## Title

The Molecular Mechanisms of Diesel Exhaust Neurotoxicity: Autophagy as a mediator of Parkinson's disease risk



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# The Molecular Mechanisms of Diesel Exhaust Neurotoxicity: Autophagy as a mediator of Parkinson's disease risk

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Lisa Marie Barnhill

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ABSTRACT OF THE DISSERTATION

The Molecular Mechanisms of Diesel Exhaust Neurotoxicity:

Autophagy as a mediator of Parkinson's disease risk

by

Lisa Marie Barnhill

Doctor of Philosophy in Molecular Toxicology

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Professor Jeff Bronstein, Chair

Parkinson's disease is the second most common neurodegenerative disorder behind Alzheimer's disease and is characterized by the loss of dopaminergic neurons in the substantia nigra. The pathological hallmark of Parkinson's disease is the accumulation of insoluble aggregates called Lewy bodies which contain the protein alpha synuclein. Because the vast majority of disease cannot be attributed to genetics alone, there is significant interest in environmental modifiers of disease risk. Epidemiological evidence is highlighting an association between long-term exposure to air pollutants and Parkinson's disease risk. This project explores

the mechanisms of toxicity that may link exposure to air pollutants and Parkinson's disease by addressing how diesel exhaust, a major contributor to urban air pollution, may induce neurotoxicity by upregulating gene expression, inhibiting protein turnover, and inducing neuroinflammation. In human cell culture, treatment with diesel exhaust particulate extract is shown to induce gene expression by upregulating promoter activity. In rat neurons and developing zebrafish larvae, synuclein protein is seen to be elevated after exposure. Using a zebrafish model for neuronal toxicity, it has been determined that exposure to diesel exhaust particulate extract causes a significant decrease in neuron number. Both the elevated protein levels and the neurotoxicity have additionally been shown, at least in part, to be due to reduced autophagic flux. When taken together, it can be seen that exposure to traffic-related air pollution can cause synuclein dysregulation, induce neuronal toxicity, and disrupt proteostasis in ways that contribute to neurotoxicity *in vivo*. Such dysregulation may, in part, explain how long-term exposure to traffic-related air pollution increases Parkinson's disease risk and opens up new avenues for exploring disease-modifying therapeutics that may mitigate environmental toxin exposures and promote neuroprotection.

The dissertation of Lisa Marie Barnhill is approved:

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List of Abbreviations

aSyn- alpha synuclein

BCA- bicinchoninic acid

BSA- bovine serum albumin

CMV- cytomegalovirus

CNS- central nervous system

DAPI- 4',6-diamidino-2-phenylindole

DEPe- diesel exhaust particulate extract

DMSO- dimethyl sulfoxide

dpf- days post fertilization

elfa- elongation factor 1B

ELISA- enzyme linked immunosorbent assay

FACS- fluorescence activated cell sorting

Fbxo7- F-box protein 7

GBA- beta-glucocerebrosidase

GWAS- genome-wide association study

$H_20_2$- hydrogen peroxide

hpf- hours post fertilization

IHC- immunohistochemistry

KCN- potassium cyanide

LC3I/II- microtubule-associated proteins 1A/1B light chain 3B

LRRK2- leucine-rich repeat kinase 2

mpeg1- macrophage expressed gene 1

MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTS- mitochondrial targeting sequence

NGS- normal goat serum

PAHs- polycyclic aromatic hydrocarbons

P/E- pepstatin A and E64D

PFA- paraformaldehyde

PI- propidium iodide

PrP- prion protein

ROS- reactive oxygen species PD- Parkinson's disease

SN- substantia nigra

SNARE- soluble NSF attachment protein receptor

sncg1- synuclein gamma 1

SNP- single nucleotide polymorphism

SRM- standard reference material

TBP- TATA binding protein

TH- tyrosine hydroxylase

UCH-L1- ubiquitin C-terminal hydrolase L1

UPS- ubiquitin proteasome system

vmat2- vesicle monoamine transporter

WT- wild type

ZF- zebrafish

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# Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SN). The pathological hallmark of PD is the accumulation of intracytosolic inclusions called Lewy bodies and Lewy neurites, which contain the neuronal protein alpha synuclein (aSyn) and are heavily ubiquitinylated (Ohama et al 1976, Spillantini et al 1997). This neuronal loss and protein accumulation leads to progressive motor symptoms including resting tremor, rigidity, and postural instability and results in cognitive decline over time. Although dopaminergic neurons appear to be particularly vulnerable to cell death in PD, other types of neurons have also been shown to accumulate alpha synuclein and may be involved in non-motor symptoms typical of the disease including slower gut motility, loss of smell, sleep disturbances, and hallucinations (Edwards et al 1991, Hawkes et al 1997, Stiasny-Kolster et al 2005).

PD has been described as a prion-like disease where the templating of misfolded alpha synuclein begins in the periphery, likely the gut and/or olfactory bulb, and spreads through a network of interconnected neurons similar to other neuronal proteins such as prion protein (PrP) (Del Tredici et al 2016, Goedert et al 2015). This is consistent with the Braak model of PD, which describes a staging of disease based on the progression of alpha synuclein pathology beginning in the brain stem and olfactory bulb in stage 1, moving through the SN leading to motor symptoms in stage 3, and eventually, in the most severe manifestation of disease, can be found throughout the neocortex and is associated with a variety of mental deficits in stage 6 (Braak et al 2003, Hawkes et al 2009).

The pathological spread and mistemplating of alpha synuclein has been supported experimentally in several ways. Adding misfolded aSyn to neurons in culture, for example, can lead to the misfolding of endogenously expressed aSyn and also spread in fibrillary form from neuron to neuron (Freundt et al 2012). The transplantation of dopaminergic neurons has been attempted in order to relieve motor symptoms in PD patients and, within a relatively short period of time, these newly grafted neurons can be seen to have Lewy body pathology (Li et al 2008, Kordower et al 2008). Additionally, toxin and synuclein-based mouse models of PD initiated in the gut show at least partial rescue of motor symptoms by severing the vagus nerve, which connects the gut and the brain (Pan-Montojo et al 2012, Holmqvist et al 2014). Lastly, there has been evidence that individuals who have had a vagotomy are less likely to develop PD (Svensson et al 2015, Liu et al 2017). This evidence is indicative of a prion-like spread of protein misfolding from peripheral neurons into the brain stem and strongly supports the Braak model of PD. Indeed, with the knowledge that aSyn pathology can begin in the gut and olfactory bulb, there is increasing evidence to suggest that environmental exposures may play an important role in peripheral aSyn dysregulation that may spread in a prion-like fashion toward to substantia nigra (Klingelhoef and Reichmann 2015).

*The Role of Alpha Synuclein in PD*

The normal role of aSyn in neurons is not well understood, but probably involves facilitation of neurotransmitter release and synaptic vesicle recycling as aSyn is often found in synaptic vesicles and is associated with SNARE complex assembly (Burre et al 2010, Bendor et al 2013) Although the accumulation of aSyn inclusions represents the pathological hallmark of PD, little is known about the exact role of aSyn in causing dopaminergic neuron death (Baba et

al 1998). One of the early animal models of PD involved exposing mice to the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine also known as MPTP. MPTP is a potent neurotoxin that acts through mitochondrial complex I inhibition and selectively kills dopaminergic neurons (Langston et al 1983, Javitch et al 1985). In this exposure model, aSyn null mice have shown resistance to the neurotoxicity induced by MPTP exposure (Dauer et al 2002). It appears that soluble, monomeric aSyn as well as insoluble, aggregated aSyn do not directly cause neurotoxicity. Rather, current research suggests that an intermediate oligomeric species which is present in the brain of those with PD represents the toxic moiety of misfolded aSyn (Conway et al 2000, Bertoncini et al 2005, Karpinar et al 2009, Winner et al 2011, Bartels et al 2011, Roberts et al 2015). There also exist many known post-translational modifications of aSyn that alter the tendency of the protein to aggregate and many are known to be associated with disease (Barrett et al 2015). The protein aSyn exists in combination with molecular chaperones that prevent misfolding. In addition, it is degraded both by autophagy and the ubiquitin proteasome system (UPS) (Webb et al 2003, Mak et al 2010, Ebrahimi-Fakhari et al 2011). Disturbances in the level of aSyn protein, post-translational modification, or rate of degradation can perturb the balance of aSyn within neurons and is thought to be associated with toxic aggregate formation (Barrett and Greenamyre 2015.

It is also known that the total amount of aSyn protein is closely associated with risk of developing disease. This is evidenced most clearly by the fact the aSyn gene duplication and triplication along with polymorphisms that cause increased expression are directly linked to familial PD (Singleton et al 2003, Chartier-Harlin et al 2004, Hadjigeorgiou et al 2006). More protein leads to increased risk of aggregation and increased formation of toxic species. One of the stronger models of PD in rodents involves the overexpression of human aSyn protein in the

brain, which suggests that aSyn overexpression alone is sufficient to lead to dopaminergic neuron death and the accumulation of insoluble aSyn aggregates indicative of disease (Masliah et al 2000, Giasson et al 2002, Kirik et al 2002, Lo Bianco et al 2002). In rodents, injecting aSyn peripherally is sufficient to cause motor deficits and CNS pathology (Sacino et al 2014).

The dysregulation of aSyn is known to be associated with other phenomena linked to PD, such as mitochondrial disfunction and neuroinflammation (Rocha et al 2017). It has been shown that aSyn contains a mitochondrial targeting sequence (MTS) and that pathologic forms of aSyn can prevent protein import and eventually cause mitochondrial toxicity (Devi et al 2007, Di Maio et al 2016). Associated with this toxicity is an increase in reactive oxygen species (ROS) and changes in mitochondrial bioenergetics that have been shown to occur in the brains of PD patients (Winklhofer and Haass 2010, Rocha et al 2017). As aSyn protein aggregates, it has also been shown to activate microglia, which represent the resident immune cells in the brain, and this activation leads to further ROS production and the release of pro-inflammatory cytokines thought to further facilitate the disease progression (Gao et al 2011, Hirsch and Hunot 2009, Hoffmann et al 2016, Zhang et al 2016).

*Disease Etiology and Genetics*

The causes of PD are complex and much remains unknown regarding disease etiology. In general, the greatest risk factor for PD is age (Hindle 2010). The average age of diagnosis with PD is 60 and at this point, dopaminergic neuron loss in the SN is already well underway. The latency period for PD is also substantial and it is thought that, at diagnosis with motor symptoms typical of dopaminergic neuron deficit in the SN, the disease has likely been progressing for a decade or longer.

Much of what is known about PD etiology comes from several infrequent genetic mutations that are associated with familial forms of PD. Genetic causes, in general, can only account for approximately 10% of disease incidence, but these fairly rare mutations give insight into the most important molecular pathways that confer susceptibility, including alterations in aSyn, disruption of degradative pathways, and alterations in mitochondrial homeostasis.

The gene most clearly associated with familial PD is aSyn. Gene duplication and triplication are known causes of rare inherited forms of Parkinson's disease (Chartier-Harlin et al 2004, Singleton et al 2003). Additionally, polymorphisms in the promoter region of aSyn that lead to an increase in protein levels, such as the Rep1 repeat, and non-coding regions of the gene, confer risk of developing PD and also can reduce the age of disease onset (Hadjigeorgiou et al 2006). There are also several mutations in the coding region of aSyn that are thought to impact protein modification, aggregation, and stability. These mutations include A53T, A30P, and E46K, among others, which are thought to increase protein phosphorylation and alter the ability of aSyn to oligomerize and form aggregates (Polymeropoulos et al 1997, Kruger et al 1998, Nahri et al 1999, Zarranz et al 2004, Choi et al 2004, Ono et al 2011).

In addition to alterations in aSyn, mutations in genes associated with protein degradation pathways have been implicated in PD as well. One of the biggest risk factors for developing PD is a mutation in the β-glucoceribrosidase (GBA) gene that causes Gaucher's disease (Nichols et al 2009), which is a lysosomal storage disorder. Mutations in the genes coding for parkin1 (Abbas et al 1999, Farrer et al 2001) and LRRK2 (Zimprich et al 2004, Di Maio et al 2018) have the potential to alter turnover of aggregation-prone proteins like alpha synuclein and are also associated with increased disease risk, implicating the process of autophagy in disease etiology.

From this genetic information, it is clear that altering synuclein homeostasis in a way that allows excessive protein accumulation can, in and of itself, lead to manifestation of disease.

Lastly, and in a similar way to MPTP, many known genetic causes of disease involve mitochondrial health. When mitochondria are compromised, neurons are often the first to suffer because of their long life, extensive processes, and energy-intensive synaptic activity. The fundamental processes of mitochondrial mobility, turnover, and electron transport chain activity serve critical functions in the brain (Devine and Kittler 2018). Mutations that impact any of these aspects of mitochondrial health have been implicated in PD (Moon and Paek 2015). One of the most important aspects of maintaining a healthy pool of mitochondria is their ability to conduct fission and fusion. Indeed, mitochondria with deficits in this process become impaired and are unable to efficiently generate ATP. Inhibition of complex I of the electron transport chain has been seen in post-mortem brains of PD patients, indicating that mitochondrial impairment is involved in disease (Schapira et al 1990, Parker et al 2008). PINK1/Parkin are important proteins involved in the process of mitophagy, or the turnover of impaired or dysfunction mitochondria. When mitochondria become impaired, the activity of PINK1/Parkin acts to target the damaged mitochondria for degradation (Narendra et al 2010, McLelland et al 2013). Mutations in PINK1 and Parkin are associated with young-onset, familial PD (Jin et al 2013, Kitada et al 1998, Valente et al 2004, Pridgeon et al 2007). Additionally, when mitochondria are under stress, they often release dangerous reactive oxygen species (ROS), which then activates the protein DJ-1. Mutations in DJ-1 associated with PD have been shown to decrease total autophagic activity and also to increase the number of dysfunctional mitochondria within cells, indicating that proper function of DJ-1 is essential for responding to oxidative stress insults (Bonifati et al 2003, Krebiehl et al 2010, Lev et al 2008). It is becoming more evident that, although several discrete

molecular pathways are involved in monogenic forms of PD, the pathways are more overlapping than once thought. When mitochondrial dysfunction occurs, for example, there is a coordinate dysregulation of aSyn protein accompanied by changes in protein turnover (Dawson et al 2003, Hoglinger et al 2003, Betarbet et al 2006). Individual mutations such as those described above do not account for a large percentage of disease likely because there are many ways to disrupt the various pathways and it is very likely to involve gene/environment interactions that have yet to be understood.

*Gene Environment Interactions*

Although a variety of pathways have been genetically associated with disease, only a small subset of PD can be attributed to genetic causes alone. Given the extent of the genetic data, it is increasingly apparent that there remains a significant but little understood environmental component contributing to disease (Elbaz et al 2007). Some of the known risk-associated exposures in PD include common lifestyle factors such as caffeine consumption and smoking, but it is not clear what connects this epidemiological finding mechanistically (Hernan et al 2002, Tanner et al 2002, Hancock et al 2007). Some of the best studied environmentally associated risk factors for PD include a variety of pesticides that disrupt pathways associated with PD (Ritz et al 2000, Wang et al 2011). Epidemiological studies have elucidated specific exposures that contribute to disease, including pesticides such as rotenone, paraquat, maneb, ziram, and benomyl (Costello et al 2009, Wang et al., 2011). However, in a pattern similar to the genetic causes of PD, these implicated pesticides do not necessarily converge upon a single mechanism or pathway of toxicity. The heterogeneity in structure and function of the toxins implicated in PD explain why it is difficult to predict what may or may not act as an environmental influencer of PD risk.

One reason why it has proven difficult to understand the causes of idiopathic PD may be because genetic susceptibilities predispose individuals to developing PD only in the presence of a second insult or environmental exposure. Many gene/environment interactions have been discovered through rigorous and targeted testing, but it is very difficult to unearth these interactions without copious amounts of data and genetic screening. As of now, it has been seen that genetic variants can alter ones risk of developing PD after pesticide exposure (Zschiedrich et al 2009, Rhodes et al 2013, Fitzmaurice et al 2014, Narayan et al 2015). Understanding the mechanisms behind neurotoxicity can shed light on the possible gene/environment interactions that may be of interest and facilitate the discovery of new risk-modifying factors in PD.

*Adverse Outcome Pathways Involved in PD*

From what we know about the genetic and environmental risk factors associated with PD, it is possible to propose a set of pathways that are relevant to the study of disease risk. These include but are not limited to, increases in aSyn protein, alterations in autophagic flux, microglial activation and neuroinflammation, and mitochondrial inhibition and oxidative stress. It is likely that these features of disease are connected and disruption of one pathway can cause a cascade of disease-associated events. Current thinking suggests that increased synuclein aggregation leads to mitochondrial dysfunction, causing release of ROS, which can lead to the down-regulation of protein degradation through the auto-lysosomal pathway, thereby perpetuating a further increase in aSyn. These pathways can be further disrupted and perpetuated by chronic inflammation caused by aggregated synuclein and neuron death. The focus on these molecular mechanisms of disease allows us to explore adverse outcome pathways associated with PD and neurodegenerative disease without waiting for the development of motor symptoms or

dopaminergic neuron loss. By focusing on pathway disruption, we can more quickly identify environmental influencers of disease risk and also explore ways to disrupt this self-perpetuating cycle of protein accumulation, synuclein toxicity, and ROS production that leads to neuron death and the continued propagation of pathology in disease.

*Air Pollution as an Environmental Risk Factor in PD*

Pesticide studies indicate that there likely remain many undiscovered environmental and lifestyle factors that must be contributing to disease etiology. Even combining known genetic and environmental risk factors, there is a significant percentage of PD cases for which cause remains unknown. Expanding beyond pesticide exposures, epidemiological data from the United States suggest an association between rural living and PD, which seems to correspond with data regarding pesticide exposure. However, studies being done around the world do not seem to find the same correlation with rural living (Chen et al 2009, Bhidayasiri et al 2011, Totaro et al 2005, Taba & Asser, 2002,Hristova et al 2009, Gatto et al 2009). Multiple studies have even discovered an inverse association, with urban living more often associated with PD. Recent studies focusing on large cohorts and including long latency periods have uncovered correlations between neurodegeneration and air pollution. More specifically, these studies are beginning to explore the connections between long-term exposure to urban air pollutants and eventual development of neurodegenerative disease. Indeed, it is thought that two of the earliest sites of synuclein pathology are the gut and the olfactory bulb, which are also the primary routes of environmental exposure. These studies are intriguing because air pollution is a very ubiquitous exposure and has the potential to affect a very large proportion of the population and also offers

an explanation for the seemingly contradictory urban/rural studies done around the world (Heusinkveld et al 2016, Jayaraj et al 2017).

Epidemiological studies exploring environmental influencers of neurodegenerative disease risk are particularly tricky because of relatively long latency period between disease initiation and disease diagnosis. For Parkinson's disease, there is thought to be many years or even decades between presymptomatic disease onset and diagnosis. Additionally, because air pollution represents such an ubiquitous, but also heterogeneous, exposure, finding proper control groups and comparing the results of various studies can prove difficult. As a result, there have been many epidemiological studies attempting to understand the connection between exposure and risk, but some of them have found that exposure to air pollution increases risk of PD and others have found no significant effect (Palacio et al 2014, Palacio et al 2017, Ritz et al 2015, Oudin et al 2015, Kioumourtzoglou et al 2015, Lee et al 2016, Liu et al 2016, Chen et al 2017). These studies attempt to identify what components of air pollution (particulate matter, nitric oxide level, ozone exposure, etc) may be of highest interest, but there is not great consensus among the literature at this preliminary juncture. One way to complement these studies and to further understand why they may differ is to utilize small animal models to determine the molecular effects of exposure to various components of air pollution, which may inform future studies and clarify existing discrepancies in the literature.

Although little is known about the mechanisms of neurotoxicity caused by air pollution, much research has been done regarding exposure to air pollution and increased risk of developing asthma, cardiovascular disease, stroke, and dementia, among other disorders (Guarnieri and Balmes 2014, Hoek et al 2001, Mustafic et al 2012, Lee et al 2018, Di et al 2017). In light of recent evidence, it is important to understand the potential neurotoxic effects of air

pollution and how these pollutants may exacerbate adverse outcome pathways associated with disease. One of the major components of air pollution in urban environments comes from diesel exhaust. The particulate matter released from diesel engines represents an incredibly heterogeneous mixture of compounds and contains a variety of hydrocarbons, metals, and inorganic constituents. Intriguing data has come out of regions with heavy air pollution suggesting that exposure to high levels of diesel exhaust may be associated with protein aggregation and markers of neurodegeneration (Calderón et al 2008, Levesque et al 2011, Chen et al 2015). Although these studies do not directly link exposure to air pollution and neurodegenerative disease, they are of particular interest because of their environmentally relevant exposures in humans and animals and they provide necessary correlative evidence connecting markers of disease with regions exposed to heavy urban air pollution.

*Existing Molecular Evidence Linking Air Pollution and PD*

What has been established in animal model systems is limited, but also suggests reason to investigate further. For example, ultrafine particles with high levels of polycyclic aromatic hydrocarbons (PAHs) have been shown to induce neuroinflammation (Campbell et al 2009), reactive oxygen species (ROS) and mitochondrial damage (Li et al 2003, Cheng et al 2016), all of which are associated with neurodegeneration. Additionally, studies exposing rodents to particulate matter have shown an accumulation in the olfactory bulb and regions of the brain (Oberdörster et al 2004), suggesting that translocation of ultrafine particles can access and affect the brain (Lucchini et al 2012) in a manner that is consistent with the pathological spread seen in Parkinson's disease. Other animal studies using diesel exhaust have shown increases in neuroinflammation and microglial activation as well as alterations in dopamine metabolism in

rodents (Yokota et al 2013, Suzuki et al 2010), and potentiation of later environmental insults in adult rodents (Allen et al 2014). In addition, it is known that inflammation is associated with neurodegenerative diseases and that aberrant activation of neuroimmune cells can potentiate neuron loss (Block et al 2007, Amor et al 2014).

*Utilizing Zebrafish to Study Neurodegenerative Disease Pathways*

One of the limiting factors in PD research currently is the difficulty of finding an appropriate disease model that can allow for screening of environmental toxins in a short period of time while still remaining relevant to a long-term disease of aging. We have decided to utilize a zebrafish model of neurotoxicity for several reasons including the ability to quickly identify and screen toxins of interest and confirm existing epidemiological data as well as the ability to accurately and easily quantify neurons of interest due to their small size, transparent appearance, and very rapid neurodevelopment.

Because very little is known about the mechanisms of neurotoxicity associated with exposure to air pollution or diesel exhaust, a zebrafish disease model allows us to explore a variety of endpoints and dissect multiple pathways of interest using various transgenic lines and reporter genes in an efficient manner (Bandmann and Burton 2010).

*Research Direction*

What this study explores are the mechanisms connecting diesel exhaust exposure to eventual neurotoxicity phenotypes in order to better understand how long-term exposure to this ubiquitous environmental toxin may predispose individuals to developing PD. More specifically, does exposure to diesel exhaust alter adverse outcome pathways related to PD in ways that can alter synuclein homeostasis, possibly mimicking the impact of gene multiplication or

overexpression. Diesel exposures were all conducted using an organic extract from a single source of diesel exhaust particulate (DEP). These exposures to diesel exhaust particulate extract (DEPe) are an effort to understand what pathways may be dysregulated through long-term exposures to air pollution and therefore contribute, along with genetic and other environmental factors, to eventual disease onset.

Chapter I will consist of modeling DEPe exposure in the human neuroblastoma cell line SK-N-MC as well as zebrafish (ZF) larvae *in vivo* in an attempt to understand how DEPe alterst aSyn gene expression and protein level changes in physiologically relevant systems. Because overexpression of aSyn is sufficient to cause PD in humans, if exposure to DEPe alters aSyn homeostasis, it is likely that long-term environmental exposure in humans would increase risk of disease as suggested by epidemiological studies. Chapter II will focus on in vivo modeling of disease-relevant mechanisms of toxicity including neuronal loss, behavioral effects, and increased inflammation. All of these characteristics of environmental toxins provide relevant insight into possible pathways of PD related neurotoxicity. Lastly, Chapter III will explore more closely the importance of protein turnover and the autophagy-lysosomal pathway in order to explain and eventually prevent the neurotoxicity identified in earlier chapters. By understanding how autophagy functions in vivo after exposure to DEPe, it is possible to identify possible therapeutic interventions that can upregulate protein degradation and therefore prevent the initial disruption of proteostasis. Because there currently exist no disease-modifying therapies for PD, any potential drug target would be of high pharmaceutical interest.

Dissertation: Chapter 1

Diesel Exhaust Particulate Extract Exposure and aSyn Expression Levels

**Introduction**:

The accumulation of amyloidogenic proteins is a hallmark of many neurodegenerative diseases. Indeed, culprit amyloid-forming proteins such as amyloid beta, huntington, alpha synuclein, and tau can accumulate into toxic oligomers and insoluble fibrils due to gene mutations, increases in protein levels, aging, and cellular stress (Lotz et al 2013). Because of the significant genetic evidence linking alpha synuclein expression and disease risk, one of the most direct ways of influencing PD risk is by altering either the amount of the transcript or the level of protein within the brain. In fact, individual surviving dopaminergic neurons in the brains of PD patients with idiopathic disease were shown to have elevated aSyn expression levels even in the absence of known mutations (Grundemann et al 2008). Very little work has been done to link toxic environmental exposures to intracellular aSyn levels even though there is epidemiological evidence to suggest an association with disease and there is suggestive work in rodents that long-term exposure to diesel exhaust can increase markers of neurodegenerative disease including aSyn protein (Levesque et al 2011).

In addition to gene duplication and triplication, which both significantly increase the level of aSyn transcript/protein, there are several other known genetic variants that can increase transcript level and are disease-associated. The human aSyn promoter is quite long and there exist known regulatory regions that have been identified through GWAS to be associated with disease and increased transcript level (Pals et al 2004). One of the most well-known promoter risk factors is the Rep1 allele, which leads to an increase in gene expression and is associated

with disease risk and age of onset (Farrer et al 2001, Chiba-Falek et al 2001, Hadjigeorgiou et al 2005). This is one of the rare polymorphisms associated with a gene-environment interaction that increases risk in the presence of head trauma, suggesting that having more aSyn within the brain predisposes one to greater neuronal damage after inflammatory insult (Goldman et al 2012, Lee et al 2015). In light of this evidence, it is highly likely that there are undiscovered gene-environment interactions that can behave in a similar fashion to increase disease risk.

To understand more about how the human aSyn promoter functions and can be altered by toxic exposures, a luciferase assay was developed to identify substances that significantly alter promoter activity in human cell culture (Chiba-Falek et al 2001). In order to use this assay to understand environmental influencers of PD risk, we screened various pesticides and other environmental toxins using this luciferase construct and two of the stronger inducers of promoter activity identified were the pesticide rotenone and diesel exhaust particulate extract (DEPe). Rotenone has been used in animal models of PD (Betarbet et al 2000, Alam and Schmidt 2002, Sherer et al 2003, Fleming et al 2004, Cannon et al 2009) and is a known mitochondrial toxin and complex I inhibitor. It was recently shown that, in human neuroblastoma cells, exposure to rotenone induced aSyn gene expression approximately 1.5 fold by rtPCR, which corroborates the results of the luciferase assay and confirms an as of yet unknown role for rotenone in connection to alpha synuclein expression (Chorfa et al 2013, Sala et al 2015). Because nothing is known about the effect of DEPe exposure on aSyn expression, this unique finding was tested in neuronal culture using rat primary mescencephalic neurons and extracting RNA after treatment. When taken together, the data presented here showing the impact of exposure to DEPe on aSyn transcript suggests that it may be an environmental influencer of PD risk and should be further explored even in the absence of known disease-associated SNPs or gene multiplication.

**Methods**:

**Diesel exhaust particulate extract characterization**

The organic extract of diesel exhaust particles (DEPe) used for these studies was generated by sonication in methanol and then was resuspended in DMSO as described previously (Lawal et al 2015). The extract was submitted for further component analysis by Dr. James Schauer from the University of Wisconsin-Madison. Total yield for each component was calculated in a spiked sample as well as the experimental sample and adjusted for dilution factor. The % recovery of each compound was determined using a spiked sample and this was used to adjust the final concentration of each compound identified in the diesel exhaust extract. The presence of 120 compounds was analyzed and final concentration calculated. The total weight of all analyzed compounds makes up about 4.2% of the weight of the extract.

**Cell culture**

Both SK-N-MC and SH-SY5Y were grown at 37$^{o}$C and 5% $CO_2$ in DMEM with 10% FBS and supplemented with penicillin/streptomycin. For RNA experiments, cells were adhered in 6-well plates for treatment and rinsed in PBS before direct lysis with Trizol. All treatments were done in a final volume of 3-5mL and were normalized to controls containing DMSO (dimethyl sulfoxide) at concentrations equivalent to the treatment wells.

**Luciferase assay**

SH-SY5Y cells were plated in 24-well plates until 70-85% confluent in DMEM/F12 media with 10% FBS + Penicillin/Streptomycin. Media was changed immediately before transfection with FuGene6. Plasmids transfected include the control Renilla plasmid pRL-SV40 and pASP10.7

generously provided by the Nussbaum lab (Chiba-Falek et al 2001), which includes 10.7Kb of the human alpha synuclein promoter driving firefly luciferase expression including the Rep1 variant region. Protocol was conducted as per the Dual-Luciferase Reporter Assay System (Promega E1910) and is described briefly as follows. Plasmids were added 50:1 (pASP10.7:pRL-SV40) and incubated with lipid (6ul/1ug DNA) for 15 minutes at room temperature before adding to cells in 24-well plate (50uL/well with a final volume of 500uL). Incubate DNA with cells for 24hrs before adding drug treatments. After 24hrs drug exposure, cells were lysed in 1X Passive Lysis Buffer and 20uL of lysate was transferred to a black-walled 96-well plate. LARII added before firefly luciferase read and Stop & Glo reagent added before Renilla luciferase read. All data was normalized to internal Renilla control relative to vehicle treated control samples.

**Trizol RNA extraction**

Cells were harvested in 0.5-1mL cold Trizol (Thermo Fisher Scientific 15596026). RNA purification was conducted according to the product specifications; briefly, 1/5 volume chloroform was added and samples were centrifuges at 11,000xg for 15min at 4$^{o}$C and aqueous layer was separated into a fresh tube. To aqueous layer, 50% original volume of isopropanol was added followed by centrifugation at 11,000xg for 10 minutes at 4$^{o}$C. Supernatant was removed from pellet and samples were washed in 500uL 75% ethanol followed by centrifugation at 9,000xg for 5 minutes at 4$^{o}$C. Supernatant was removed and pellets were air-dried for 5-10 minutes before resuspension in 30-100uL of nuclease free $H_2O$ followed by incubation at 55$^{o}$C for 15 minutes.

**cDNA preparation**

Between 0.5 and 1ug of purified RNA was converted into cDNA using BioRad iScript Reverse Transcription Supermix (BioRad 1708841) as recommended by the manufacturer. Briefly, RNA was reverse transcribed in a final volume of 20uL at 46$^{o}$C followed immediately by 1 minute of enzyme inactivation at 95$^{o}$C. All samples within one experiment were internally normalized to DMSO as a vehicle control.

**rtPCR primers and assay**

Quantitative rtPCR was conducted in 96-well plates using a BioRad CFX Connect and SsoAdvanced Universal SYBR Green Supermix (Biorad 1725271). All data was normalized to housekeeping control genes; human and rat cDNA samples were normalized to TATA binding protein transcript (TBP). Primers used as follows:

TBP Forward Primer: 5'-TGCACAGGAGCCAAGAGTGAA-3'
TBP Reverse Primer: 5'-CACATCACAGCTCCCCACCA-3'

Human aSyn Forward Primer: 5'-TGCACAGGAGCCAAGAGTGAA-3'
Human aSyn Reverse Primer: 5'-CACATCACAGCTCCCCACCA-3'

Rat aSyn Forward Primer: 5'-GAGTTCTGCGGAAGCCTAGAGAGC-3'
Rat aSyn Reverse Primer: 5'-GTTTTCTCAGCAGCAGCCACAACTCC-3'

Samples were run for quantitative rtPCR using conditions recommended by the manufacturer. All data was calculated and analyzed using double delta Ct and normalized to internal housekeeping gene expression.

**Rat primary culture preparation**

Rat primary neuronal cultures were established as previously described (Chou et al 2008). Briefly, rat mescencephalic neurons were extracted from 1-2 days postnatal pup brains by dissecting the substantia nigra and digested in papain. Neurons were then plated in MatTek 50mm glass-bottom dishes on a glial bed and allowed to adhere for 5-7 days before treatment. Neurons were treated with DEPe for an additional 24-48 hours. Upon completion of the treatment, cultures were either fixed in 4% PFA for 15 minutes and then rinsed in PBS for immunocytochemistry or collected in 0.5mL Trizol for RNA extraction.

**Rat primary culture antibody labeling protocol**

Immunocytochemistry on rat primary culture neurons was conducted according to previously established protocol (Chou et al 2008). In brief, dishes were washed 3x in PBS and blocked for 1hr in 5% goat serum + 0.5% Triton X 100. Primary antibody was diluted as follows: rabbit α tyrosine hydroxylase (abcam #ab112) at a concentration of 1:1000. Incubate in primary antibody overnight at 4$^{o}$C. Wash cultures in PBS + 0.1% goat serum 4x for 5min each rotating gently and add the following secondary antibody: goat α rabbit IgG Alexa Fluor 568 (Invitrogen #A11011) at a concentration of 1:1000. Incubate cultures in the dark for 2 hours at room temperature and wash as described previously. Add 6 drops of VectaShield with dapi (Vector Labs H-1200-10) to each dish and coverslip. Each dish was imaged for tyrosine hydroxylase positive neuron counts at 10x with approximately 50 images covering the majority of the well. Blinded counts were conducted to determine the number of dopaminergic neurons per culture

**Results**:

Studying heterogenous environmental exposures like air pollution presents several unique challenges. First, it is very difficult to model inhalation as a route of exposure in many systems, such as cell culture and non-rodent animal models. In addition to exposure, air pollution originating from different sources is often composed of very different compounds, which may significantly alter the toxicity of the mixture. This heterogeneity and variability poses a challenge when attempting to understand what specific components of air pollution are responsibility for a given toxic outcome. Because much of the epidemiology around PD and air pollution focuses on urban, traffic-related pollution, one source of relevant toxic material can be found in diesel exhaust particulate (DEP). Due to the logistical constraints of model systems as well as the limits of understanding regarding lung absorption, this study will utilize an organic extract of DEP (DEPe) that allows for the study of carbon-based compounds without the distribution issues that come with whole particulate. Much of the extract is composed of long-chain hydrocarbons as well as polycyclic aromatic hydrocarbons (PAHs), many of which are known to be toxic.

The particular source of DEPe used in these studies originates from a single diesel engine and has been previously published in cell culture and animal models (Sagai et al 1993, Hiura et al 1999, Lawal et al 2015). Because little is known about the components of this DEPe source, a partial characterization of the constituent compounds was conducted. Of the total weight of the extract, approximately 4.2% by weight was identified by mass spectrometry. These compounds consisted of PAHs, acids, and long chain hydrocarbons (Table 1). With this knowledge, future studies on the neurotoxicity of this mixture can further differentiate which compounds may be toxic in isolation and also allows for comparison of relative toxicity between mixtures.

**Table 1: DEPe component analysis**

| Analyte | DEP Sample | Lab Blank | (Sample minus Blank) | Sample/% Recovery |
|---|---|---|---|---|
| Phenanthrene | 132.66 | 0 | 132.66 | 142.46 |
| Anthracene | 0 | 0 | | |
| Fluoranthene | 403.88 | 0 | 403.88 | 420.5769031 |
| Acephenanthrylene | 0 | 0 | | |
| Pyrene | 172.41 | 0 | 172.41 | 191.2267081 |
| Benzo(GHI)fluoranthene | 106.21 | 0 | 106.21 | 93.2320927 |
| Cyclopenta(cd)pyrene | 0 | 0 | | |
| Benz(a)anthracene | 0 | 0 | | |
| Chrysene | 94.05 | 0 | 94.05 | 93.86227545 |
| 1-Methylchrysene | 0 | 0 | | |
| Retene | 0 | 0 | | |
| Benzo(b)fluoranthene | 0 | 0 | | |
| Benzo(k)fluoranthene | 0 | 0 | | |
| Benzo(j)fluoranthene | 0 | 0 | | |
| Benzo(e)pyrene | 0 | 0 | | |
| Benzo(a)pyrene | 0 | 0 | | |
| Perylene | 0 | 0 | | |
| Indeno(1,2,3-cd)pyrene | 0 | 0 | | |
| Benzo(GHI)perylene | 0 | 0 | | |
| Dibenz(ah)anthracene | 0 | 0 | | |
| Picene | 0 | 0 | | |
| Coronene | 0 | 0 | | |
| Dibenzo(ae)pyrene | 0 | 0 | | |
| 17A(H)-22,29,30-Trisnorhopane | 0 | 0 | | |
| 17A(H)-21B(H)-30-Norhopane | 0 | 0 | | |
| 17A(H)-21B(H)-Hopane | 0 | 0 | | |
| 22S-Homohopane | 0 | 0 | | |
| 22R-Homohopane | 0 | 0 | | |
| 22S-Bishomohopane | 0 | 0 | | |
| 22R-Bishomohopane | 0 | 0 | | |
| 22S-Trishomohopane | 0 | 0 | | |
| 22R-Trishomohopane | 0 | 0 | | |
| AAA-20S-C27-Cholestane | 0 | 0 | | |
| ABB-20R-C27-Cholestane | 0 | 0 | | |
| AAA-20R-C27-Cholestane | 0 | 0 | | |
| ABB-20R-C28-Ergostane | 0 | 0 | | |
| ABB-20S-C28-Ergostane | 0 | 0 | | |
| ABB-20R-C29-Sitostane | 0 | 0 | | |
| ABB-20S-C29-Sitostane | 0 | 0 | | |

| | | | | |
|---|---|---|---|---|
| Undecane | 0 | 0 | | |
| Dodecane | 0 | 0 | | |
| Tridecane | 0 | 0 | | |
| Tetradecane | 0 | 0 | | |
| Pentadecane | 0 | 0 | | |
| Hexadecane | 1040.07 | 0 | 1040.07 | 956.2982714 |
| Norpristane | 0 | 0 | | |
| Heptadecane | 538.56 | 0 | 538.56 | 632.3353293 |
| Pristane | 0 | 0 | | |
| Octadecane | 901.18 | 0 | 901.18 | 969.0107527 |
| Phytane | 352.18 | 0 | 352.18 | 381.3122564 |
| Nonadecane | 1243.17 | 0 | 1243.17 | 1295.508545 |
| Eicosane | 1269.96 | 0 | 1269.96 | 1223.586087 |
| Heneicosane | 1060.36 | 0 | 1060.36 | 943.8006231 |
| Docosane | 696.48 | 16.28 | 680.2 | 605.1601423 |
| Tricosane | 364.64 | 13.13 | 351.51 | 360.0430196 |
| Tetracosane | 202.2 | 10.29 | 191.91 | 187.2292683 |
| Pentacosane | 234.87 | 12.52 | 222.35 | 225.0278312 |
| Hexacosane | 141.03 | 20.33 | 120.7 | 122.1907269 |
| Heptacosane | 229.29 | 34.77 | 194.52 | 195.1248872 |
| Octacosane | 411.57 | 64.48 | 347.09 | 348.5539265 |
| Nonacosane | 629.88 | 90.81 | 539.07 | 527.1562683 |
| Triacontane | 1017.15 | 140.03 | 877.12 | 872.4089914 |
| Hentriacontane | 1167.64 | 157.77 | 1009.87 | 966.8453806 |
| Dotriacontane | 1556.55 | 216.9 | 1339.65 | 1273.673702 |
| Tritriacontane | 1369.05 | 201.59 | 1167.46 | 1099.510266 |
| Tetratriacontane | 1317.22 | 187 | 1130.22 | 1107.190439 |
| Pentatriacontane | 1030.87 | 153.5 | 877.37 | 847.454844 |
| Hexatriacontane | 819.04 | 130.37 | 688.67 | 680.5712027 |
| Heptatriacontane | 714.54 | 118.13 | 596.41 | 595.1007783 |
| Octatriacontane | 683.5 | 106.11 | 577.39 | 597.1558589 |
| Nonatriacontane | 667.22 | 91.3 | 575.92 | 588.935474 |
| Tetracontane | 726.53 | 100.01 | 626.52 | 620.6855558 |
| Pentadecylcyclohexane | 0 | 0 | | |
| Hexadecylcyclohexane | 0 | 0 | | |
| Heptadecylcyclohexane | 0 | 0 | | |
| Octadecylcyclohexane | 0 | 0 | | |
| Nonadecylcyclohexane | 0 | 0 | | |
| Squalane | 0 | 0 | | |
| Hexanoic acid | 0 | | | |
| Octanoic acid | 0 | 119.92 | -119.92 | |
| Decanoic acid | 254.57 | 57.75 | 196.82 | 179.7442922 |
| Dodecanoic acid | 604.51 | 45.91 | 558.6 | 556.374502 |

| | | | | |
|---|---|---|---|---|
| Tetradecanoic acid | 890.44 | 61.18 | 829.26 | 787.8954869 |
| Pentadecanoic acid | 1101.1 | 45.47 | 1055.63 | 1055.63 |
| Hexadecanoic acid | 2919.7 | 452.25 | 2467.45 | 1869.705236 |
| Heptadecanoic acid | 778.85 | 15.57 | 763.28 | 763.28 |
| Octadecanoic acid | 2958.56 | 411.59 | 2546.97 | 1929.376562 |
| Nonadecanoic acid | 317.64 | 0 | 317.64 | 317.64 |
| Pinonic acid | 0 | 0 | | |
| Palmitoleic acid | 0 | 0 | | |
| Oleic acid | 0 | 0 | | |
| Linoleic acid | 0 | 0 | | |
| Linolenic acid | 0 | 0 | | |
| Eicosanoic acid | 192.51 | 6.39 | 186.12 | 180.5763074 |
| Heneicosanoic acid | 0 | 0 | | |
| Docosanoic acid | 0 | 0 | | |
| Tricosanoic acid | 0 | 0 | | |
| Tetracosanoic acid | 0 | 0 | | |
| Pentacosanoic acid | 0 | 0 | | |
| Hexacosanoic acid | 0 | 0 | | |
| Heptacosanoic acid | 0 | 0 | | |
| Octacosanoic acid | 0 | 0 | | |
| Nonacosanoic acid | 0 | 0 | | |
| Triacontanoic acid | 0 | 0 | | |
| Dehydroabietic acid | 0 | 0 | | |
| 7-oxodehydroabietic acid | 0 | 0 | | |
| Phthalic acid | 8192.84 | 32.26 | 8160.58 | 6930.428875 |
| Isophthalic acid | 2451.3 | 0 | 2451.3 | 1949.964203 |
| Terephthalic acid | 1335.29 | 0 | 1335.29 | 1080.768919 |
| 1,2,4-Benzenetricarboxylic acid | 10195.07 | 0 | 10195.07 | 11391.13966 |
| 1,2,3-Benzenetricarboxylic acid | 608.17 | 0 | 608.17 | 585.2853431 |
| 1,3,5-Benzenetricarboxylic acid | 0 | 0 | | |
| 1,2,4,5-Benzenetetracarboxylic acid | 10553.16 | 0 | 10553.16 | 32794.15786 |
| Methylphthalic acid | 3635.63 | 18.4 | 3617.23 | 3068.569732 |
| Succinic acid | 0 | 0 | | |
| Glutaric acid | 0 | 0 | | |
| Adipic acid | 0 | 0 | | |
| Pimelic acid | 0 | 0 | | |
| Suberic acid | 0 | 34.21 | -34.21 | |
| Azelaic acid | 0 | 0 | | |
| Sebacic acid | 0 | 0 | | |

| | |
|---|---|
| | |

Table 1: Compound quantities are given in ng/40uL. % recovery is quantified using a spiked sample of known concentration and each compound yield is adjusted for % recovery.

Based on what is known about aSyn transcript levels and disease risk, one aspect that remains unknown is how the aSyn promoter responds to environmental toxin exposure. To understand whether certain toxins can serve to modulate the human promoter and thereby alter the amount of aSyn present in the brain, one relatively simplistic assay designed to target this disease-relevant readout is a luciferase promoter activity assay. This involves utilizing human neuroblastoma cells in culture, which have an intact endogenous aSyn promoter with low-level activity and can also be induced to produce dopamine. These cells were then transfected with a construct containing 10.7kB, including the known active regions, of the human aSyn promoter driving the gene for firefly luciferase (Chiba-Falek et al 2001). Along with this construct, a control renilla luciferase plasmid which is constitutively expressed was co-transfected and served as an internal transfection control. The ratio of firefly/renilla luciferase expression is used as a readout of aSyn promoter activity after 24 hours of exposure to a toxin. This assay was used to screen a variety of pesticides that have previously been associated with PD risk, although little is known about how they may alter synuclein expression. Of the pesticides originally assayed, only rotenone was seen to potently induce promoter activity. Rotenone is often used in animal models of PD because, as a very potent mitochondrial inhibitor, it has been shown to be toxic to dopaminergic neurons and exposure is associated with PD in humans and rodents (Bywood et al 2003). In addition, rotenone has been shown in the literature to increase aSyn promoter activity in this cell line by rtPCR, further confirming the relevance of the finding and the assay (Chorfa et al 2013, Sala et al 2013). Ziram, benomyl, and paraquat have also been associated with increased incidence of PD in epidemiological studies, but in this assay, were not associated with an increased aSyn promoter activity (Fig. 1A) (Costello et al 2009, Wang et al 2011).

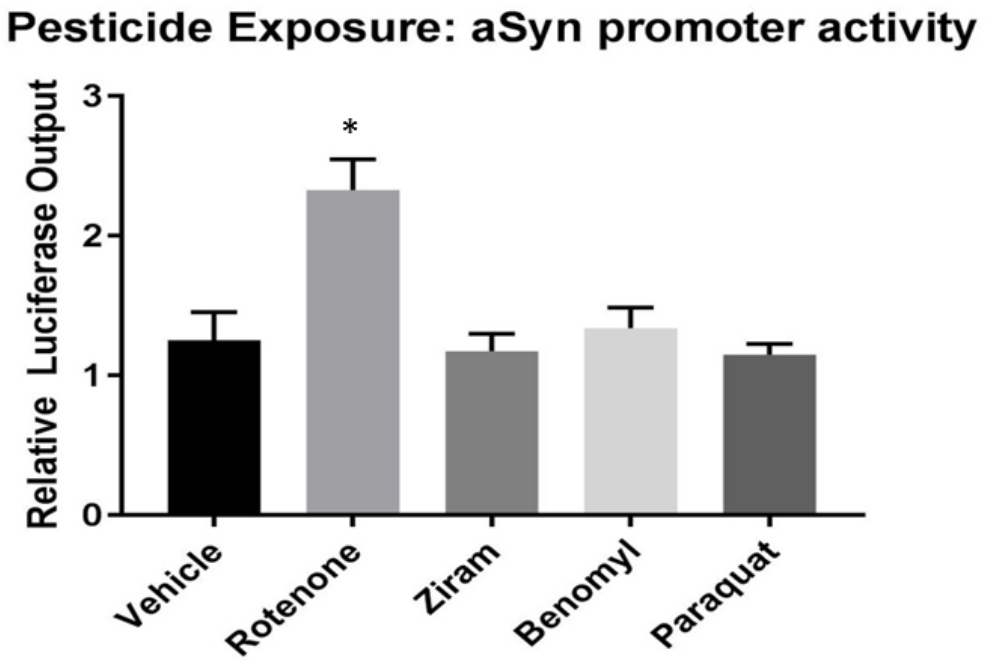
Because the toxicity of rotenone is thought to act primarily through mitochondrial complex I inhibition, one question this data raises is the possibility that aSyn promoter activity can be modulated by mitochondrial toxins or increases in oxidative stress. To test this, the luciferase assay was used in combination with inhibitors of complex I of the electron transport chain (rotenone), complex III (antimycin), complex I (pyridaben), and complex IV (KCN) along with hydrogen peroxide to mimic the reactive oxygen species (ROS) generation caused by mitochondrial inhibition. Inhibitors of complex I-III induce ROS generation and inhibition of complex IV does not. The inhibitors of complex I both showed significant increase in aSyn promoter activity via luciferase expression and inhibition of complex III by antimycin as well as ROS treatment both slightly elevated promoter activity, but not statistically significantly. Inhibition of complex IV, which does not induce ROS production, did not show a change in promoter activity in this assay (Fig 1B). This data is consistent with the theory that expression from the aSyn promoter may, in part, be driven by an oxidative environment. This is also intriguing because of the genetic evidence indicating that mitochondrial health is crucial to dopaminergic neuron survival and mitochondria in the PD brain may have reduced function (Mizuno et al 1989, Hoglinger et al 2003, Moon and Paek 2015, Rocha et al 2018). Based on this preliminary screen, environmental toxins that alter mitochondrial function may be implicated in disease risk by creating an oxidative neuronal environment.

In addition to using the luciferase assay to screen pesticides, we next used the assay to test whether DEPe may alter aSyn promoter activity at 1 and 10ug/mL. If promoter activity is altered via luciferase, it would suggest that gene expression may be altered from the endogenous promoter as well and would warrant further investigation. Although not statistically significant due to variability between experiments, the data was suggestive of an effect at the higher dose

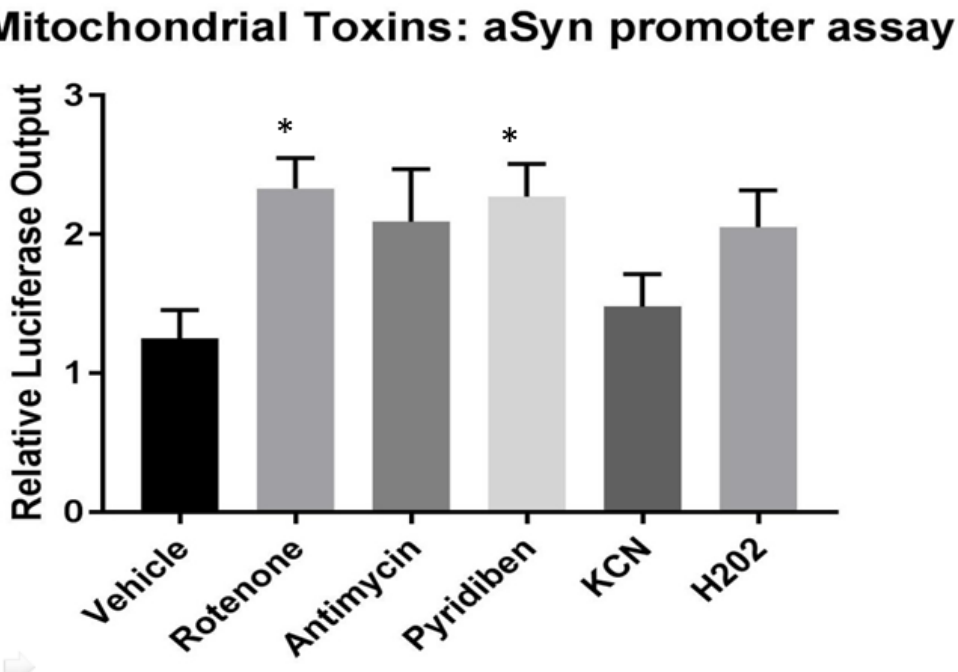
(Fig. 1C). In order to confirm in a more sensitive and endogenously relevant way, we then looked at RNA transcript levels in SK-N-MC human neuroblastoma cells by quantitative rtPCR and showed that transcript levels increased using two sources of DEPe to a degree consistent with the luciferase assay increase (Fig 1D).

**Figure 1: Alpha Synuclein Promoter Activity and Transcript Levels in Human Cells**

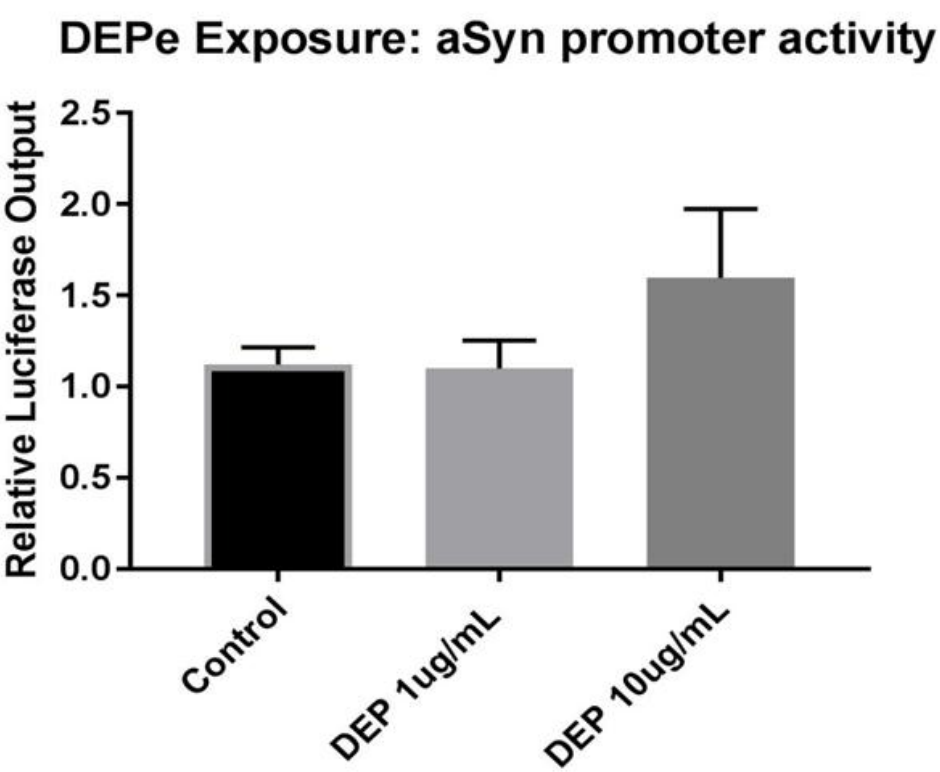
**1A**




**1B**



1C


DEPe Exposure: aSyn promoter activity
Relative Luciferase Output
2.5
2.0
1.5
1.0
0.5
0.0
Control
DEP 1ug/mL
DEP 10ug/mL

1D

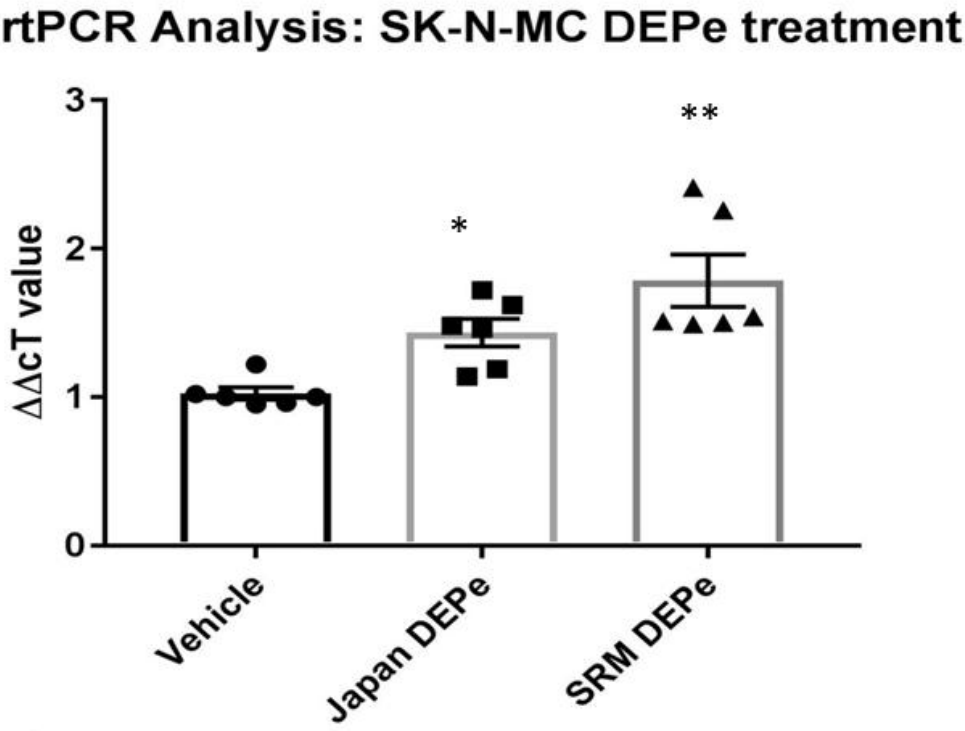

rtPCR Analysis: SK-N-MC DEPe treatment
ΔΔcT value
3
2
1
0
*
**
Vehicle
Japan DEPe
SRM DEPe

Figure 1: SH-SY5Y human neuroblastoma cells were transfected with luciferase reporters and analyzed for renilla/firefly ratios. 1A- cells treated for 24hrs with pesticides show rotenone significantly elevates aSyn promoter activity (DMSO vehicle control n=17, Rotenone at 100nM n=22, Ziram at 100nM n=5, Benomyl at 1uM n=5, Paraquat at 100nM n=5). 1B- mitochondrial toxins were tested to better understand aSyn promoter activity. Rotenone at 100nM n=22, Antimycin at 100nM n=6, Pyridaben at 100nM n=10, KCN at 2mM n=6, H202 at 10uM n=7). 1C- DEPe extract was tested for aSyn promoter activity at 1 and 10ug/mL showing increasing activity with higher exposure. Data from luciferase assay was confirmed via rtPCR in SK-N-MC endogenous aSyn RNA. Japan DEPe was added at 10ug/mL and SRM-DEPe was added at 25ug/mL along with a DMSO vehicle control. 6 wells were treated and each sample was run in duplicate and averaged for rtPCR results. Data analyzed by ΔΔcT relative to the expression of TATA binding protein (TBP) expression control. All data was analyzed with one way ANOVA. * $p<0.05$, ** $p<0.005$ with Dunnett's multiple comparisons analysis; all error bars represent standard error of the mean.

In order to determine if DEPe exposure can alter aSyn promoter activity in non-cancerous neurons, rat primary mescencephalic cultures were treated for 24hrs with DEPe in order to assess gene expression by rtPCR and total dopaminergic neuron number through immunocytochemistry staining against tyrosine hydroxylase, which is an enzyme critical for the generation of dopamine and is a specific marker of dopaminergic neurons. The neuronal cultures were plated on an astroglial bed and were composed of cells from mixed neuronal origin. Each dish contained about 200 dopaminergic neurons per well and they were labeled with antibodies toward tyrosine hydroxylase (TH) (Fig 2A). Cells were fixed, labeled, and imaged at 10x and then blinded for total neuronal counts. Neuron number after 24 and 48 hours of DEPe treatment were statistically unchanged, indicating that short term exposure to DEPe does not induce significant neuron death under these conditions (Fig 2B).

**Figure 2: Immunohistochemistry and neuron counts in rat primary mescencephalic culture**

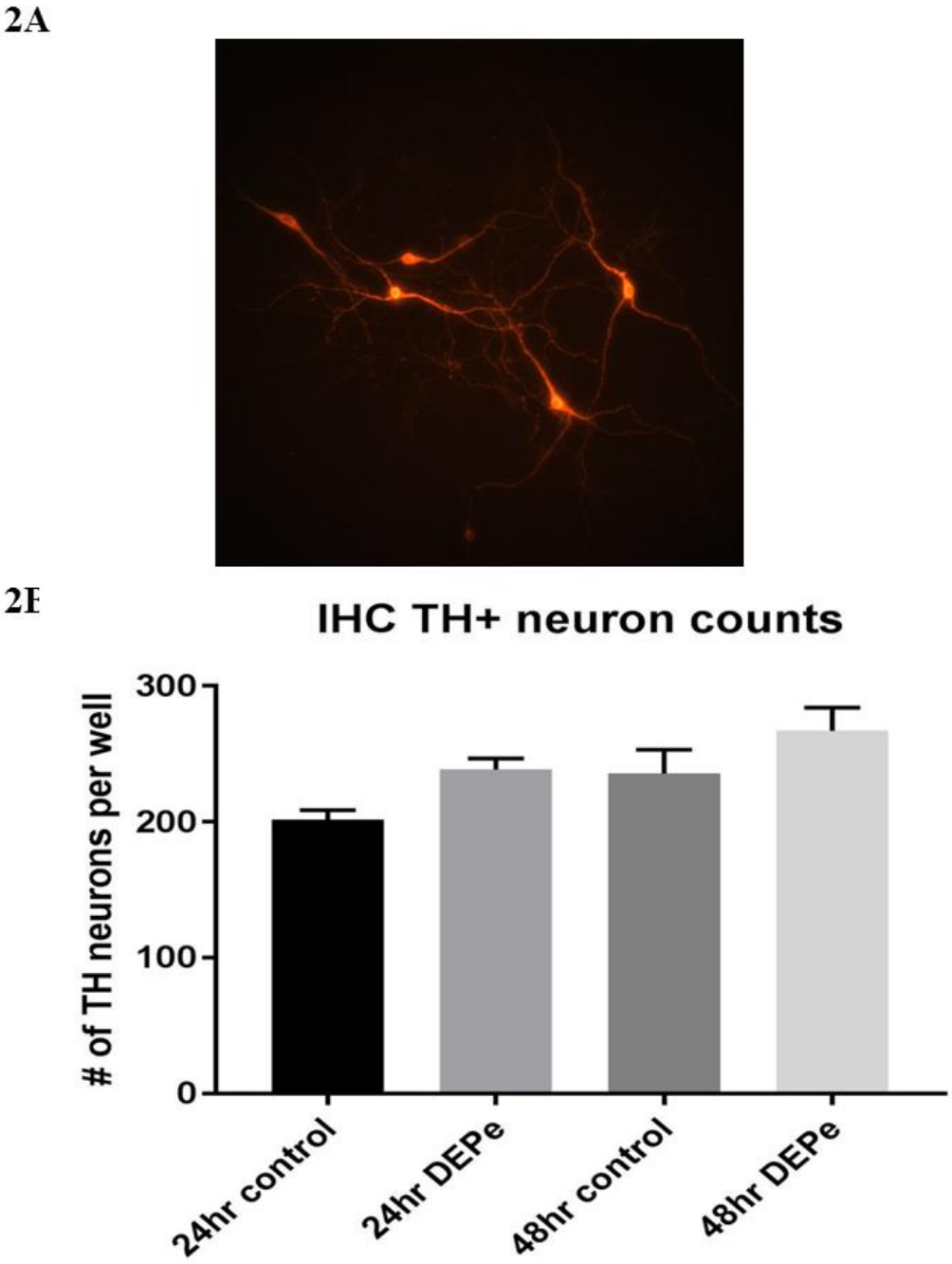



Fig 2: Dishes of rat primary mescencephalic cultures were treated with DEPe for 24 or 48 hours and then fixed for antibody labeling against tyrosine hydroxylase to label dopaminergic neurons. Wells were imaged for th1 (red) and quantified in a blinded and randomized fashion (A). The number of th1 neurons per culture plate (n=3/condition) trended up, but there was no statistical difference between groups (B).

Additionally, rat primary mescencephalic cultures were used to assess aSyn gene expression by rtPCR. Wells with mixed neuronal culture were treated with DEPe for 24 hours and collected in Trizol for RNA extraction and cDNA synthesis. Gene expression changes were assessed using primers designed against rat alpha synuclein and normalized to GAPDH gene expression. Although there was a low number of cultures tested, aSyn gene expression after DEPe was 1.2x elevated above control with a p value of 0.059 (Fig 3A). The degree of change in gene expression was consistent with the change seen by luciferase assay, but because dopaminergic neurons were a minority within the wells collected, it is possible that the gene expression changes in dopaminergic neurons were underestimated in this experiment.

**Fig 3: Level of aSyn transcript in rat primary mescencephalic cultures treated with DEPe**

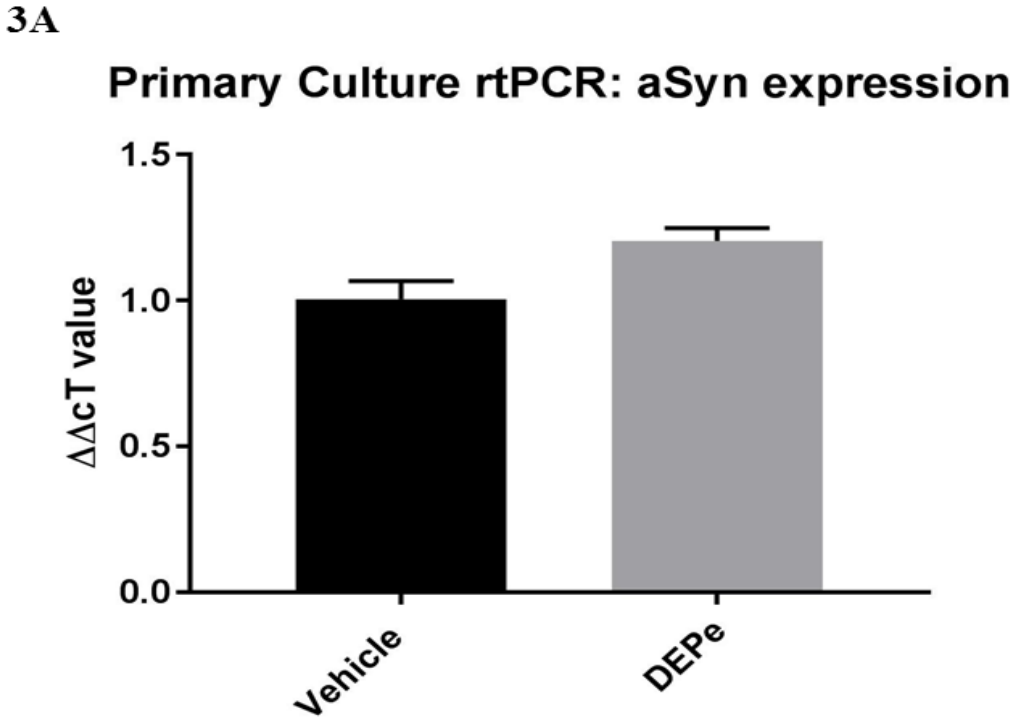



Figure 3: Rat primary mescencephalic cultures were treated with DEPe for 24hrs before harvesting RNA from the whole well. RNA purification and cDNA synthesis was conducted followed by rtPCR (n=3 dishes per condition, run in duplicate for rtPCR) normalized to GAPDH expression (p=0.059)

**Discussion:**

The importance of aSyn gene expression levels has been shown to be highly relevant to PD disease risk, primarily using genetic data. Because genetics can only account for a small percentage of PD risk, we know that there must be underexplored environmental influencers of disease risk. The human aSyn promoter is very large and carries known variations that can alter gene expression such as the Rep1 repeat. Using a luciferase reporter construct, we have shown that certain environmental toxins known to be relevant to PD from epidemiological studies can also influence promoter activity. Rotenone and other mitochondrial toxins elevate gene expression by around 1.5 fold. This data suggests that mitochondrial inhibition or oxidative stress may be responsible for inducing aSyn expression. Because mitochondrial dysfunction is also a known feature of PD, this connection may partially explain how aSyn becomes dysregulated after complex I inhibition. In addition, it has been shown that aSyn aggregation can impair mitochondrial function, possibly leading to a self-perpetuating cycle of protein aggregation and oxidative stress that, over time, has the potential to lead to increased risk of developing PD.

Exposure to DEPe alters alpha synuclein transcript levels as expressed from the endogenous promoter as well, which was determined by rtPCR in human neuroblastoma cells as well as rodent primary culture neurons. Because aSyn aggregation and accumulation is the pathological hallmark of PD, these findings of increased transcript, when extrapolated over the lifetime of an individual, have the potential to be highly significant when considering factors influencing disease risk. This is also very interesting because air pollution in general is a ubiquitious environmental pollutant and a high percentage of the population has at least some degree of exposure, unlike other established influencers of PD risk like pesticides. When looking

at potential causes of idiopathic PD, we must take a closer look at various forms of air pollution as well as the individual components that make up air pollution in order to understand how they may impact pathways involved in disease pathogenesis.

These findings are limited by the fact that they were done in isolated cells rather than an animal model, but we believe there is significant reason to screen other environmental pollutants and also to expand upon known toxins associated with PD in order to understand the molecular underpinings of aSyn transcript increases. Based on the fact that genetic mutations can only account for a small percentage of PD cases, increases in transcript of this magnitude, over an individuals' lifetime, represent significant alterations in aSyn homeostasis. It is highly likely that some combination of environmental modulators such as pesticides and diesel exhaust work in combination with genetic predispositions over a period of possibly decades in order to increase eventual neurodegenerative disease risk. These results bring up questions regarding the cause of protein accumulation. Because of the importance of this finding in particular, additional work will explore the molecular mechanisms behind aSyn build-up in order to identify specific interventions that may prove efficacious as therapeutic treatments.

Dissertation: Chapter 2

Diesel Exhaust Particulate Extract Exposure and Toxicity in Zebrafish

**Introduction**:

Zebrafish (ZF) represent a unique transitional *in vivo* model system for studying neurodegeneration and molecular pathways involved in neurotoxicity (Bandmann and Burton 2010, O'Donnell et al 2014, Stewart et al 2014, Martin-Jimenez et al 2015, Lulla et al 2016). Many genetic lines even exist to model neurodegenerative diseases such as Huntington, ALS, tauopathies, and Parkinson's disease (Anichtchik et al 2008, Bai et al 2007, Bretaud et al 2007, Flinn et al 2009, Lemmens et al 2007, Schiffer et al 2007, Wen et al 2008). Their small size, rapid development, transparent appearance, and genetic tractability all make ZF very amenable to investigation of multiple endpoints involved in toxicity induced by environmental exposures. They can be easily and reproducible treated through exposure in water and have a relatively complex nervous system that includes tyrosine hydroxylase-positive dopaminergic neurons (Wen et al 2008, Wagner and Russell 2013) that are sensitive to aSyn toxicity in PD. Some of the major advantages of the model system that make it uniquely suited to the study of air pollution and PD include: the ability to use larvae for screening toxins as well as therapeutic drugs, the transparency of young larvae that allows whole animal imaging in live and fixed ZF, and the abundance of transgenic lines that allow for a variety of endogenously expressed fluorescent markers that can be used to easily and reproducibly quantify cells of interest and to parse molecular pathways within particular cells.

ZF do not have a homolog to the human aSyn gene, but the genome does contain three synuclein family genes, synuclein gamm 1, synuclein gamma 2, and synuclein beta (Sun et al

2008, Milanese et al 2012). Based on region of expression, protein aggregation propensity, and neurotoxicity, it appears that synuclein gamma 1(sncg1) is the closest functional paralog to human aSyn in the developing ZF embryo. Expression of sncg1 is detectable in the central nervous system of ZF before 24hpf and isolated sncg1 protein has been shown to form aggregates *in vitro,* and when over expressed *in vivo,* leads to neuronal toxicity (Milanese et al 2012, Lulla et al 2016). For the duration of this chapter, all synuclein analysis in ZF will focus on sncg1. In order to test whether exposure to DEPe in larvae ZF alters synuclein, both gene expression as well as sncg1 protein levels will be assessed using quantitative rtPCR and Western blot.

Preliminary understanding of sensitivity to an environmental toxin of interest can be seen in increased mortality and/or alterations in behavioral patterns in developing ZF larvae. We have previously established and utilized a light cycling behavioral assay that measures the tendency of ZF larvae to increase spontaneous movement in dark conditions and also to respond to drugs modulating the dopaminergic system (Irons et al 2012, Lulla et al 2016). This assay gives an understanding of total movement and gross toxicity, as well as a more sensitive understanding of response to light stimuli, which may be more indicative of neurotoxicity than overall reduction in movement. The assay demonstrates that, after light exposure, ZF embryos have a brief period of movement known as a startle followed by a longer period of freezing behavior where they move significantly less. When the light is turned off, movement increases significantly and gradually moves back toward baseline over a period of several minutes (Emran et al 2008). This predictable behavioral readout can be an indication of general as well as specific toxicity present in the form of changes in total distance moved, responsiveness to light stimuli, and the ratio of movement in dark/light periods.

In addition to gross estimations of toxicity in developing ZF, of particular relevance to PD is the survival of dopaminergic neurons, which are particularly vulnerable to neuron loss in disease and also express high levels of synuclein protein relative to other neuronal subtypes. This neuron loss is also associated with behavioral changes and previous studies have utilized ZF larval models of behavior to understand dopaminergic neuron signaling after exposure to the environmental neurotoxin ziram (Lulla et al). In order to understand if DEPe exposure leads to neuron loss in ZF, the quantification of neuronal subtypes was conducted using transgenic lines expressing GFP in specific neuronal lineages (Wen et al 2008, Palanca et al 2013).

Alongside this quantification of neuronal toxicity, an important consideration is how the neuroimmune cells within the brain of the developing ZF larvae respond to exposure to DEPe and how this may or may not contribute to neuron loss. Based on what we know of microglial activation in PD, constituatively inflammatory neuroimmune cells are associated with disease progression (Joers et al 2016). In addition to well established dopaminergic neuron pathways as well as synuclein expression, ZF embryos also have robust neuroimmune colonization of the brain very early in development and transgenic models exist that allow for the visualization of these cells within the brain of the intact animal (Herbomel et al 2001, Peri et al 2008, Ellet et al 2011, Rossi et al 2015). Microglia represent the primary macrophage presence in the brain of ZF embryos and, when induced via injury or exposure to a toxin, microglia take on an activated, phagocytic morphology (Peri et al 2008, Svahn et al 2012, Xu et al 2015). In order to understand any potential inflammatory response in a ZF model, we use a transgenic line expressing the mcherry fluorophore within microglia and precursor macrophages both in the brain and the periphery (Ellet et al 2011). Inflammation in this model is determined by changes in macrophage

morphology in the central nervous system. Using this genetic line, we have explored how exposure to DEPe affects macrophages and inflammation within the brain.

These general assays were chosen because of the relevance to diesel exhaust exposure and will be described in detail here in order to characterize the toxicity of DEPe in a ZF larval model. The data collected serves to lay a foundation for better understanding the molecular mechanisms responsible for any neurotoxicity induced by exposure. After establishing the type of toxicity present in this ZF model, it is possible to parse the pathways involved in neuronal injury.

**Methods**:

## Zebrafish Treatment

ZF DEPe treatments were conducted on dechorionated embryos/larvae between 24-120 hours post fertilization (hpf) in E3 medium with a final concentration of 0.1% DMSO vehicle. ET*vmat2*:eGFP ZF which express GFP in aminergic neurons under the vesicle monoamine transporter (*vmat2*) promoter as well as Isl:GFP ZF which express eGFP in sensory neurons under the *islet1* promoter were utilized for neurotoxocity assays and *mpeg1*:mcherry ZF which express mcherry under a microglial promoter were utilized in neuroinflammation assays (Wen et al 2008, Ellet et al 2011, Palanca et al 2013). Unless otherwise indicated, DEPe was added at 10ug/mL and embryos were incubated for 24hrs with treatment before being washed out three times in E3 and grown up to 72 or 120hpf for fixation or live analysis

## ZF Behavior Assay

ZF embryos that had been dechorionated were treated between 24-48hpf as described above with DEPe and washed 3x before growing up to 7dpf. Behavior analysis was conducted as previously published using the ZebraBox and data was collected using ViewPoint ZebraLab software (Lulla et al 2016). Briefly, larvae were transferred into a square 96-well plate with 12 larvae per condition and were acclimated in the dark for 10 minutes before recording movement. Behavior was captured for 1 hour with light cycling of 10min light/10min dark for 3 cycles. Data was analyzed by averaging the distance moved per larvae in the light and the dark.

**ZF Neuron Counts**

After treatment, as described above, 3 or 5dpf ET*vmat2*:GFP ZF larvae were anesthetized in <0.01% tricaine methanesulfonate (TRICAINE-S, MS-222, Western Chemiccal) and fixed in 4% paraformaldehyde (PFA). Larvae were washed in PBS and cleared in 100% glycerol before mounting for confocal imaging at 20x magnification (Leica SPE). For neuron counts, z-stacks containing approximately 40-60 optical sections were collected for each ZF brain 1-2uM apart. Individual aminergic neurons in the telencephalon, diencephalon, and raphae regions of the brain were quantified through the stacks using imageJ in a blinded and randomized fashion (Wen et al 2008). Peripheral sensory neurons were quantified by treating Tg(isl1[ss]:Gal4-VP16,UAS:eGFP)$^{zf154}$ transgenic embryos and imaging at 10x magnification (Leica SPE) in the tail region of 5dpf larvae (Palanca et al 2013). Neuron numbers were quanitified in imageJ through z-stacks in a blinded and randomized fashion.

**ZF Neuroinflammation Antibody Labeling and Imaging**

Transgenic homozygous ET*vmat2*:GFP fish were crossed with homozygous Tg(*mpeg1*:mcherry) and heterozygous embryos were then treated with DEPe as described previously (Ellet et al 2011). At 3dpf, larvae were anesthetized and fixed as previously described before antibody labeling for GFP and mCherry as follows.

Larvae were permeablized in 10ug/mL proteinase K (), washed in PBS 3x and incubated in 10% blocking solution with BSA and NGS overnight. Larvae were incubated rotating in 1$^{o}$ antibodies in 1% blocking solution overnight. Primary antibodies were used as follows: rabbit α mcherry 1$^{o}$ antibody (1:300) and mouse α GFP 1$^{o}$ antibody (1:300). Antibodies were washed 8x in TBSTx at RT before adding 2$^{o}$ antibodies diluted as follows: AlexaFluor 568 goat α rabbit antibody (1:300)

and AlexaFluor 488 goat α mouse antibody (1:300). Secondary antibodies were washed out in TBSTx 8x at RT before clearing embryos in glycerol and mounting for whole mount confocal microscopy. When imaging the brain, cells labeled with AlexaFluor 488 represent vmat2 expressing aminergic neurons and cells labeled with AlexaFluor 568 represent mpeg1 expressing microglia.

**Results**:

ZF embryos develop very rapidly and by 24 hours post fertilization (hpf), they have a beating heart, well-established neuronal networks, and the basic body-pattern of an adult fish. By 3 days post fertilization (dpf), larvae have typically hatched from the egg chorion but are still surviving on yolk until 5-7dpf. Unless otherwise noted, all ZF exposures occur at 24hpf in embryos that have been removed from chorion for more consistent chemical exposure and experiments end by 7dpf in order to reduce the variation that comes after larvae begin feeding. Embryos are typically treated 20 per well in a final concentration of 0.01% DMSO vehicle, which was also included as a control.

**Fig. 1- Diesel exhaust particulate extract toxicity in ZF**

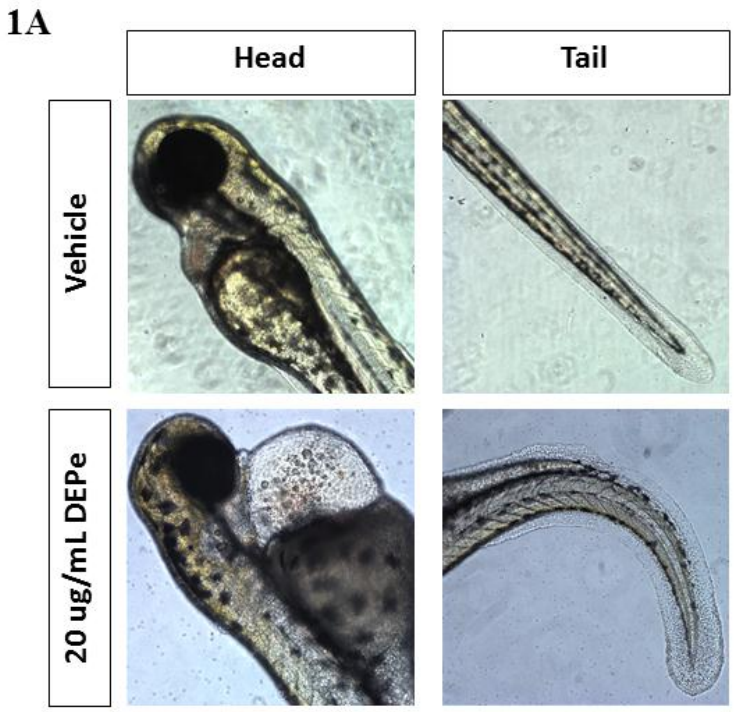
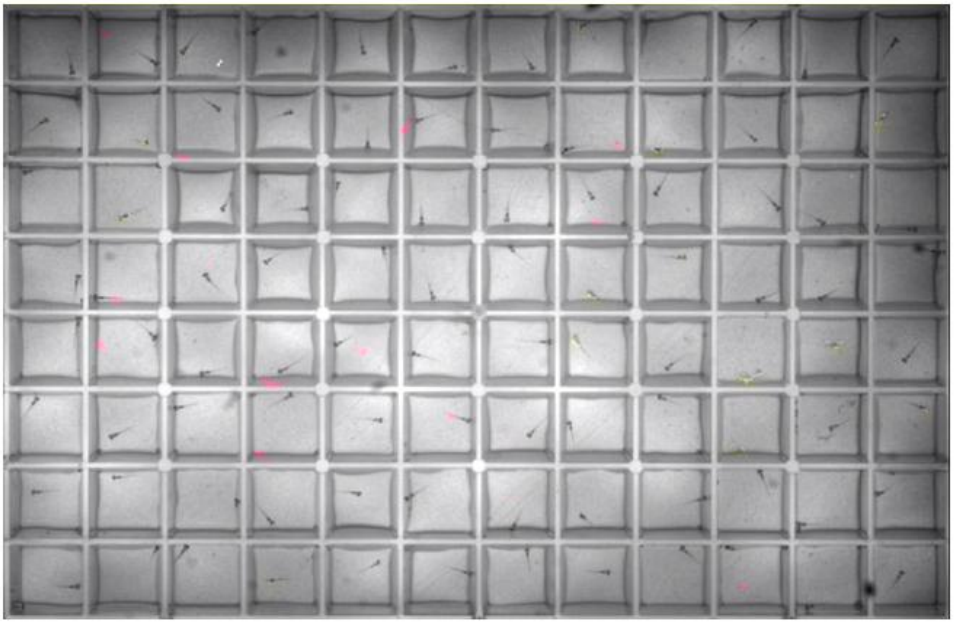



Fig.1: Dechorionated embryos were exposed to 20ug/mL DEPe in 0.01% DMSO from 24-72hpf and imaged for gross morphological defects.

General toxicity of DEPe in developing ZF embryos was determined by exposing 24 hpf dechorionated embryos to 1ug/mL up to 25ug/mL DEPe through 7dpf. Significant cardiac edema (swelling around the developing heart) as well as tail curvature was detected at 20ug/mL (Fig 1). Exposure to 20ug/mL DEPe through 7dpf was almost completely lethal to developing ZF (data not shown) and lower concentrations showed significant morphological abnormalities. Shorter exposures showed reproducible morphological defects. Because the majority of treated ZF survive through 5dpf at 10ug/mL, further experiments testing toxicity were done at this dose unless otherwise noted.

**Fig. 2- Impact of DEPe treatment on ZF behavior at 7dpf**

**2A**



**2B**

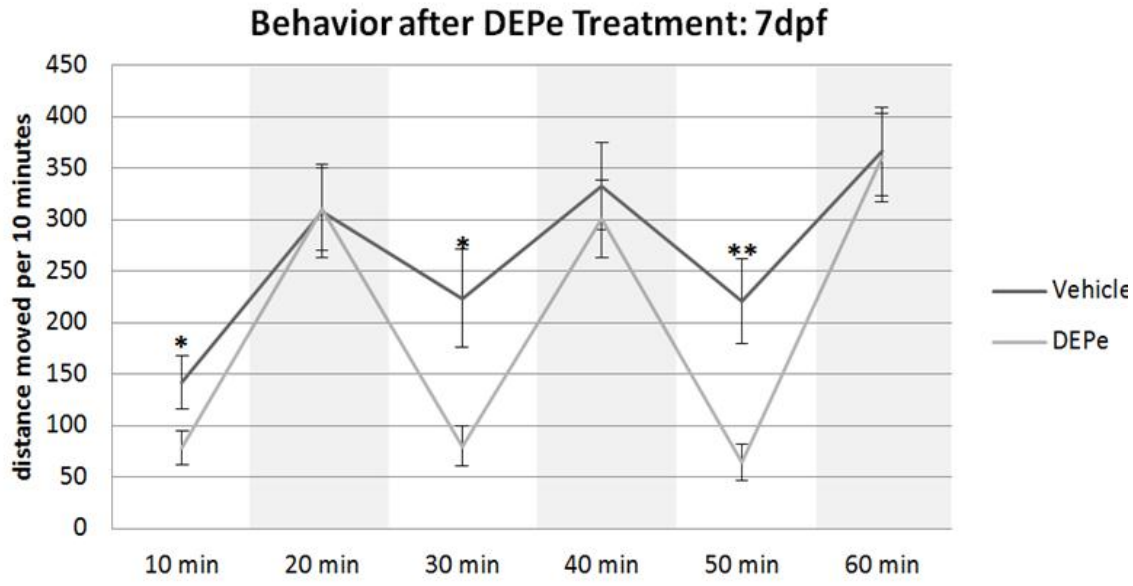



Fig 2: Square, clear-walled 96-well plates were used for behavioral analysis in 7dpf embryos treated with DEPe between 24-48hpf and washed out in E3 medium (A). Light cycling was analyzed averaging distance moved per 10 minutes in both light and dark conditions (dark conditions are shaded on graph) for 3 cycles with 12 larvae per condition (B). Statistical analysis by two-tailed Student's t-test *=$p<0.05$, **=$p<0.005$

One advantage of using an in vivo ZF model system is that treatments can be explored at the molecular level as well as the organismal level. One readout of gross toxicity in ZF larvae can be seen in behavioral deficits. Behavioral phenotypes can be directly related to neurotoxicity or represent a more global toxicity, but as a screening tool, can prove useful as has been shown in previous environmental exposures to pesticides like ziram (Lulla et al). By 7dpf, ZF larvae are hatched, have an inflated swim bladder, and respond to light/dark stimuli. In order to understand how toxic exposures may be impacting ZF larvae that are otherwise morphologically normal, a light cycling assay has been developed that demonstrates how effectively larvae respond to light/dark stimulus as well as how much total movement occurs over 60 minutes of recording. Up to 12 larvae per treatment condition are examined for 1 hour in 10 minute light/ 10 minute dark cycles in 96-well plates (Fig 2A). WT ZF show a reproducible light cycling behavior as early as 5dpf where total movement per 10 minutes increases in dark conditions relative to light conditions. After DEPe treatment at a sub-lethal dose between 24-48hpf followed by a 5 day washout, total movement in the dark condition was statistically unchanged, but in light conditions, total movement was significantly reduced in all 3 light cycles relative to untreated controls (Fig 2B). This indicates that there is some DEPe-induced toxicity that impacts the light cycling behavior of developing ZF even when the toxin has been washed out for 5 days. One possible explanation for this behavioral deficit in light cycling could be neurotoxicity originating from early DEPe exposure. Because there are other possible reasons why light cycling may be altered, it is important to do further analysis in order to determine if neurotoxicity is a valid explanation for this change in light cycling behavior.

**Fig 3 - DEPe treatment and analysis of ZF aminergic neurons**

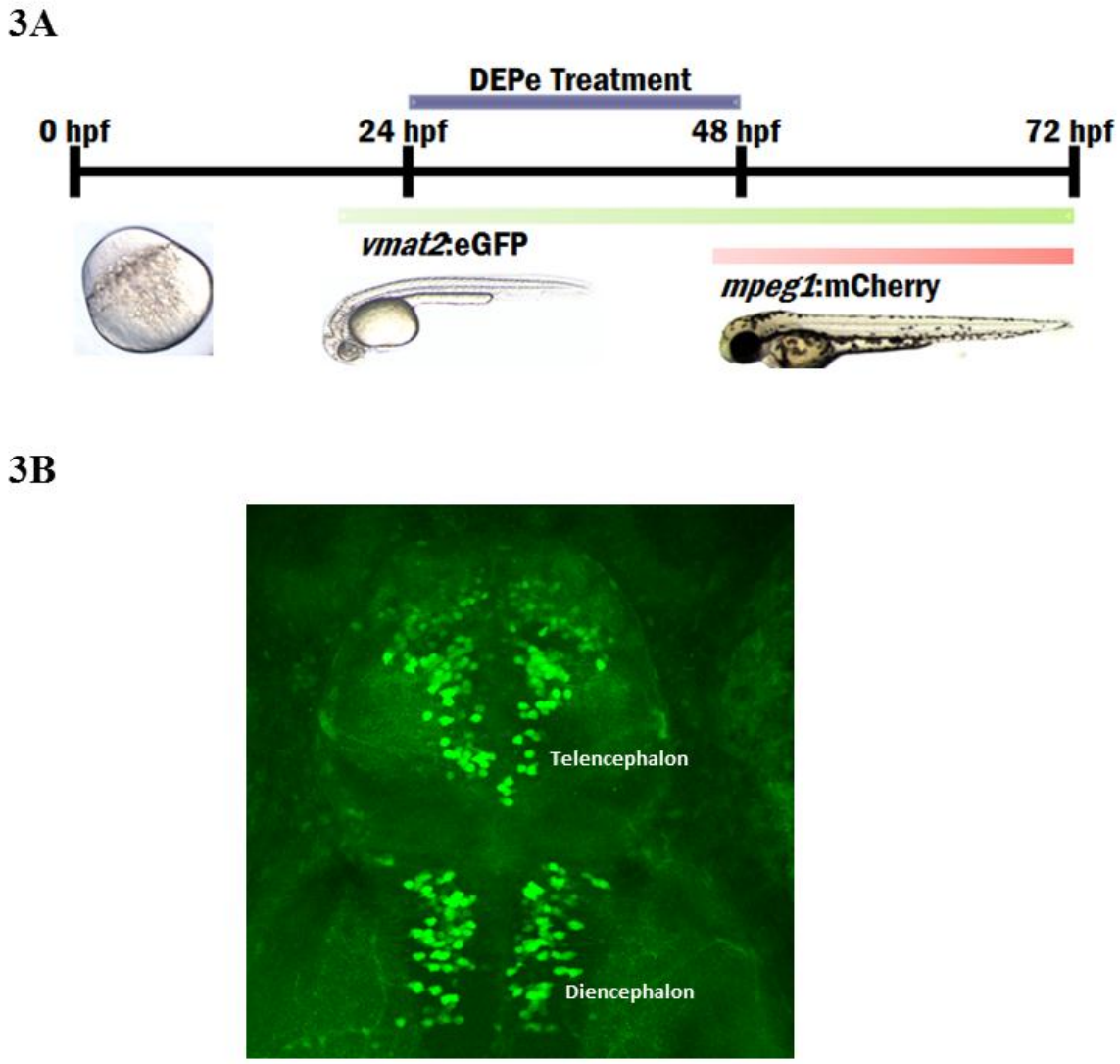
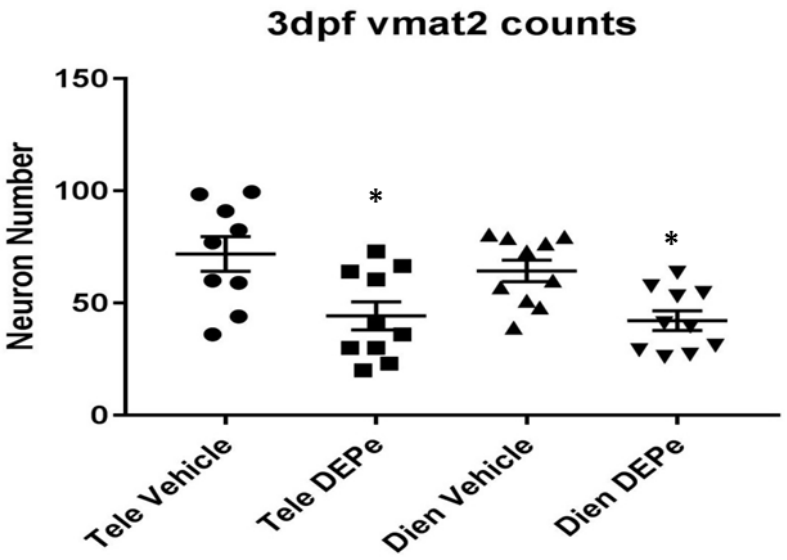



Fig 3- Treatment schematic represents the exposure period relative to the differentiation of the cells of interest (A) from fertilization to 3dpf. Representative image of the aminergic neurons quantified in the vmat2:eGFP transgenic line.

After establishing that exposing ZF larvae to DEPe does induce behavioral toxicity, it is important to explore if this correlates with a neurological toxicity that may be relevant to PD. Mortality and behavioral toxicity can be due to a variety of mechanisms, but it is possible that developmental exposure to DEPe causes a decrease in neuron number that may be relevant to neurodegenerative disease. Under the treatment conditions used in developing ZF, mainly treating between 24-48hpf followed by a toxin wash-out, aminergic neurons are present and continuing to differentiate in the developing ZF during the time of DEPe treatment. Importantly, during this early window of exposure, the neuroimmune cells known as microglia have not yet migrated to take residence in the brain, but aminergic neurons are well established (Fig 3A). To address the possibility of DEPe-induced neurotoxicity, ZF embryos from the transgenic line ET*vmat2*:GFP, which expresses GFP in aminergic neurons (dopaminergic, noradrenergic, and serotonergic) under the control of the vesicle monoamine transporter (vmat2) promoter, were treated between 24-48hpf, washed out in E3, and grown up to 3 or 5dpf where larvae were then fixed and imaged via confocal microscopy for quantification of neuronal clusters that are of interest in PD within the telencephalon and diencephalon (Fig 3B). The telencephalic and diencephalic clusters imaged in the ET*vmat2*:GFP line contain, but are not exclusively composed of, dopaminergic neurons. Quantification of these neuronal clusters showed an approximately 25-50% loss of GFP positive neurons that appears progressive over time even after toxin washout (Fig 4A-B). The toxicity seen here in aminergic neurons is highly significant and is maintained over an extended period of time, even worsening 3 days after removing the DEPe. This indicates that there must be underlying mechanisms of dysregulation that are initiated during this early developmental period that lead to continued neurotoxicity.

**Fig 4: Aminergic neuron counts after DEPe exposure in vmat2:eGFP larvae**

**4A**




**4B**

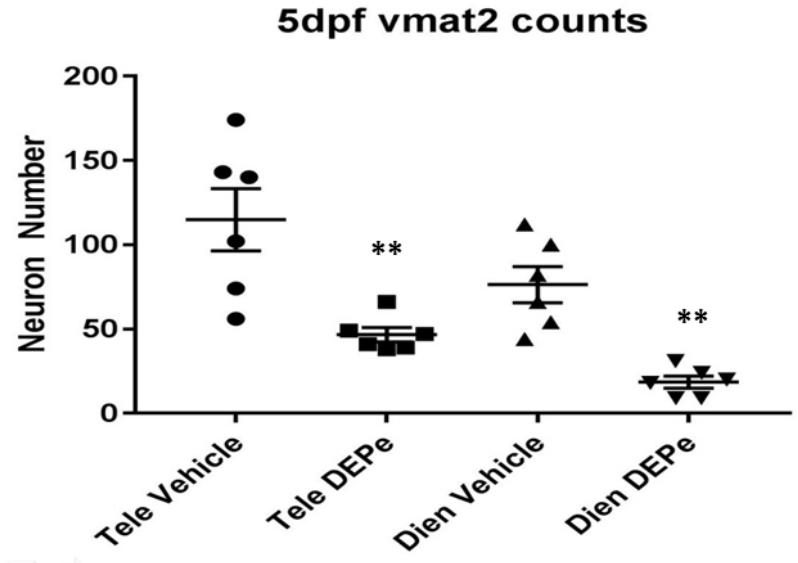



Fig 4- Neurons expressing GFP in vmat2+ neurons were quantified after DEPe treatment at A) 3dpf or B) 5dpf via confocal microscopy in fixed embryos. Tele represents aminergic neurons in the telencephalon and dien represents aminergic neurons in the diencephalon. Counts from 3 and 5dpf show a statistically significant decrease in neurons after treatment. (*=$p<0.05$, **=$p< 0.005$ as determined by one way ANOVA with Sidak's multiple comparisons analysis)

**Fig 5: Serotonergic neuron counts of vmat2:eGFP neurons in raphae cluster**

5A


5B

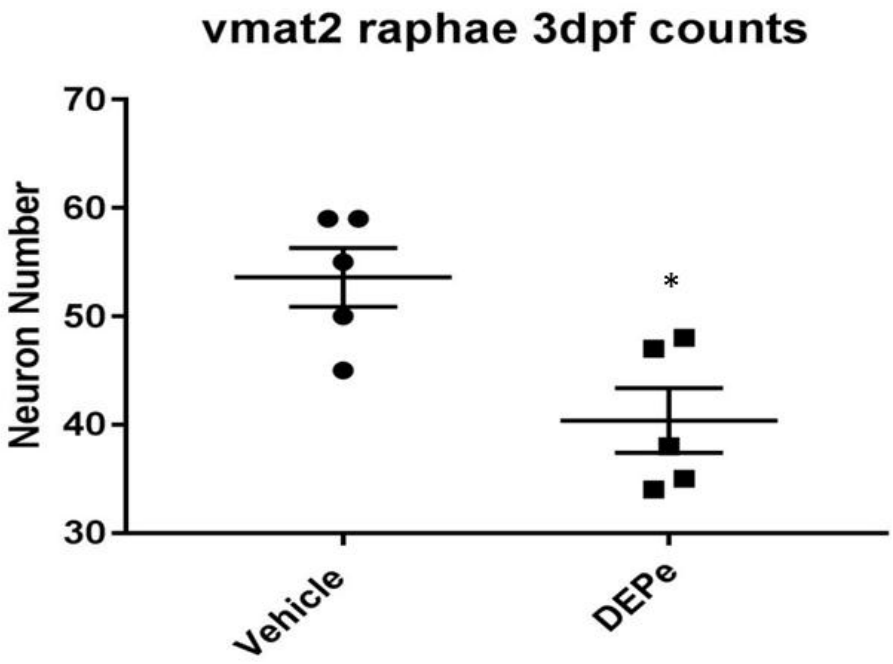
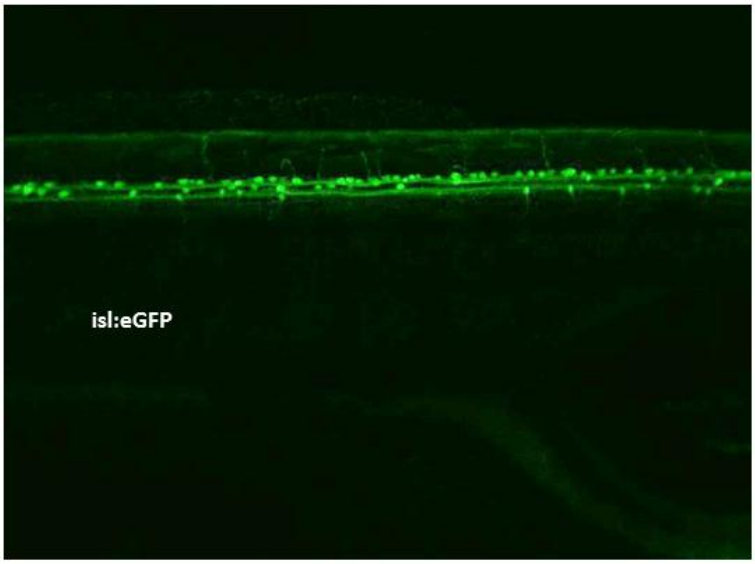



Fig 5: Neuron counts done on confocal images of vmat2:eGFP positive embryos in the raphae cluster (A) represent primarily serotinergic neurons. There is a statistically significant loss of neurons within this cluster at 3dpf as quantified by unpaired Student's t-test *=$p<0.05$ (B).

Given the considerable aminergic neuron loss in regions of the brain containing dopaminergic neurons, it is important to test the specificity of DEPe-induced neurotoxicity. To accomplish this, a non-dopaminergic cluster of neurons seen in the ZF line ET*vmat2*:GFP that primarily consist of serotonergic neurons within the raphae was quantified after DEPe exposure and washout as described above (Fig 5A). The quantification of this cluster resulted in loss of GFP positive neurons comparable to the clusters that contain dopaminergic neurons (Fig 5B). This suggests that exposure to DEPe results in loss of a variety of neuron subtypes and is not restricted to dopaminergic neurons.

**Fig. 6- Sensory neuron counts after DEPe on non-aminergic neurons**

**6A**




**6B**

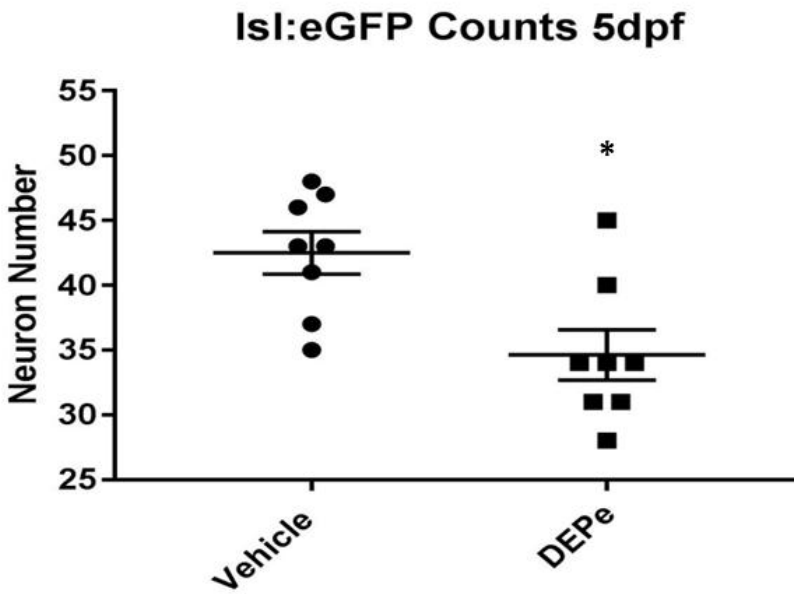



Fig 6- Neurons expressing GFP from the islet1 promoter were imaged and quantified at 5dpf after 24hours of DEPe treatment (A) in order to determine the selectivity of neurotoxicity induced by DEPe exposure. Quantification of blinded confocal images showed a statistically significant loss of GFP positive sensory neurons in the tail of Isl:GFP embryos after treatment (B). Statistical analysis by unpaired Student's t-test *=$p<0.05$.

One additional neuronal toxicity control was conducted to further understand how selective this toxin is in developing ZF larvae. Sensory neurons expressing GFP from the *islet1* promoter were quantified using the transgenic line Tg(isl1[ss]:Gal4-VP16,UAS:eGFP)$^{zf154}$, which labels both central and peripheral sensory neurons and is present in early developing ZF embryos (Fig 6A). After washout and imaging via confocal microscopy, quantification of peripheral sensory neurons in the tail of 5dpf larvae demonstrated a similar loss of GFP positive neurons compared to the loss seen in *vmat2*:GFP larvae (Fig 6B).

**Fig 7: Time window of neuronal susceptibility to DEPe exposure**

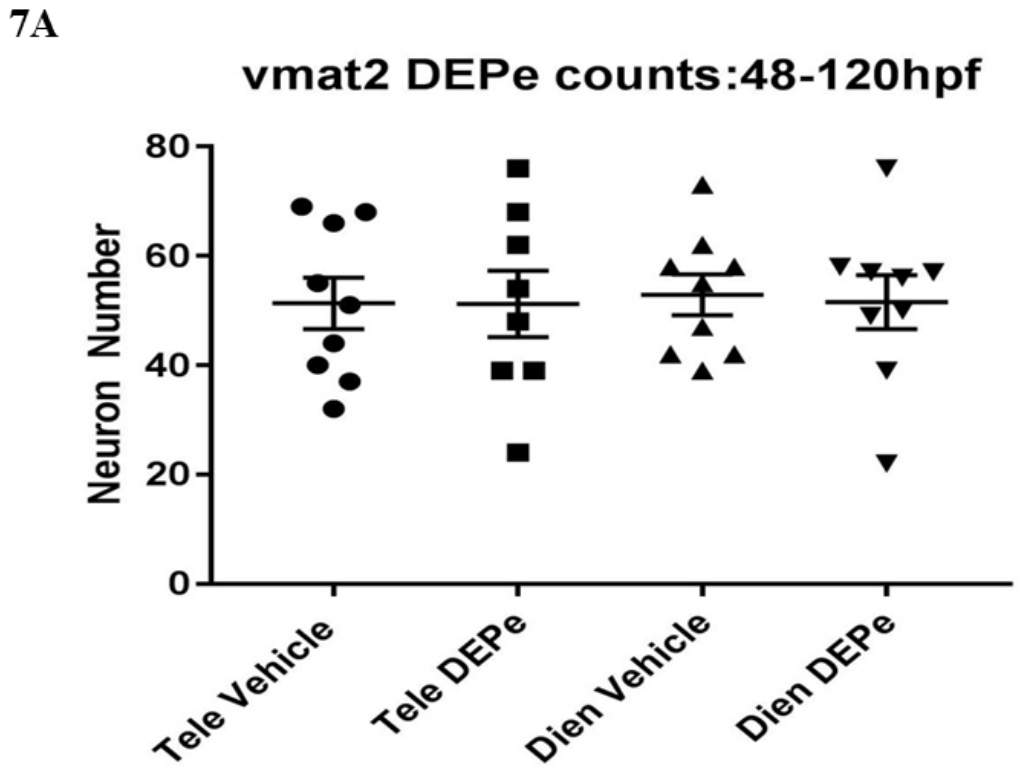



Fig 7: No significant change in neuron number was seen in aminergic neurons in the telencephalic and diencephalic regions of the vmat2:eGFP expressing larvae when they were treated between 48-120hpf with no wash-out period (Fig 8A). Statistical analysis one-way ANOVA.

Additionally, in order to determine when in development neurons are most susceptible to the toxic exposure, larvae were exposed between 48-120hpf to the same dose of DEPe and vmat2 GFP positive neurons were quantified. Under these exposure conditions, vmat2 GFP positive neurons were not lost, even in the absence of toxin washout (Fig 7A). When taken together, these experiments suggest that, during development, neurons of many types are sensitive to the exposure of DEPe and this is a very sensitive model of developmental neurotoxicity that can be used to identify windows of susceptibility to environmental neurotoxins. Additionally, when exploring what molecular mechanisms may be responsible for the neurotoxicity, we can utilize this neuron loss model to better understand how to rescue this toxicity. One major advantage of this *in vivo* model is also that it does not require many months of toxin exposure in order to establish a behavioral phenotype and significant neuron loss in order to provide information relevant to disease.

**Fig 8- Effect of DEPe treatment on neuroimmune response**

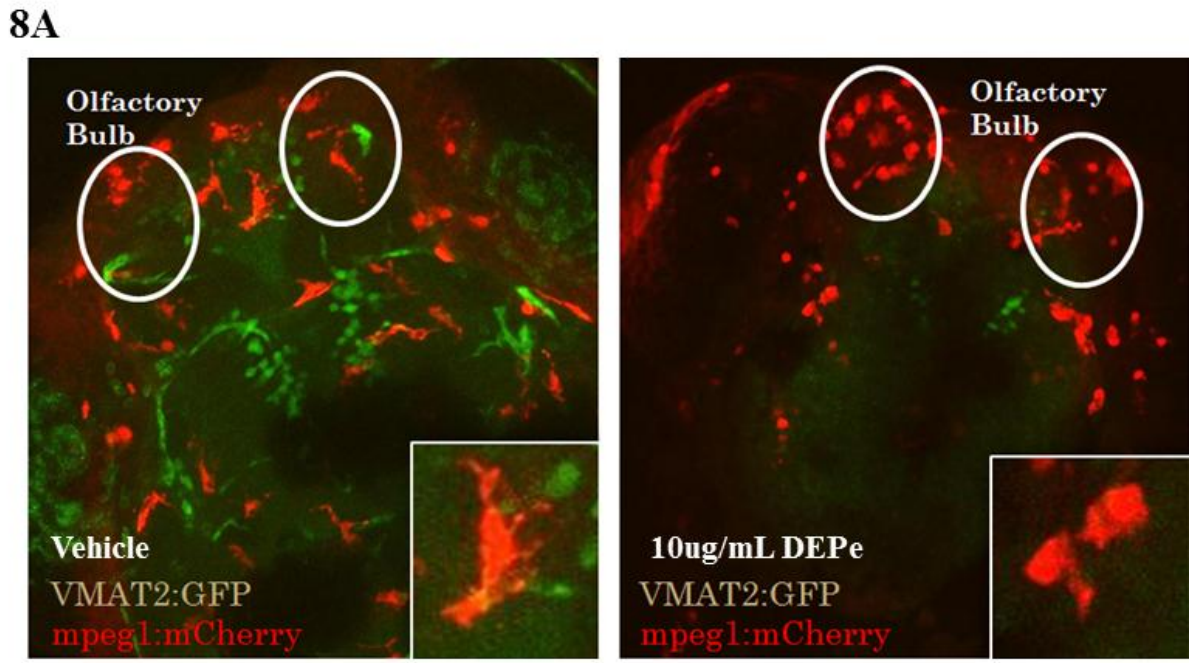



Fig 8: Embryos heterozygous for vmat2:eGFP and mpeg1:mcherry were fixed and antibody labeled in order to image both microglia (red) and aminergic neurons (green) in larvae treated with DEPe.

It has been established in the literature that exposure to air pollution can increase neuroinflammation. Inflammation is also a feature common to many neurodegenerative disorders. Therefore, understanding what exposure to DEPe does to the inflammatory cells in the brains of ZF embryos is of importance. DEPe treatment was conducted as described in Fig 3, where treatment was washed out before the brain was colonized by microglial cells. At 3dpf after exposure DEPe, transgenic ZF larvae expressing GFP in aminergic neurons (ET*vmat2*:GFP) and mcherry in microglia (*mpeg1*:mcherry) were fixed, antibody labeled, and imaged via confocal microscopy. A clear morphological change was observed in the mcherry-expressing microglia within the developing brain toward a more activated and inflammatory state (Fig 8A). This is

indicated by a more rounded cell body and retracted projections. Additionally, it is notable that there appears to be an aggregation of macrophages in the region of the olfactory bulb, which represents the primary interface between the central nervous system and the environment. This could indicate that any inflammatory response may be concentrated at the region of the brain in closest contact to the toxin.

**Discussion:**

Using a ZF animal model system to understand the neurotoxicity of exposure to DEPe allows for rapid, reproducible, and quantitative data regarding behavioral outputs, neuronal survival, and neuroimmune response. This chapter presents a variety of assays used to assess global as well as neuronal toxicity and describes specifically the developmental loss of neurons after exposure to the environmental pollutant DEPe. Gross toxicity after exposure has been described with increased mortality as well as decreased movement in light conditions using a behavioral light cycling assay. Both indicate that this animal model is sensitive, particularly early in development, to exposure to the toxin of interest.

The neuronal toxicity assays conducted using transgenic ZF expressing GFP in aminergic or sensory neurons have demonstrated significant neuronal loss across various neuronal subtypes when exposed to DEPe between 24-48hpf. With the understanding that the window of susceptibility to DEPe represents a developmental exposure, it is possible that neurons within the developing ZF embryo are uniquely sensitive to the toxin. In particular, the mechanisms of toxicity induced during this early and acute exposure model may not precisely mimic the processes that go on in neurodegenerative disorders. Although these are limitations of the model system, there are significant advantages that come with such an exposure. We have the ability to quickly assess and screen compounds for rescuing toxicity and we can also look more closely at the pathways induced after toxin exposure.

Within the exposure time window included in these studies, aminergic neurons are already colonizing the brain of the developing embryo which gives increased relevance to disease, but the cells that will eventually populate the brain as resident neuroimmune

macrophages are not yet functional and fully established. This suggests that the neuron loss quantified as the number of GFP positive neurons, which occurs very early, is likely not due to microglia within the brain, as these cells only become fully differentiated starting around 72hpf (Svahn et al 2012). Using a toxin wash-out model of exposure also means that the DEPe is removed before the neuroimmune cells would be functional in the brain. When viewed together, this fact makes it unlikely that the toxicity demonstrated above is due to inflammatory processes, as neuron loss has already begun to occur as early as 72hpf.

Although much remains to be understood regarding the neurotoxicity induced by exposure to DEPe, much of the data presented here suggests that there is significant *in vivo* neurotoxicity induced by exposure to DEPe in zebrafish. Given the epidemiological evidence suggesting an association between long-term exposure to traffic-related air pollution and PD, the findings in this chapter suggest that this uniquely sensitive model system may prove of great interest when studing the mechanisms of toxicity induced by such exposures and to understand how the disruption of specific pathways may lead to neuron loss.

Dissertation: Chapter 3

Diesel Exhaust Particulate Extract Exposure and Proteostasis

**Introduction**:

One common feature of most adult-onset neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Lewy body disease, is the accumulation of aggregate-prone proteins within neurons. Because many diseases share this common mechanism of toxicity, increasing focus has been put on upregulating protein turnover as a possible therapeutic intervention (Rubinsztein et al 2012). Understanding how environmental insults may inhibit protein turnover in ways that facilitate protein aggregation is of great interest in many neurodegenerative diseases. In PD, genetic evidence demonstrates that increase in aSyn protein over many decades is sufficient to cause disease. In order to understand the role of environmental exposures in altering the total burden of toxic aSyn in neurons, it is critical to understand proteostasis as a whole. Understanding how exposures can impact protein degradation is essential in order to utilize this knowledge for targeted therapeutic intervention to prevent neurotoxicity.

Because soluble aSyn can be degraded by the UPS or via autophagy, both of these cellular processes are of interest when understanding the mechanisms of action of environmental toxins associated with PD (Mak et al 2010, Ebrahimi-Fakhari et al 2011). From GWAS, it is known that many disease-associated SNPs involve protein degradation pathways. Several genetic mutations associated with PD reside in genes involved in ubiquitinylation and the UPS, including Parkin, UCH-L1, and Fbxo7 (Leroy et al 1998, McNaught et al 2001, Walden & Muqit et al 2017). In a similar way, exposure to the pesticide ziram, which is an environmental risk factor

for PD, is also known to cause inhibition of E1, an enzyme involved in the ubiquitin proteasome system (UPS) (Chou et al 2008). It is also likely that increasing aSyn protein alone is enough to inhibit the UPS, which may further potentiate aSyn accumulation and allow aggregates to form (Tanaka et al 2001, Bence et al 2001). This information suggests that one mechanism to explore when attempting to understand aSyn protein accumulation is inhibition of proteasomal degradation.

In addition to the UPS, aSyn protein can also be degraded via chaperone-mediated or macro-autophagy (Webb et al 2003, Vogiatzi et al 2008). Many diseases are known to be associated with reduced basal autophagy, including neurodegenerative disorders like Alzheimers and Parkinson's and markers of autophagy have been shown to be reduced in neurons within the brains of PD patients (Martinez-Vincente et al 2007, Levine & Kroemer et al 2008, Chu et al 2009, Dehay et al 2010). Indeed, one of the largest genetic risk factors for PD is a mutation in GBA, which causes a global lysosomal storage defect and increases PD risk by as much as 5-fold and those that develop disease have a lower average age of onset (Sidransky et al 2009, Ran et al 2016, Robak et al 2017). In addition, individuals with PD have been shown to have a slightly lower GBA activity level, even in the absence of mutation, when compared to age-matched controls (Murphy et al 2014, Alcalay et al 2015). Mechanistically, it has also been shown that GBA mutation may increase the formation of exosomes, facilitating the spread of aSyn oligomers that accumulate when degradation is inhibited (Papadopolous et al 2018). Studies are continuing to find rare SNPs involved in lysosomal function to be associated with disease (Robak et al 2017). In mouse models, inhibiting autophagy is enough to cause neurodegenerative phenotype and induces the formation of insoluble, ubiquitinylated aggregates (Hara et al 2006, Komatsu et al 2006). This represents strong evidence of the importance of protein degradation

and turnover in long-term neuronal health and the auto-lysosomal pathway represents an important mechanism to study when examining PD associated environmental risk factors that impair aSyn degradation. By preventing normal degradation of aSyn, inhibition of autophagy may increase the formation of oligomeric and insoluble species, cause the spread of misfolded aSyn through the brain, and eventually lead to the death of neurons carrying an abnormal burden of toxic aSyn (Mazzulli et al 2011).

Inducing autophagy to reduce the levels of aSyn in the brains of individuals with PD has recently become of interest for therapeutic purposes (Menzies et al 2017). In rodent models of PD, treatment with the drug nilotinib has been shown to be neuroprotective and to activate the clearance of aggregated protein (Hebron et al 2013, Karuppagounder et al 2014, Lonskaya et al 2014). Nilotinib is a c-Abl tyrosine kinase inhibitor first generated to treat chronic myeloid leukemia (CML). In the brains of PD patients, it has been shown that c-Abl is elevated and that c-Abl activation leads to aSyn posttranslational phosphorylation and decreased levels of parkin, both of which impair the degradation of aSyn via macroautophagy (Winslow et al 2010, Schlatterer et al 2011, Mahul-Mellier et al 2014, Brahmachari et al 2016). Additionally, this has led to the development of effective c-Abl inhibitors that have more favorable pharmacokinetic properties and may be utilized as a treatment for PD by clearing aSyn (Lee et al 2018). Although still in preliminary stages of investigation, this class of drugs has shown promise in several model systems and lends credence to the idea that impaired autophagy is a primary molecular mechanism of neurotoxicity in PD. Additionally, if drugs targeting autophagic flux prove effective for synucleinopathies, there is a strong possibility that they would provide efficacious reduction of aggregated proteins in other neurodegenerative diseases such as Alzheimer's, amyotrophic lateral sclerosis, Huntington's, and tauopathies.

Because protein turnover is a very rapid and dynamic process, measuring flux within the autophagic system has proven difficult (Klionsky et al 2016). In order to understand how exposures are impacting total lysosomal degradation in a living system, it is necessary to develop new and more sensitive tools with which to parse this pathway. Additionally, if protein turnover is altered after exposure to environmental toxins, it is important to understand how such alterations in flux may contribute to neurotoxicity. The following studies look at how exposure to diesel exhaust particulate extract (DEPe) alters proteostasis by particularly examining how DEPe affects the UPS and autophagic flux both in cells and in an *in vivo* ZF model system. Lastly, of significant importance for disease is the identification of therapeutics that may work to counteract the molecular mechanisms underlying disease progression. If autophagy inhibition is a major contributor to neurotoxicity, it is of great interest to test compounds designed to combat this toxicity pharmacologically.

**Methods**:

**rtPCR primers and assay**

Quantitative rtPCR was conducted in 96-well plates using a BioRad CFX Connect. All data was normalized to housekeeping control gene elfa (elongation factor 1B). Primers used as follows:

Zebrafish elfa Forward Primer: 5'-CTTCTCAGGCTGACTGTGC-3'
Zebrafish elfa Reverse Primer: 5'-CCGCTAGCATTACCCTCC-3'

Zebrafish sncg1 Forward Primer: 5'-ATGGTGGTATGGAAGGAGGA-3'
Zebrafish sncg1 Reverse Primer: 5'-GGGCTCAGGGAAAGTCTTTT-3'

Samples were run using the BioRad SsoAdvanced SYBR Green Supermix under conditions recommended by the manufacturer. All data was calculated using double delta Ct relative to internal housekeeping gene expression.

**Western blot for ZF**

Between 25-50ug of total protein was loaded onto a 12% SDS-PAGE gel with 1x loading dye and 2-mercaptoethanol in a final volume of 25uL. Gel was run and transferred to nitrocellulose membrane using the XCell-II blotting system (Life Technologies). Membrane was blocked in 5% non-fat milk and probed with antibodies: rabbit α sncg1 $1^o$ antibody (1:1000) followed by donkey-α-rabbit HRP $2^o$ antibody (1:2500); mouse α tubulin $1^o$ antibody (1:10000) followed by goat α mouse HRP $2^o$ antibody (1:10000). Blots were developed in chemiluminescent substrate ECL Plus (ThermoFisher 32132) and exposed to film for band visualization. Quantification of bands was done with ImageJ using the gel analysis feature. For sncg1 quantification tetramer (~55kD), all bands were normalized to α-tubulin (~50kD).

**FACS analysis of SK-GFPu**

Human neuroblastoma SK-N-MC cells stably transfected with CMV:GFPu were grown as described previously (Wang et al 2006). Cells were plated in 24-well plates for assay and adhered until 60-75% confluent. Cells were treated with DMSO as a vehicle (0.5% DMSO) and lactacystin was used as a positive control at a concentration of 2.5uM. DEPe was added between 10-25ug/mL and all treatments were incubated at 37$^{o}$C for 24 hours before FACS analysis. After 24 hours of treatment, propidium iodide (PI) was added to wells for 30 minutes at a concentration of 1ug/mL in order to sort for viable cells. Cells were released in trypsin and immediately sorted using fluorescence activated cell sorting (FACS). Cells of interest fall below PI threshold and exhibit an increase in GFP fluorescence after lactacystin treatement in quadrant A4.

**Autophagy Western blot assay in SK-N-MC**

Cells were plated and treated in 6-well plates for 24hrs with DMSO vehicle control at 0.1%, 10ug/mL DEPe, or pepstatin A (Cat. No. P5318-5MG, Sigma Aldrich) at 10 ug/mL with E64D at 5ug/mL (Cat. No. BML-PI107-0005, Enzo Life Sciences). Cells were washed in PBS 1x before direct lysis in 1x RIPA with protease inhibitors (80-200uL/sample). Samples were sonicated briefly on ice before centrifugation at 11,000xg for 15 minutes at 4$^{o}$C. Supernatants were transferred to a clean microfuge tube and protein concentrations were established by BCA assay. All lysates were stored at -80$^{o}$C.

Between 25-50ug of total protein was loaded onto a 12% BOLT bis-tris gel with 1x loading dye and 2-mercaptoethanol in a final volume of 25uL. Gel was run and transferred to nitrocellulose membrane using the XCell-II blotting system (Life Technologies). Membrane was blocked in

5% non-fat milk and probed with antibodies: rabbit α lc3B $1^{o}$ antibody (1:1000) followed by biotinylated goat-α-rabbit $2^{o}$ antibody (1:2500) and streptavidin HRP diluted 1:250; mouse α tubulin $1^{o}$ antibody (1:10,000) followed by goat α mouse HRP $2^{o}$ antibody (1:10,000). Blots were developed in chemiluminescent substrate ECL Plus (ThermoFisher 32132) and exposed to film for band visualization. Quantification of bands was done with ImageJ using the gel analysis feature. For lc3II/lc3I ratios, band intensity for both lc3I (~17kD) and lc3II (~15kD) was measured and displayed as a ratio.

**Zebrafish autophagy assay**

For autophagy studies, transgenic ZF expressing GFP tagged lc3 under the neuronal promoter *elavl3,* Tg*(elavl3:eGFP:map1lc3b)*$^{LA50016}$ were utilized, known as *huc:eGFP:lc3* onwards. For autophagy studies, experiments were done as previously published. Briefly, ZF were treated between 48-72hpf before being live imaged via confocal microscopy (Leica SPE) and analyzed for autophagosome number.

Single larvae were anesthetized with <0.01% tricaine methanesulfonate (TRICAINE-S, MS-222, Western Chemiccal) and mounted in 1% agarose into a 35/12mm glass-bottom culture dish (Cat. No. 64-0757, Warner Instruments). For dorsal views used for quantification of punctae, z-stacks of eGFP-Map1lc3b positive optic tectum regions were acquired using a 40x oil0immersion objective (NA=1.15) and a 488 nM excitation laser line. Each z-stack was comprised of 13 1024x1024 pixel sections with a z-step size of 2 microns. 3 non-consecutive sections from the optic tectum z-stack were quantified for punctae number.

**Nilotinib rescue**

Rescue experiments were conducted by co-treating embryos with DEPe as described above and the autophagy-inducing drug nilotinib (Sigma-Aldrich CDS023093) at a concentration of 10uM along with a DMSO vehicle control. After 48hpf DEPe washout, embryos were retreated with nilotinib for the remainder of the experiment and vmat2 neuron counts were analyzed via confocal microscopy in imageJ as previously described.

**Results**:

Based on the findings after DEPe treatment in human and rodent cells, it is important to understand if there is any alteration in ZF sncg1 transcript or protein after exposure to DEPe during development. Of significant note, although sncg1 is the functional equivalent of human aSyn, the promoters of these to genes vary widely. In order to understand whether DEPe exposure alters sncg1 transcript level in ZF larvae in a manner similar to that seen in human and rodent cells, embryos were treated between 24-48hpf followed by immediate RNA extraction from whole embryos in Trizol. Expression of sncg1, th1, and the pan-neuronal gene HuC all showed a significant reduction in gene expression after exposure to DEPe (Fig 1A). This is in contrast to the elevated synuclein expression seen in human cell culture as shown in the previous chapter. Because th1 and HuC gene expression were also significantly reduced (Fig 1A), it is possible that this decrease in neuronal gene expression is due primarily to neurotoxicity induced by DEPe exposure rather than promoter changes that were shown previously via luciferase and rtPCR in neuroblastoma cell lines that may be more resistant to death after exposure to a toxin.

In addition to sncg1 gene expression, ZF larvae were also treated and harvested for protein in order to look at the total amount of sncg1 in the brain. Rather than extract protein from the whole embryo, this study was done on protein extracted from the heads of decapitated embryos at 5dpf. After exposure to DEPe from 48-120hpf, the protein level of sncg1 was significantly increased relative to control embryos, particularly when looking at the species that corresponds with roughly tetrameric synuclein (Fig 1B). This is particularly interesting when viewed in light of the gene expression changes shown in Fig 1A. Because the protein increases cannot be due to increased promoter activity or transcript, there must be alternative mechanisms that lead to the accumulation of protein in these embryos.

**Figure 1: ZF sncg1 expression and protein level**

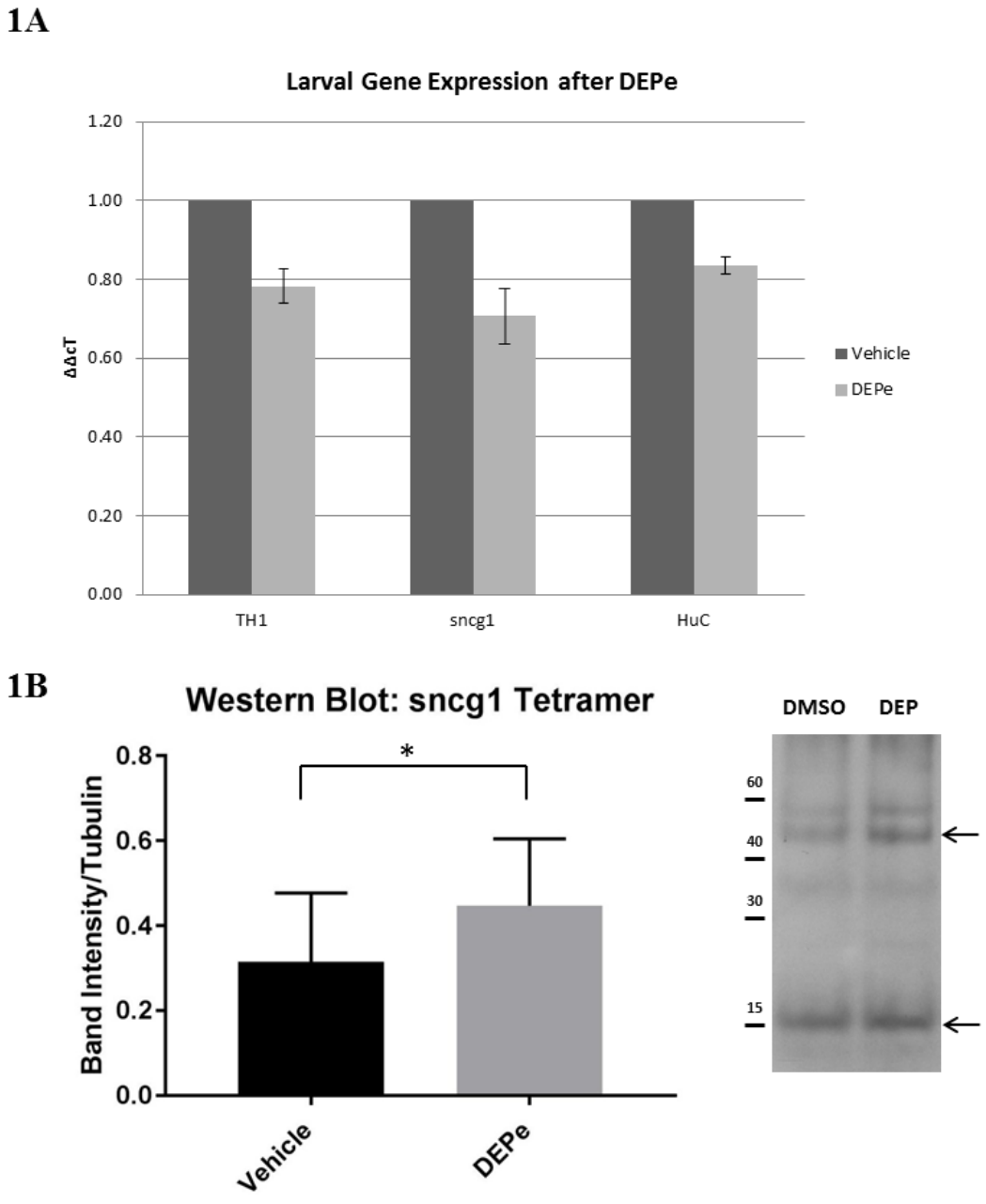
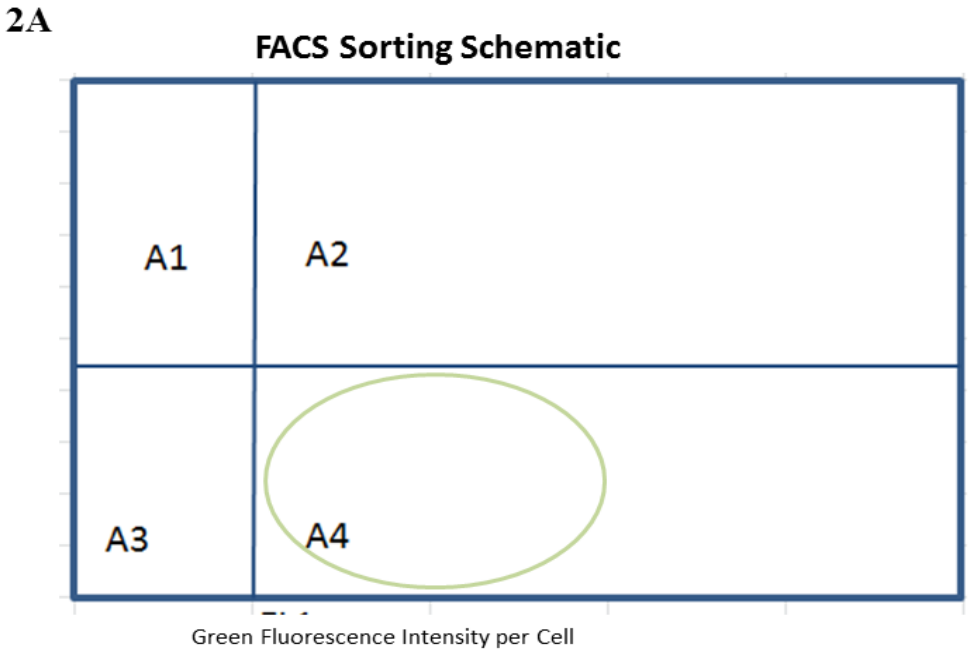



Fig.1- A) approximately 20 embryos per condition were treated between 24-48hpf and harvested in Trizol for RNA extraction and first-strand cDNA synthesis. rtPCR was conducted (N=2 per condition run in duplicate) to test expression of th1, sncg1, and HuC relative to the expression of control gene elfa. B) Western blot run on protein extracted from the heads of approximately 50 embryos per treatment (N=5 treatments) in DEPe for 48-120hpf and normalized to tubulin intensity. Band intensity measured for 55kD (tetrameric synuclein) and quantified for sncg1. (Paired Student's t-test p=0.027)

Due to the lack of increase in *sncg1* gene expression in treated ZF larvae, it is important to interrogate whether or not defects in protein degradation pathways may explain the protein accumulation observed. Using human neuroblastoma cells, proteasome efficiency was tested. Transformed SK-N-MC cells (SK-GFPu) expressing a ubiquitin proteasome degron called GFPu, which targets the protein for rapid degradation via the UPS, were used in the assay. Under normal conditions, GFPu is degraded very quickly by the proteasome and therefore GFP fluorescence is low. Upon inhibition of the proteasome, GFP accumulation can be identified via FACS as an increase in total cell fluorescence (Fig 2A). Cells were treated for 24 hrs with increasing doses of DEPe or the positive control, lactacystin, which is known to induce proteasome inhibition. Treatment with lactacystin showed 2.5 fold higher GFP fluorescence when compared to control, which is an indication that the cells were sensitive to UPS inhibition. Up to a concentration of 25ug/mL, DEPe treatment did not result in any detectable change in total cell fluorescence (Fig 2B), suggesting DEPe does not have an impact on proteasome function, at least during the exposure timeframe of 24 hours.

**Figure 2: Proteasome inhibition in cell GFPu accumulation**



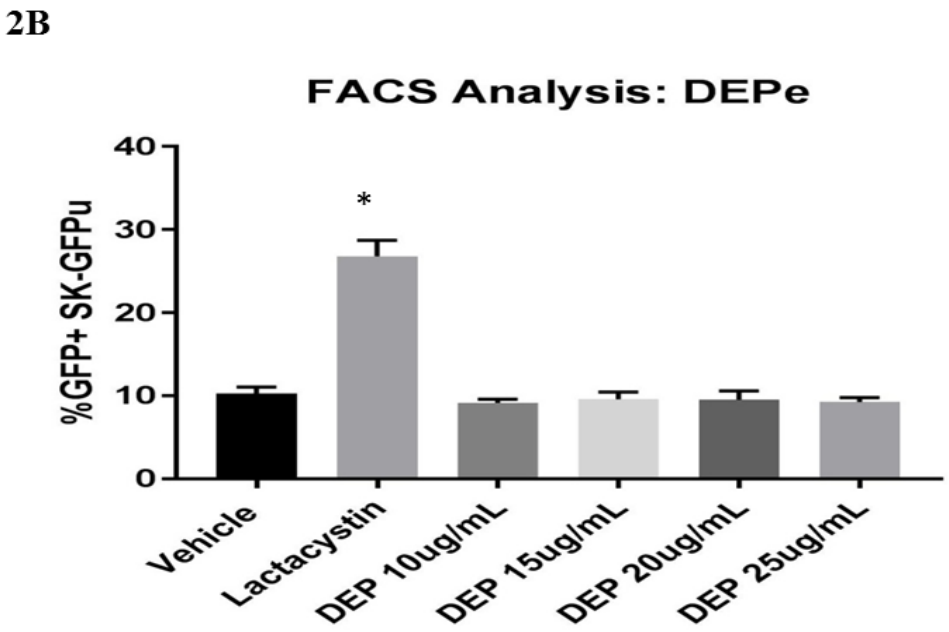




Fig 2: Schematic of the FACS sorting data (A). Shift in cell fluorescence to the right indicates increase in GFP resulting from inhibition of the UPS (quadrant A4). Quantification of the % of cells in quadrant A4 represents the degree of proteasome inhibition. Lactacystin resulted in a statistically significant increase in GFP+ cells relative to control (2.5 fold increase), but no dose of DEPe altered GFP fluorescence (B). Data analysis: one way ANOVA with Dunnett's multiple comparisons analysis *p=0.0001

**Figure 3: Autophagy rate in cells via Western blot**

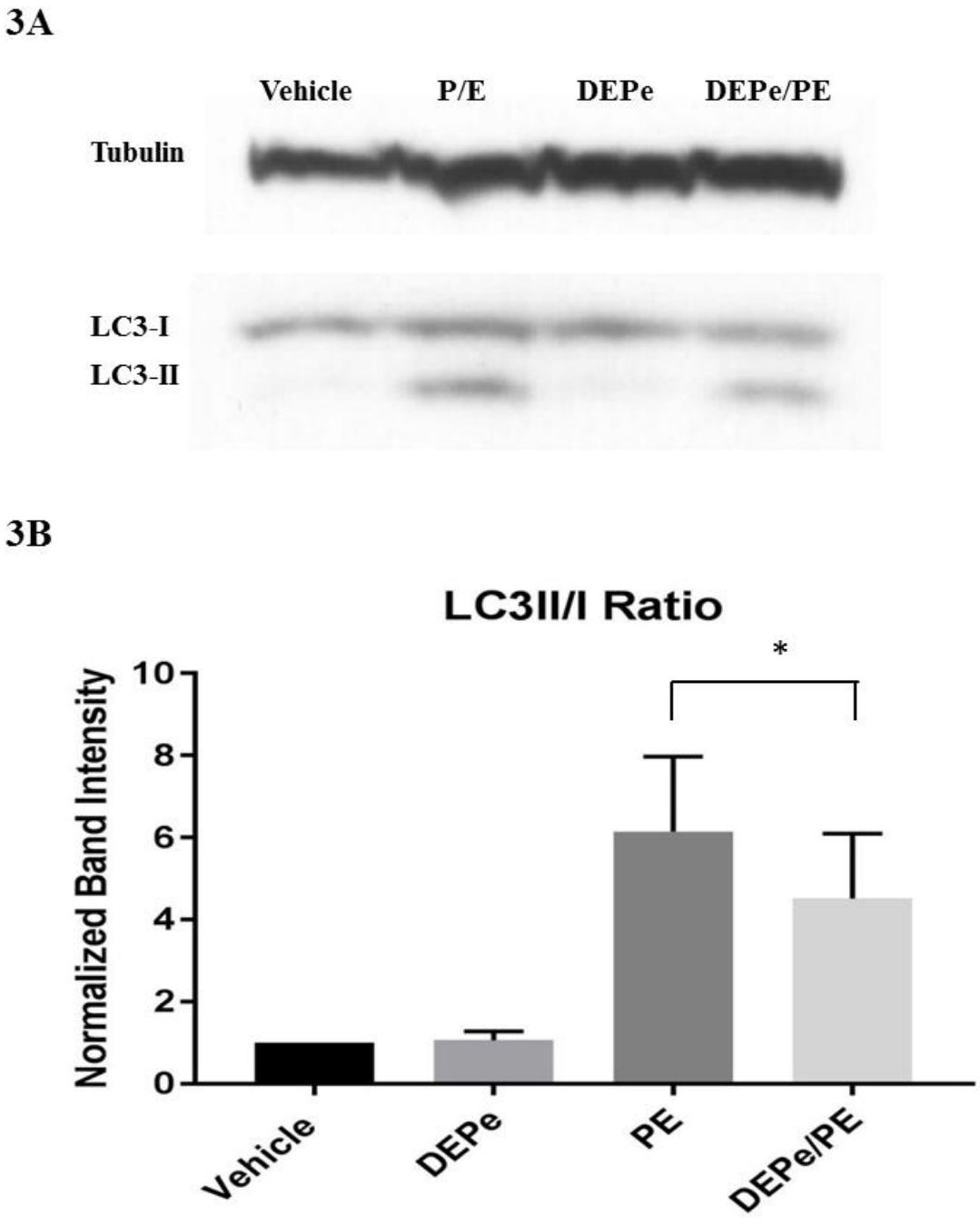



Fig. 3: SK-N-MC cells treated with DEPe, pepstatin/E64D, or both were collected for protein purification after 24hrs of treatment and run on a Western blot (A). Lysosomal inhibition caused a significant increase in LCII/I ratio, but when combined with DEP, showed a decrease in LC3II/I, indicating lower autophagic flux (B). Quantification of N=5 Western blots; *p=0.044 by paired Student's t-test.

Because the increase in sncg1 protein in ZF larvae is likely not due to inhibition of the UPS system, it is necessary to explore the process of autophagic turnover to determine if treatment with DEPe could be disrupting proteostasis in a manner consistent with sncg1 accumulation seen by Western. To test this, treatments were conducted in SK-N-MC human neuroblastoma cells for 24hrs and levels of lc3II/lc3I were quantified via Western blot (Fig 3A). To determine if DEPe may impact autophagy, cells were treated with DEPe and the lysosomal protease inhibitors Pepstatin A and E64D (P/E). As described in the literature, treatment with P/E alone lead to an increase in the ratio of lc3II/I, as shown by Western blot, because turnover was blocked. After treatment with DEPe alone, the ratios of lc3II/I were unchanged, but after treatment in combination with Pepstatin A and E64D (P/E) to inhibit lysosomal proteases, there was a reduction in lc3II/lc3I levels compared to P/E treatment alone, suggesting that the rate of autophagosome formation during the treatment was reduced in a manner consistent with a reduction in autophagic turnover (Fig. 3B). When taken together, this data suggests that the increase in sncg1 protein levels originates not from increased expression or decreased proteasome activity, but in reduced turnover through the autophagic pathway.

**Figure 4: ZF autophagic flux assay**

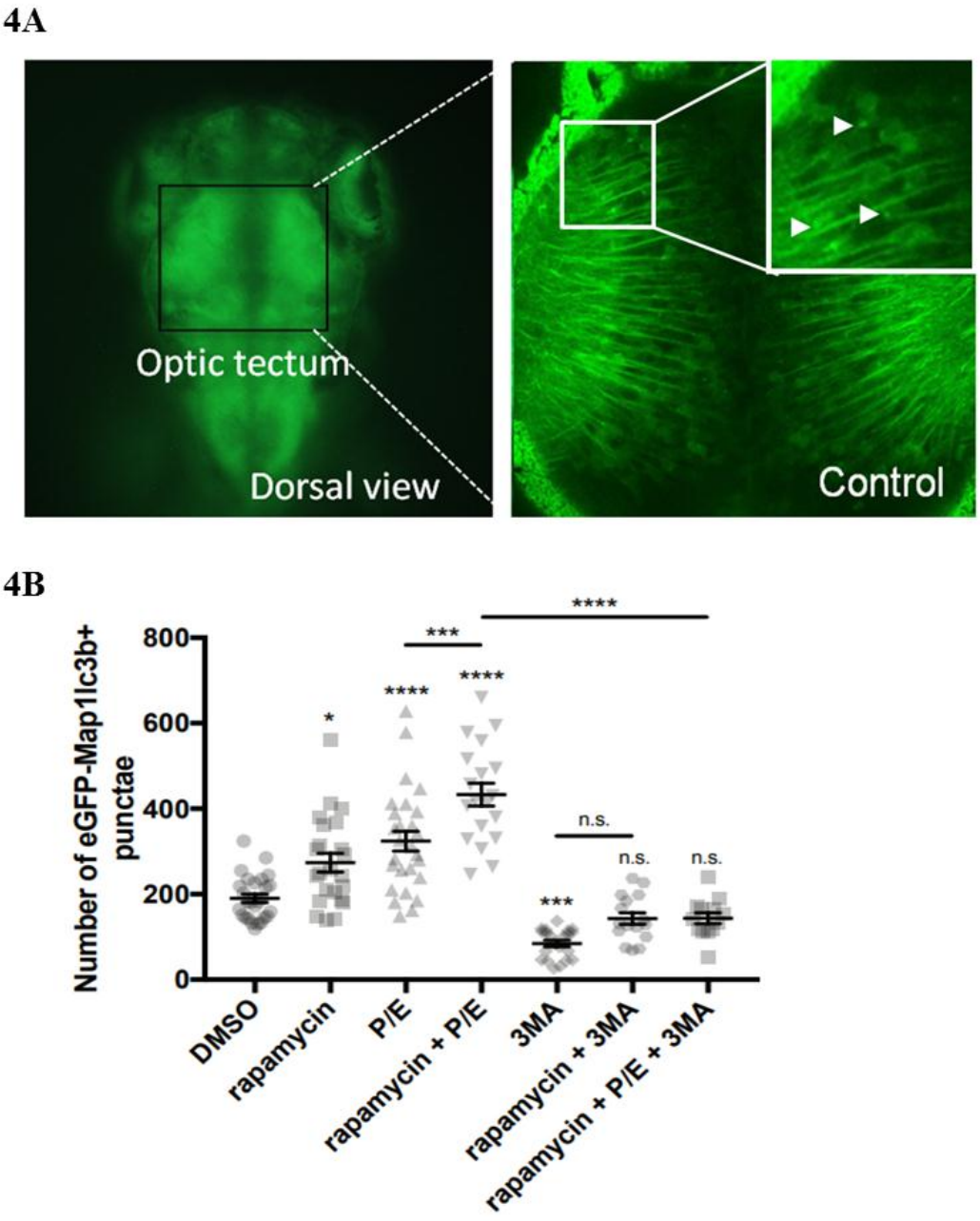



Fig 4- Confocal image of a life (Tg*(elavl3:eGFP:map1lc3b)*$^{LA50016}$) ZF optic tectum showing GFP expression from the HuC promoter and labeling autophagosomes (A). Quantification of punctae in (Tg*(elavl3:eGFP:map1lc3b)*$^{LA50016}$) larvae after treatment with autophagy-modifying chemicals including rapamycin (increases autophagosome formation), P/E (lysosomal inhibitor), and 3MA (inhibitor of autophagosome formation) (Khuansuwan et al 2018). Statistical analysis by one-way ANOVA with Tukey's multiple comparison analysis n>12 embryos per group. *=p<0.05, ***=p<0.001, ****=p<0.0001

To confirm the reduction in autophagic flux seen previously in an animal model, drug treatments and quantification was conducted in transgenic ZF larvae expressing eGFP fused to lc3 in the brain (Tg*(elavl3:eGFP:map1lc3b)*$^{LA50016}$) referred to as HuC:eGFP:lc3 (Khuansuwan et al 2018). Under normal conditions, eGFP:lc3 protein is fairly diffused and relatively ubiquitous within neurons. Autophagosomes can be seen as small, intracellular, eGFP-positive punctae when visualized in the live animal by confocal microscopy (Fig. 4A). Counting the number of autophagosomes in the region of the optic tectum can be an indicator of autophagy, particularly when paired with inhibitors of autophagic flux as described previously in cell culture. This line was tested for proof of concept using drugs known to upregulate autophagy (mTOR inhibitor rapamycin), prevent autophagosome formation (3-methyl adenine), and decrease autophagic carrier flux (lysosomal protease inhibitors pepstatin A and E64D), both of which have been shown to alter the number of autophagosomes in the ZF brain as predicted by their mechanism of action (Fig 4B). Rapamycin significantly increases the number of autophagosomes, consistent with increased formation, P/E treatment significantly increases punctae by inhibiting lysosomal function, and the combination of an inducer of autophagy and an inhibitor of turnover yields an even greater increase in punctae as they build up and cannot be degraded. Treatment with the inhibitor of autophagy, 3MA, shows a significant decrease in the number of autophagosomes, consistent with the proposed mechanism of action.

**Figure 5: Effect of DEPe on autophagic turnover in ZF**

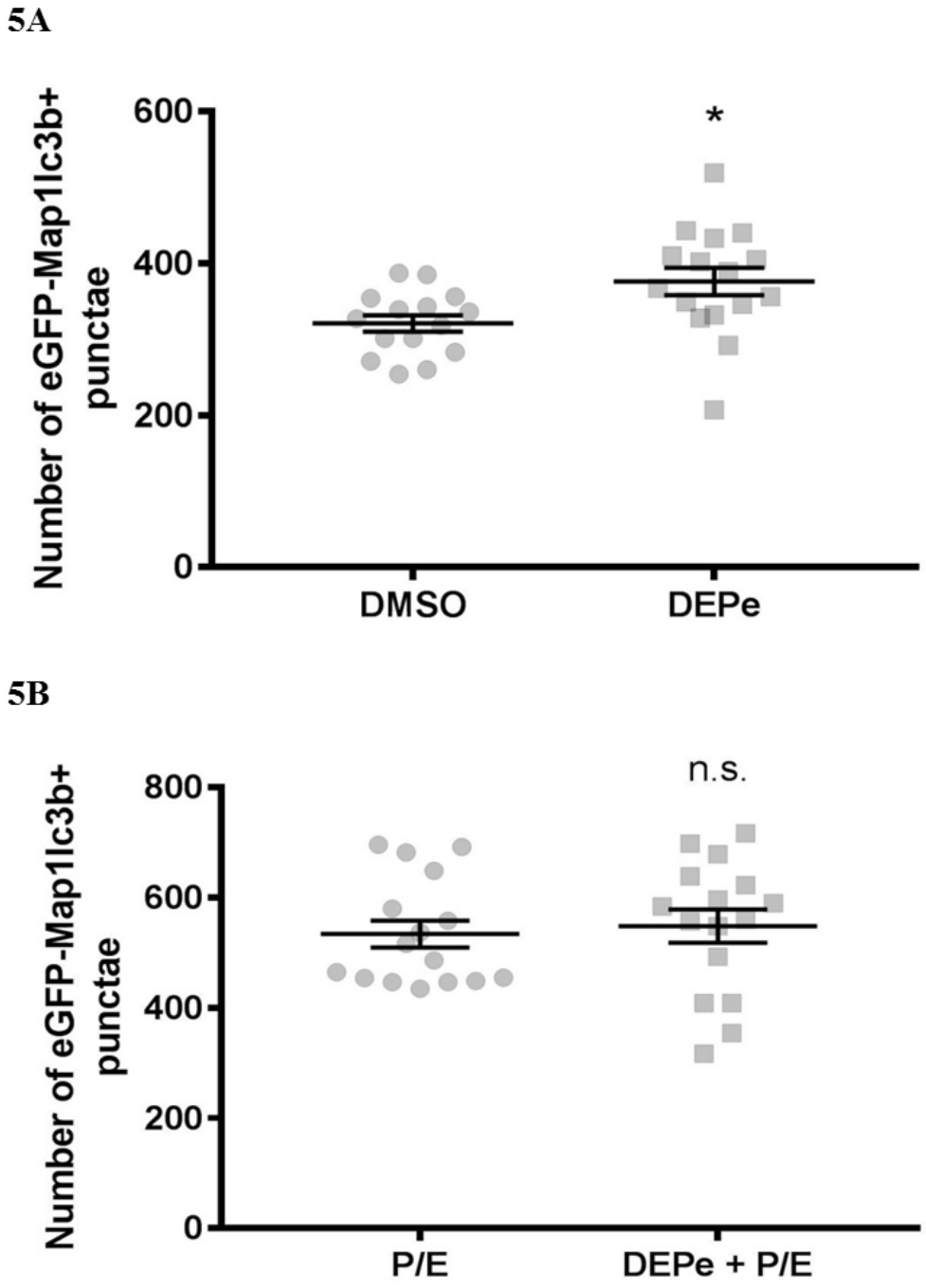



Fig 5- Punctae quantification from confocal images of life embryos treated with DEP (A) show an increase in punctae as analyzed by student ttest ($p>0.05$). When paired with a lysosomal inhibitor, DEPe does not show any additional punctae formation (B), suggesting that the underlying dysfunction in autophagy seen in Fig 4A originates from decreased autophagic flux. Statistical analysis by unpaired Student's t-test, *=$p<0.05$.

To determine if DEPe acts as an inhibitor of autophagic flux *in vivo,* we performed similar experiments in the *huc:eGFP:lc3* transgenic zebrafish with DEPe and lysosomal inhibitors P/E to quantify autophagosome turnover. When *huc:eGFP:lc3* embryos are treated with DEPe for 24hrs, there is an increase in the total number of autophagosomes present at 3dpf (Fig 5A). This can be interpreted as either an increase in autophagsome formation (like rapamycin) or a decrease in turnover (like P/E treatment). When combined with lysosomal inhibition by blocking turnover with the protease inhibitors P/E, there is no difference in the total autophagosome accumulation seen after 24hrs (Fig 5B). This, when compared with drugs that have known mechanisms of action (Fig 4B), suggests that the initial increase in autophagosome number is not due to increased formation, but rather is consistent with inhibited turnover. These findings can only be explained by a decrease in turnover consistent with what was seen by Western blot in human cell culture described previously in Fig 3B.

**Figure 6: ZF autophagy rate after nilotinib treatment**

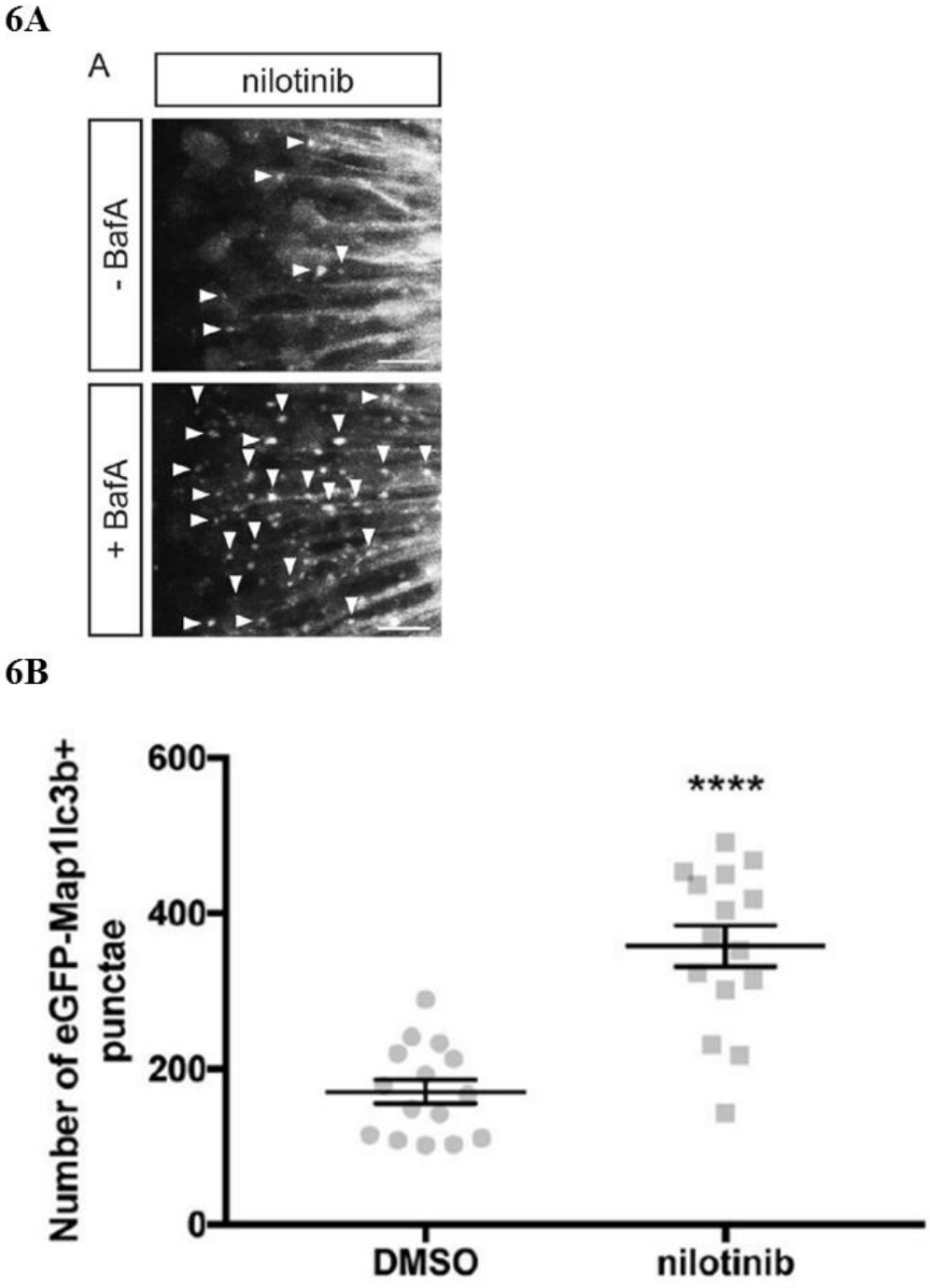



Fig 6- Example of a drug known to induce autophagy as quantified using the ZF autophagic flux assay (A). The drug nilotinib is a clear and potent inducer of punctae, consistent with the known mechanism of action (B) (Khuansuwan et al 2018). Statistical analysis by unpaired Student's t-test, ****=$p<0.0001$.

With a functional ZF assay that can screen compounds for alterations in autophagic flux, we next used this transgenic line to test a compound that has been increasingly studied as a possible therapeutic for neurodegenerative diseases. Nilotinib is a known inducer of autophagy that acts through inhibiting cAbl. Treating *huc:eGFP:lc3* transgenic zebrafish with nilotinib for 24 hours before live imaging via confocal followed by punctae counts demonstrated a significant increase in autophagosomes within the ZF optic tectum and, when treated with a drug that inhibits autophagosome/lysosome fusion known as bafilomycin A, causes a very dramatic increase in the number of punctae (Fig 6A-B). This provides additional support for the assay as an appropriate tool to screen for autophagy-modifying compounds and also demonstrates that nilotinib may be an appropriate therapeutic when attempting to rescue ZF with autophagy impairment.

Given the evidence in fish and cell culture that DEPe reduces autophagy, it can be interrogated whether or not this reduction leads to neurotoxicity *in vivo* and may explain the neuron loss seen in developing zebrafish larvae. To test this, we used to drug nilotinib previously shown in ZF to increase autophagic flux, and treated in combination with DEPe treatment in an attempt to rescue the aminergic neuron loss seen in fish as shown in chapter 2. Treatment with DEPe as described previously  under conditions that lead to neuron loss plus the addition of nilotinib between 24-72hpf can be used to test the efficacy of autophagic induction to reduce neuron loss. Embryos treated and fixed at 72hpf were analyzed for *vmat2*:GFP expression and neuron counts via confocal microscopy as previously described. Treatment with DEPe significantly reduced the number of neurons in the diencephalic aminergic cluster, but in the nilotinib or nilotinib+DEPe treatment conditions, there was not a significant decrease in the

diencephalon (Fig 7A). This suggests that upregulating autophagic flux may have a neuroprotective effect against DEPe induced toxicity.

**Figure 7: Testing neuroprotection with nilotinib after DEPe treatment**

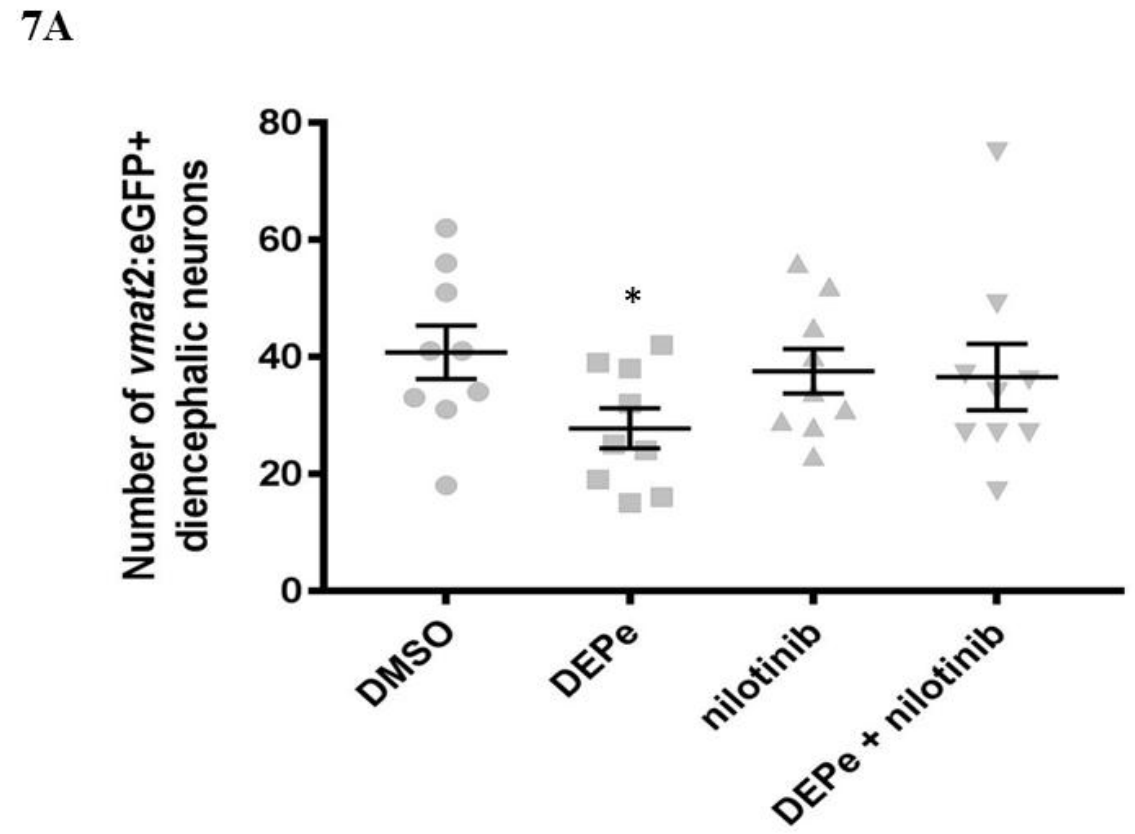



Fig 7- vmat2:eGFP neuron counts from blinded confocal images after treatment with DEPe and/or nilotinib. Data presented shows only diencephalic counts. DEPe alone shows a significant reduction by unpaired Student's t-test (*=$p<0.05$) in the number of aminergic neurons in the diecephalon. Neither nilotinib alone nor the combined nilotinib/DEPe treatments shows a significant change in neuron number.

**Discussion**:

Given the importance of protein turnover in many neurodegenerative diseases, understanding how environmental exposures can impact the degradation of aggregate-prone neuronal proteins is crucial. Although much is known about the genetic causes leading to inhibition of the UPS and autophagic clearance, the role of exogenous toxins has not been thoroughly explored. This study is an attempt to understand how environmental exposures can disrupt pathways known to cause neurotoxicity and neurodegenerative disease and lead to the accumulation of aSyn. Exposure to DEPe is shown here to increase sncg1 protein, but not transcript, in ZF embryos. From a mechanistic standpoint, this may be the result of impaired proteostasis. Cell-based data provides evidence that the proteasome is not inhibited after DEPe exposure, but Western blot for LC3II/I ratios indicate that autophagic flux may be the cause of protein increases. When testing this theory in ZF, confocal counts of GFP-positive LC3 punctae indicate that autophagosome turnover is likely impaired. In order to determine if this decrease in protein degradation causes neurotoxicity, vmat2:eGFP neurons were quantified with and without the autophagy inducing drug nilotinib. The reduction in neurons loss seen in this model supports the fact that decreased autophagic flux can cause toxicity in neurons. In cells as well as ZF brains, the process of autophagy appears to be downregulated or impaired after treatment with DEPe in a manner that is critical for neuron survival.

Genetics have provided an abundance of evidence to link lysosomal function and protein turnover to PD. Upregulating autophagic flux pharmacologically is currently of interest in clinical trials for PD. The drug nilotinib, which induces autophagosome formation, appears, at least preliminarily, to be of interest in treating patients with disease and is currently in phase II clinical trials. Although this is a very short term toxicity/rescue study, the link between

neurotoxicity and protein turnover is of significant therapeutic interest and provides insight into the mechanisms of neuron loss after environmentally relevant toxin exposure. Because nilotinib is a selective tyrosine kinase inhibitor that is functional as a chemotherapeutic agent, future experiments should focus on identifying drugs that can target autophagic flux more precisely and can be used to mitigate any off-target effects in order to be suitable for long-term treatment of individuals with Parkinson's disease.

Of additional interest to the field of neurodegenerative diseases, this study provides evidence that disruption of proteostasis is critical in the study of neurotoxicity. This is a pathway that is clearly impaired in the brains of those with neurodegenerative diseases and one which may serve a critical role in preventing disease initiation and early spread. Preventing protein aggregation and pathology early in disease may prove to be an important therapeutic strategy in both genetic and idiopathic forms of disease and pharmaceutical agents that can target these pathways likely have a role in many common neurodegenerative disorders.

# Dissertation Conclusion

The etiology of PD remains, in large part, unknown. Genetic and environmental evidence suggests that a variety of pathways, when disrupted, can cause disease. The data presented here demonstrates possible links between diesel exhaust exposure and adverse outcome pathways associated with neurodegenerative diseases including toxic protein accumulation and reduction in autophagic turnover. The chronic disruption of these pathways may provide relevant insight into how environmental pollutants can serve to exacerbate or mimic genetic predispositions toward development of PD. Of particular interest to this study is the connection between exposure to diesel exhaust and dysregulation of synuclein homeostasis leading to the accumulation of protein and eventual neurotoxicity. Alterations in one or more of these pathways, when extrapolated over a lifetime of exposure to such a ubiquitous pollutant, likely contribute to the slow and progressive neuron loss characteristic of this disease of aging.

This body of work highlights the importance of maintaining proteostasis and sheds new light on how exposure to environmental toxins may contribute to disease by altering autophagic flux in neurons. Tracing the cause of protein accumulation to a reduction in total autophagy brings another level of disease-relevance given that there are genetic associations implicating impaired autophagy to neurodegeneration as well as mitochondrial dysfunction. Connecting these adverse outcome pathways to long-term exposure to air pollution may, at least in part, explain the increased risk of PD that has been shown with prolonged exposure to urban air pollutants.

In addition to this increased understanding of how the environment can contribute to disease risk by altering autophagic flux, the conclusion of this work also indicates that a

potentially underexplored avenue of disease intervention by inducing or upregulating autophagic flux. Of interest in both genetic causes of disease as well as environmentally modulated risk factors, the fact that nilotinib, a potent inducer of autophagosome formation, can serve to protect neurons from exposure to environmental toxins demonstrates that therapeutic intervention focused on autophagic flux may prove to be widely efficacious for idiopathic as well as genetic forms of PD.

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