocrine disrupting chemicals bind to a novel receptor, microtubule-associated protein 2, and positively and negatively regulate dendritic outgrowth in hippocampal neurons. Journal f Neurochem. 1;114(5):1333-43. doi: 10.1111/j.1471-4159.2010.06847.x

Montoya, V., Gutierrez, C., Najera, O., Leony, D., Varela-Ramirez, A., Popova, J., Rasenick, M. M., Das, S., Roychowdhury, S. (2007). G protein betagamma subunits interact with alphabeta- and gamma-tubulin and play a role in microtubule assembly in PC12 cells. Cell Motil. Cytoskeleton. *64,* 936–950.

Palop J.J., Chin J., Mucke L. (2006) A network dysfunction perspective on neurodegenerative diseases. Nature. 443:768.

Przedborski S., Vila M., Jackson-Lewis V. Neurodegeneration: what is it and where are we? J. Clin. Invest. 2003;111:3–10.

Roychowdhury, S., Rasenick, M. M. 1997. G protein beta1gamma2 subunits promote microtubule assembly. *J. Biol. Chem.* 272, 31476–31581.

Scott, W.K., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Lyons, K. et al, Complete genomic screen in Parkinson disease: evidence for multiple genes. *JAMA*. 2001;286:2239–2244

Sise S (2017) Nonylphenol in Human Breast Milk in Relation to Sociodemo-graphic Variables, Diet, Obstetrics Histories and Lifestyle Habits in a Turkish Population

Sierra-Fonseca, J.A, Najera, O., Martinez-Jurado, J., Walker, E.M., Varela-Ramirez, A., Khan, A.M., Miranda, M., Lamango, N.S., and Roychowdhury, S. (2014). BMC Neuroscience 15: 132 doi: 10.1186/s12868-014-0132-4.

Tas. R. P., Kapitein L. C. (2018)Exploring cytoskeletal diversity in neurons. Science. Vol 361 – 6399. 231-232. 10.1126/science.aat5992

U.S. Environmental Agency (2010). Overview Nonylphenol (NP) and Nonylphenol Ethoxylates (NPEs) Action Plan [RIN 2070-ZA09].

Wang, K., Wong, Y.H. (2009). G protein signaling controls the differentiation of multiple cell lineages. Biofactors. *35*, 232-238.

Wang, X., Huang, T., Bu, G., and Xu, H. (2014). Dysregulation of protein trafficking in neurodegeneration. Molecular Neurodegeneration. *9*, 31. doi: 10.1186/1750-1326-9-31

Witte, H., Bradke, F. (2008). The role of the cytoskeleton during neuronal polarization. *Curr. Opin. Neurobiol.* 18, 479–487.

Wu, E.H., Wong, Y.H. (2005). Pertussis toxin-sensitive Gi/o proteins are involved in nerve growth factor-induced pro-survival AKT signaling cascade in PC12 cells. Cell Signal. *17*, 881-890.

Xu, Jie, Yang, Wang, Jie, Yu, Hisham Hisham, Jamal, Liu, Xiao-Yun, Fan, Qi-Yuan and Yan, Li. (2010). Toxic Effect of Gestational Exposure to Nonylphenol on F1 Male Rats. 3United Nations University-International Institute for Global Health. Birth Defects Research (Part B) 89:418–428

Yoshikawa Y. (2005). Experimental behavioral tests using monkey and rat offspring born from mothers exposed perinatally to EDCs. Psychopharmacol 25:115–124.

Zhang Y-Q , Mao Z, Zheng Y-L, Han B-P, Ling-Tong Chen Jing Li 2 and Fei Li. Elevation of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 Expression in the Mouse Brain after Chronic Nonylphenol Exposure. Int. J. Mol. Sci. 2008, 9, 1977-1988; DOI: 10.3390/ijms9101977

Zhang Y-X, Yang X, Zou P, Du P-F, Wang J, Jin F, et al. Nonylphenol Toxicity Evaluation and Discovery of Biomarkers in Rat Urine by a Metabolomics Strategy through HPLC-QTOF-MS. Int J Environ Res Public Health. 2016;13

Zhou, F.Q., Zhou, J., Dedhar, S., Wu, Y.H., Snider, W.D. (2004). NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron. 42, 897-912.

Zhou, W., Zhu, M., Wilson, M.A., Petsko, G.A., Fink, A.L. The oxidation state of DJ-1 regulates its chaperone activity toward α-synuclein. *J Mol Biol*. 2006;356:1036–1048

Curriculum Vita

Michelle Alejandra Aranda-Barroso was born on April 16, 1994 in Juarez, Chihuahua, Mexico. The first daughter of Laura Barroso and Javier Aranda. She graduated from Bachillerato 2 of the Universidad de Colima in Colima, Mexico in the spring of 2012 and enetered the Univeristy of Texas at El Paso in spring 2013 with PASE scholarship. While persuing her bachelor's degree in Microbiology, she started working as a volunteer in Dr. Roychowdhury's laboratory. After grauating with her bacherlor's degree from The University of Texas at El Paso in spring 2017, 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 as a graduate assistant in the Business department.

This thesis was typed by Michelle Alejandra Aranda Barroso