IDENTIFICATION OF NEUROTOXIC TARGETS OF DIVERSE CHEMICAL CLASSES OF DIETARY NEUROTOXINS/NEUROTOXICANTS

by

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To mom and dad. You are the people I strive to be. Thank you for everything you have done for me.

To Papa. I know you'll be watching over me the rest of my career. I'm so glad I got to celebrate my defense with you.

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*This dissertation contains published articles as part of its final work. Below is a list of the articles and book chapters that have been used. Each chapter that contains portions of articles or is published as an article has a note at the beginning of the chapter that specifies the citation.

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5-HIAA:	5-hydroxyindoleacetic acid
5-HT:	5-hydroxytryptamine, serotonin
6-OHDA:	6-hydroxydopamine
8-MeIQx:	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
α-syn:	alpha-synuclein
AαC:	2-amino-9H-pyrido(2,3-b)indole
ACh:	acetylcholine
AIA:	aminoimidazoaazarine
ALDH:	aldehyde dehydrogenase
Bcrp1/Abcg2:	breast cancer resistance protein
BSA:	bovine serum albumin
cAMP:	cyclic adenosine monophosphate
COMT:	catechol-O-methyltransferase
CYP:	cytochrome P450
DA:	dopamine
DAB:	3,3'-diaminobenzidine
DCPIP:	2,6-Dichlorophenolindophenol
DOPAC:	3,4-dihydroxyphenylacetic acid
DOPAL:	3,4-dihydroxyphenylacetaldehyde
ECD:	electrochemical detector
GABA:	γ-aminobutyric acid
GBA:	glucocerebrosidase
GTPase:	guanosine triphosphate hydrolase
HAA:	heterocyclic aromatic amine
HNE:	4-hydroxynonenal
HPLC:	high performance liquid chromatography
HVA:	homovanillic acid
ip:	intraperitoneal
IQ:	2-amino-3,8-dimethylimidazo[4,5-f]quinoline

iv:	intravenous
kDa:	kilodalton
L-DOPA:	l-3,4-dihydroxyphenylalanine
LB:	Lewy body
LHM:	liver homogenization medium
LRRK2:	Leucine-rich repeat kinase 2
MAO:	monoamine oxidase
MeAαC:	2-amino-3-methyl-9H-pyridole[2,3-b]indole
MeIQ:	2-amino-3,4-dimethyl-3H-imidazo[4,5-f]quinolone
MPP+:	1-methyl-4-phenylpyridinium
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MAO:	monoamine oxidase
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH:	nicotinamide adenine dinucleotide
NE:	norepinephrine
NT:	nitrotyrosine
OPA:	o-phthaldehyde
PBS:	phosphate buffered saline
PD:	Parkinson’s disease
PFAS:	per- and polyfluoroalkyl substances
PFBS:	perfluorobutane sulfonate
PFHxA:	perfluorohexanoate
PFHxS:	perfluorohexane sulfonate
PFOA:	perfluorooctanoate
PFOS:	perfluorooctane sulfonate
PFPeA:	perfluoro-n-pantanoic acid
PhIP:	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PINK1:	PTEN-induced putative kinase protein 1
ppb:	parts per billion
ppm:	parts per million
ROS:	reactive oxygen species

SN:	substantia nigra
SNpc:	substantia nigra pars compacta
str:	striatum
TH:	tyrosine hydroxylase
TOMM:	translocase of outer mitochondrial membrane
Trp-P-1:	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
Trp-P-2:	3-amino-1-methyl-5H-pyrido[4,3-b]indole
TUNEL:	terminal deoxynucleotidyl transferase dUTP nicked end labeling

ABSTRACT

Neurological disorders are a major public health concern due to prevalence, severity of symptoms, and impact on caregivers and economic losses. While genetic susceptibility likely has a role in most cases, exposure to toxicants can lead to neurotoxicity, including potentially developmental origins of adult disease or increased risk of disease onset. These exposures are not necessarily large, acute exposures, but could accumulate, with a chronic low-dose exposure, causing toxicity. This research focuses on the potential neurotoxicity of two classes of dietary toxins/toxicants, heterocyclic aromatic amines (HAAs) and per- and polyfluoroalkyl substances (PFAS). HAAs, such as PhIP, harmane, and harmine, are formed in charred or overcooked meat, coffee, tobacco, and other foods. PFAS are largely used in making household materials, but are found in small amounts in eggs and dairy products and largely in contaminated water. While these two classes are diverse in terms of structure, common neurotoxic targets and mechanisms often exist. Therefore, we tested the effects of these chemicals on cell viability and neurotoxicity. In the first aim, we aimed to elucidate the mechanism of toxicity of harmane and harmine, focusing on their ability to cause mitochondrial dysfunction. The second aim was to determine the effects of either harmane or PhIP on the nigrostriatal motor systems and motor function of rats and mice, respectively. The third aim determined the effects of PFAS on neurodevelopment of Northern leopard frogs, focusing on changes in neurotransmitter levels and accumulation in the brain. Harmane did not cause motor dysfunction, but potentially affected the nigro-striatal motor system in an age- or sex-dependent manner. PhIP had differential effects on dopamine levels over time and caused motor dysfunction after subchronic exposure in mice. Perfluorooctane sulfonate (PFOS) accumulated in the brains of frogs and PFAS caused changes in neurotransmitter levels that were dose- and time-dependent. Overall, this research shows that toxins/toxicants humans are exposed to over their whole lives through their diet and contaminated water can cause neurotoxicity, potentially leading to or increasing risk of disease states.

*This dissertation contains published articles as part of its final work. Below is a list of the articles and book chapters that have been used. Each chapter that contains portions of articles or is published as an article has a note at the beginning of the chapter that specifies the citation.

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CHAPTER 1. INTRODUCTION

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1.1 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting more than 10 million people worldwide. In the following sections, we will briefly discuss the etiology, symptoms, and risk factors for PD.

1.1.1 Etiology

PD is diagnosed postmortem, when protein aggregates known as Lewy bodies (LBs) are found in the brain (Braak et al., 2003). LBs are composed of various proteins but contain large amounts of alpha-synuclein (a protein that can cause PD that will be discussed in section 1.3) and are found throughout the brain and enteric nervous system of patients with PD (Rietdijk, Perez-Pardo, Garssen, van Wezel, & Kraneveld, 2017).

Aside from accumulation of LBs, PD is also characterized by loss of dopaminergic (DAergic) neurons, specifically those in the substantia nigra pars compacta (SNpc), which are an important part of the nigrostriatal system. The nigrostriatal system consists of DAergic neurons in the SNpc with processes that extend into the striatum; the striatal γ-aminobutyric acid (GABA)ergic neurons are activated by the DAergic neurons and release GABA in the globus palladus, inhibiting the GABAergic neurons in the globus palladus, leading to movement. Therefore, when the DAergic neurons in the SNpc are lost, it takes much more for the GABAergic neurons in the globus palladus to allow movement to occur, which leads to the motor deficits found in PD.

1.1.2 Symptoms

Classic signs of PD are motor symptoms, such as stooped posture, postural instability, rigidity, and resting tremors, which can also occur as postural tremors or kinetic tremors, although the latter tend to be associated more with essential tremor (Hernandez, Reed, & Singleton, 2016; Ross et al., 2004) (Lavita, Aro, Kiss, Manto, & Duez, 2016). Motor symptoms generally do not occur until loss of about 60% of the DAergic neurons in the SN, which is too late for most therapeutics to work well, so most therapies for PD are to help symptoms and don't actually stop or slow progression of the disease.

While motor symptoms are most commonly studied, PD also causes non-motor symptoms. Gastrointestinal issues, such as trouble swallowing (dysphagia), nausea, and constipation generally occur prior to motor dysfunction (Rietdijk et al., 2017). Besides intestinal problems, most PD cases also include olfactory dysfunction and problems with regulation of heart rate caused by decreased sympathetic innervation (Scorza, Fiorini, Scorza, & Finsterer, 2018). These symptoms generally occur in the early stages of PD, prior to motor dysfunction; however, PD is not generally thought to be the cause until motor symptoms occur, due to the large number of potential causes of these nonmotor symptoms.

1.1.3 Genetic susceptibility

Research has found many genetic mutations that occur in PD. These include proteins important for many different functions. The following paragraphs will briefly discuss the main genetic mutations that are known to cause PD.

Alpha-synuclein

Alpha-synuclein (α-syn) is a 14 kiloDalton (kDa) cytoplasmic protein found in the synapse of neurons that is alpha-helical in the N-terminus and is unstructured in the C-terminus (Villar-Pique, Lopes da Fonseca, & Outeiro, 2016). It is still unclear what the native function of α-syn is, but it is thought to play a role in DA storage and release (Tarasova, Lytkina, Roman, Bachurin, & Ustyugov, 2016). Due to α-syn accumulation in LBs, it is one of the main proteins studied in PD. Certain mutations in α-syn are known to cause PD, such as the most common, A53T, along with E46K, H50Q, G51D, A30P, or overexpression due to multiple copies, which are common causes

of familial PD (Table 1.1) (Hernandez et al., 2016). α-syn mutations or overexpression are commonly dominant and tend to cause early-onset PD with dementia, autonomic dysfunction, and cognitive decline, but good response to treatment (Hernandez et al., 2016).

Table 1.1Genes known to play a role in PD. Genes, along with their type of mutations that can cause PD, and the form of PD are shown.

Gene	Mutation	Dominant/Recessive	Reference
SNCA	Duplication, triplication, single nucleotide polymorphisms	Mostly dominant	(Hernandez et al., 2016; L. Xu & Pu, 2016)
LRRK2	Single nucleotide polymorphisms	Dominant	(Billingsley, Bandres-Ciga, Saez-Atienzar, & Singleton, 2018; Hernandez et al., 2016; Karuppagounder et al., 2016; Marras et al., 2016)
PINK1	Single nucleotide polymorphisms	Recessive	(Healy et al., 2004; Hernandez et al., 2016; Valente et al., 2004)
Parkin	Deletion, duplication	Recessive	(Grunewald et al., 2010; Hernandez et al., 2016; Khan et al., 2003; Periquet et al., 2003)
DJ-1	Single nucleotide polymorphism	Recessive	(Bonifati et al., 2003; Hernandez et al., 2016; Mitsumoto & Nakagawa, 2001)
GBA	Single nucleotide polymorphisms	Dominant	(Malek et al., 2018; Sidransky & Lopez, 2012)
ATP13A2	Single nucleotide polymorphism, deletion	Recessive	(Bruggemann et al., 2010; Hernandez et al., 2016; Ramirez et al., 2006)
PLA2G6	Single nucleotide polymorphisms	Recessive	(Doherty & Hardy, 2013; Hernandez et al., 2016)
FBX07	Single nucleotide polymorphisms	Recessive	(Hernandez et al., 2016; Shojaee et al., 2008)
VPS35	Single nucleotide polymorphisms	Dominant	(Haugarvoll & Wszolek, 2009; Hernandez et al., 2016; Kumar et al., 2012; Vilarino-Guell et al., 2011)

Leucine-rich repeat kinase

Leucine-rich repeat kinase 2 (LRRK2), is a 288kDa kinase with guanosine triphosphate hydrolase (GTPase) activity in one domain that plays a role in vesicle trafficking, synaptic transmission, endosome maturation, and cytoskeletal dynamics (Volta et al., 2015). Mutations in LRRK2 that lead to a gain of function are the most common genetic mutation that are known to cause PD, with G2019S being the main cause of PD in certain nationalities, such as North African and Ashkenazi Jewish, and G2385R being the most prominent (Table 1.1) (Karuppagounder et al., 2016; Volta et al., 2015). LRRK2 mutations are dominant and tend to cause late-onset PD with a slower progression mostly affecting olfactory, autonomic, and cognitive function (Karuppagounder et al., 2016; Marras et al., 2016).

PTEN-induced putative kinase protein 1 and Parkin

PTEN-induced putative kinase protein 1 (PINK1) and parkin are both implicated in genetic recessive PD (Table 1.1) (Seirafi, Kozlov, & Gehring, 2015). PINK1 is a mitochondrial-targeted kinase that is brought into the mitochondria through translocases of outer mitochondrial membranes, TOMM40 and TOMM20, and cleaved prior to export into the cytosol, which has low levels (Seirafi et al., 2015). When the mitochondrial membrane potential is lost, PINK1 is not imported into the mitochondria and phosphorylates parkin, leading to its activation (Jin et al., 2010; Lazarou, Jin, Kane, & Youle, 2012; Seirafi et al., 2015). Parkin is a cytosolic E3 ubiquitin ligase that tags proteins on the outer mitochondrial membrane for degradation after dysfunction (Seirafi et al., 2015). Due to both PINK1 and parkin's importance in mitophagy, the degradation of dysfunctional mitochondria through autophagy, mutations that affect their functions lead to accumulation of dysfunctional mitochondria (Seirafi et al., 2015). Mutations in parkin leading to dysfunction cause autosomal recessive juvenile onset PD, which is a special form of PD with disease progression usually starting before the age of 20, or early-onset recessive PD (Grunewald et al., 2010; Hernandez et al., 2016; Khan et al., 2003; Periquet et al., 2003). Symptoms of these, besides early onset, generally include foot dystonia and psychiatric problems (Grunewald et al., 2010; Hernandez et al., 2016; Ibanez et al., 2006; Khan et al., 2003; Periquet et al., 2003). After parkin mutations, PINK1 mutations are the second most common autosomal recessive cause of

early-onset PD, but it differs compared to PD caused by parkin because it does not include dementia (Healy et al., 2004; Hernandez et al., 2016; Ibanez et al., 2006; Valente et al., 2004).

Glucocerebrosidase

Glucocerebrosidase (GBA) is a 57kDa lysosomal protein that plays a role in metabolism of glucocerebroside by hydrolysis, which is important for lipid metabolism (Do, McKinney, Sharma, & Sidransky, 2019). This protein was originally linked to Gaucher's disease, which is prominent in people of Ashkenazi Jewish ancestry (Malek et al., 2018; Sidransky & Lopez, 2012). Interestingly, there was an increase in the risk of PD in this group of people (Sidransky & Lopez, 2012). Further research has found that mutations in GBA, such as N370S and L444P are risk markers for PD (Table 1.1) (Malek et al., 2018; Sidransky & Lopez, 2012). Mutations in GBA tend to cause PD symptoms such as cognitive decline, earlier onset, and faster disease progression, although many symptoms, such as effects of l-3,4-dihydroxyphenylalanine (L-DOPA) treatment are inconsistent among individuals with GBA mutations (Malek et al., 2018; Sidransky & Lopez, 2012)

Other genetic factors

There are many more genetic factors that have been linked to PD which are beyond the scope of this publication. Therefore, a brief list of the main factors has been included in Table 1.1.

1.1.4 Environmental toxins/toxicants – established risk factors

While there are genetic mutations that cause PD, these mutations are known causes of less than 10% of cases; therefore, research has started to focus on environmental factors that could increase risk of PD. These include toxins and toxicants, which are formed naturally in the environment or man-made, respectively. There are three main compounds that have been shown to cause or increase risk of PD and that are used for modeling PD in the laboratory (Tanner et al., 2011). These compounds are rotenone, paraquat, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (J. R. Cannon & Greenamyre, 2010; Jason R. Cannon et al., 2009). The following paragraphs will give a summary of the known pathways of toxicity of these three compounds as well as metals that are thought to cause PD or PD-like diseases.

MPTP

MPTP was a contaminant of synthetic heroin that caused parkinsonism in people who used the tainted heroin (Langston, 2017). MPTP, although not an environmental concern, is important because it has placed emphasis on the ability of environmental toxicants to cause PD. MPTP is metabolized to its cationic form, 1-methyl-4-phenyl-1,2,3-dihydropyridinium by monoamine oxidase B (MAO-B), sporadically oxidizes to 1-methyl-4-phenylpyridinium (MPP+) in astrocytes, and is taken up into DAergic neurons by the dopamine transporter (Heikkila, Hess, & Duvoisin, 1985; Heikkila, Manzino, Cabbat, & Duvoisin, 1984; Singer & Ramsay, 1990). Once it is inside the DAergic neuron, MPP+ accumulates in the mitochondria in an ATP-dependent manner and inhibits mitochondrial complex I, nicotinamide adenine dinucleotide (NADH):ubiquionone oxidoreductase (Ramsay, Salach, & Singer, 1986) .

Rotenone

Rotenone is a pesticide found in certain types of plants but is also frequently used as an insecticide and piscicide. Rotenone inhibits mitochondrial complex I throughout the brain (Sherer et al., 2003). This inhibition causes increased reactive oxygen species (ROS) production, oxidative stress, and causes DAergic neuron death in the midbrain, probably due to the increased sensitivity of these neurons to oxidative stress (Sherer et al., 2003). Because of its inhibition of complex I, its mechanism of action is similar to MPTP, but it does not need to be metabolized and is not specifically taken up into DAergic neurons like MPTP (Sherer et al., 2003; Tsukada et al., 2016).

Paraquat

Paraquat is another herbicide that is implicated in PD. Paraquat reacts with NADH and causes formation of superoxide radicals (Bus, Aust, & Gibson, 1976). These radicals then cause lipid peroxidation, which leads to destruction of the membrane (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973). Interestingly, although paraquat is structurally similar to MPTP and MPP+, it does not affect mitochondrial respiration at all (Dranka, Zielonka, Kanthasamy, & Kalyanaraman, 2012).

Metals

It is debated as to the role of heavy metals in PD. Both iron and copper can undergo the Fenton reaction, leading to oxidative stress; however, meta-analyses have not shown a link between increased iron or copper consumption and PD (P. Cheng et al., 2015). Manganese is a heavy metal that is necessary in small amounts, but high exposure can occur through the environment or through occupational work, such as welding and smelting (Kwakye, Paoliello, Mukhopadhyay, Bowman, & Aschner, 2015). Low-dose chronic exposure can lead to motor dysfunction such as in PD, but due to loss of neurons in the globus palladus instead of the SN, which is considered manganese-induced parkinsonism (Kwakye et al., 2015).

While there are many environmental models of PD, and some compounds known to cause it, the exposure to these compounds is not prominent enough to cause most cases of PD. Therefore, other compounds that humans are exposed to chronically throughout their lifetime are of interest to determine their roles in susceptibility to PD. We are interested in compounds found in the diet, which would cause chronic exposures throughout the lifetime, that we think are increasing risk of PD or other neurodevelopmental or neurological diseases. My research focuses on two classes of compounds: heterocyclic aromatic amines (HAAs) found in the food we eat, and per- and polyfluoroalkyl substances (PFAS) which are contaminants found in water and food.

1.2 Formation and Exposure to Heterocyclic Aromatic Amines

1.2.1 Formation of HAAs in food

HAAs are chemicals that have at least one ring structure with both carbon and nitrogen present, and are present in the diet (Felton et al., 1984). Of the original 20 HAAs identified, the first to be studied were 3-amino-1, 4-dimethyl-5H-pyrido[4, 3-b]indole (Trp-P-1), and 3-amino-1-methyl-5H-pyrido[4, 3-b]indole (Trp-P-2), 2-amino-9H-pyridole[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyridole[2,3-b]indole (MeAαC), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx) 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) (Fig. 1.1) (Robert J. Turesky, 2007). Most of these are mutagenic and may cause colorectal cancer (Lang et al., 1994; Lin, Lang, & Kadlubar, 1995). With the large number of HAAs in the diet, there are various subclasses. The most common subclasses are aminoimidazoaazarines (AIAs) and

carbolines, including α-. B-, and γ-carbolines. AIAs, such as IQ, its derivatives, and PhIP, are formed through Maillard reactions with amino acids and sugar (Skog, Johansson, & Jagerstad, 1998). α-carbolines, such as AαC, and β-carbolines, such as harmane and norharmane, are formed by pyrolysis of glutamate and tryptophan (Matsumoto, Yoshida, & Tomita, 1981).

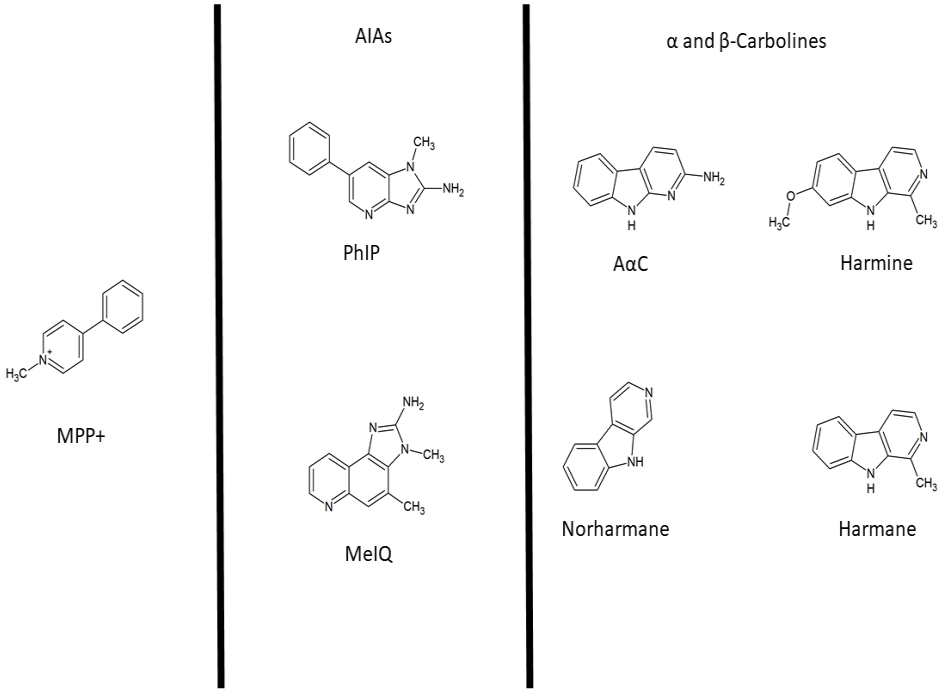

Figure 1.1 Structures of the main heterocyclic aromatic amines studied. MPP+ is a known toxicant that causes PD. AIAs are represented by PhIP and MeIQ, while carbolines studied are AαC, harmine, norharmane, and harmane.

Most HAAs are found in meat (Ni, McNaughton, LeMaster, Sinha, & Turesky, 2008). PhIP is the most abundant HAA in meat, with the highest amount detected in well-done chicken, reaching 304.71 µg/g chicken (Gibis & Weiss, 2015; Ni et al., 2008). Meat also contains lower amounts of other HAAs, such as IQ, AαC, and 8-MeIQx (Ni et al., 2008). Harmane and norharmane are also present in meat, but in much smaller quantities, averaging 26.4 ng/g and 82.3 ng/g well-done meat, the most being in venison, veal, pork, and beef (Gibis & Weiss, 2015; Herraiz, 2004). They are also present in other food, however, being especially prominent in coffee (averaging 420 ng/g and 2100 ng/g ground coffee, respectively), wine, soy sauce, and some grain products, such as cereals and bread, especially toast (Herraiz, 2004).

1.2.2 Distribution of HAAs in the human body

Harmane is found throughout the body, especially in the kidney, duodenum, the contents in the duodenum, the stomach, adrenal glands, and urine, but is also found in the brain (N. J. Anderson et al., 2006). HAAs are transported from plasma into different organs, where export is regulated through the breast cancer resistance protein (Bcrp1/Abcg2) (van Herwaarden et al., 2006; Vlaming et al., 2014). Knockdown of Bcrp1/Abcg2 decreases the levels of IQ, Trp-P-1, and PhIP in various organs, including brain, while increasing levels in the plasma (van Herwaarden et al., 2006; Vlaming et al., 2014). HAAs have been found inside the brains of mice systemically treated after 2-24 hours (Enokizono, Kusuhara, Ose, Schinkel, & Sugiyama, 2008; Teunissen et al., 2010). Research has shown that PhIP and its metabolite N-OH PhIP accumulate in the brain, indicating that the brain may be susceptible to adverse effects (Enokizono et al., 2008). Furthermore, the size (molecular weight <400-500 kDa), and the lipophilicity of PhIP and N-OH PhIP indicate that they would be likely to cross the blood-brain barrier (Butcher, Minchin, Kadlubar, & Ilett, 1996; Pardridge, 2005).

1.2.3 Metabolism of HAAs

Cytochrome P450s (CYPs) metabolize various compounds, usually by hydroxylation, oxidation, or dealkylation, (often referred to as Phase I metabolism) (Martignoni, Groothuis, & de Kanter, 2006). After Phase I metabolism, Phase II metabolism usually occurs, such as sulfonation, glucuronidation, and adduct formation (Cheung et al., 2005). CYP1As are well-conserved throughout species used for research, with at least 80% homology among humans, rats, mice, dogs, and monkeys (Martignoni et al., 2006). There are two enzymes in the CYP1A family, CYP1A1, which is mostly found in the small intestine, lung, placenta, and kidney, with only small levels present in the liver, and CYP1A2, which is mostly hepatic (Martignoni et al., 2006). While CYP1A1 and some other CYPs do metabolize HAAs, the predominant enzymes for this are CYP1A2 and CYP2D6 (C. Chen et al., 2007; Guan, Louis, & Zheng, 2001; A. M. Yu, Idle, Krausz, Kupfer, & Gonzalez, 2003; Zhao et al., 2012) (Fig. 1.1). CYP1A expression differs based on induction through different drugs (Martignoni et al., 2006). One factor that affects CYP1A1 expression is the diet, where people who ate chargrilled meat had higher expression of CYP1A1 (Martignoni et al., 2006). Because HAAs are present at higher levels in charred meat, it is possible

that HAAs are inducing expression of CYP1A1, which could lead to increased metabolism to their toxic forms.

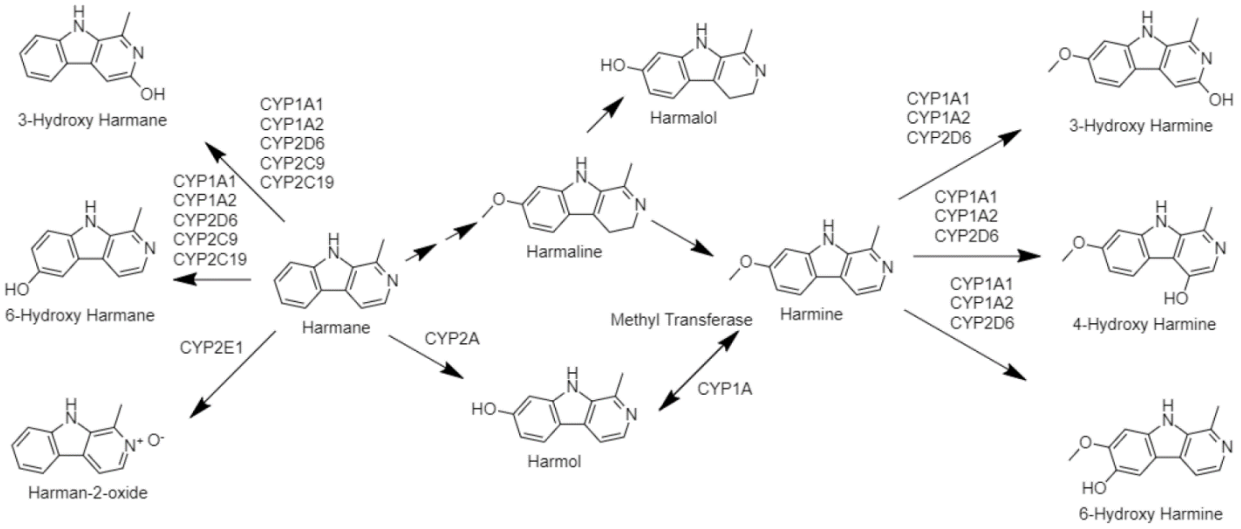

Figure 1.2 Metabolism of harmane.

Research has focused on the metabolism of HAAs and how this affects cancer risk. There are two phenotypes for CYP1A2, a fast metabolizing phenotype, and a slow (Lang et al., 1994). The fast phenotype of CYP1A2 increased the risk of cancer by itself (Lang et al., 1994). It further increases the odds ratio of cancer or polyps when the person had a preference for charred meat (Lang et al., 1994). Therefore, metabolism of HAAs could be increasing the toxicity of these compounds.

PhIP is metabolized by microsomes from human hepatocytes to two primary metabolites: N-OH PhIP and 4'-OH PhIP, predominantly through CYP1A2 (Lin et al., 1995). N-hydroxylation is ≥13-fold less efficient in rodents vs. humans (rodent CYP1A2 converts far more PhIP to 4'-OH-PhIP) (C. Chen et al., 2007; Cheung et al., 2005; Turesky, Constable, Fay, & Guengerich, 1999). This is corroborated by mice expressing the human version of CYP1A2 had increased N-OH PhIP and decreased 4'-OH PhIP compared to wild-type mice (Cheung et al., 2005). In humans, N-OH PhIP is produced mostly by CYP1A2; however, one study found that CYP1A1 is the main enzyme that produces N-OH PhIP in mice, which further shows that, although CYP1As have high homology, there are differences between human and mouse enzymes (Ma et al., 2007).

CYP1A2 also metabolizes harmane and norharmane (Herraiz, Guillen, & Aran, 2008) (Fig. 1.2). However, it is thought that CYP1A1 and CYP2D6 are the most important enzymes for harmane and norharmane metabolism (A. M. Yu et al., 2003). The proposed mechanism of metabolism is that harmane can be transformed by CYPs to 6-OH harmane, 3-OH harmane, or harman-2-oxide (Fig. 1.2) (Herraiz et al., 2008). It can also be metabolized to harmol or harmaline, which can be further transformed to harmalol or harmine (Guan et al., 2001; A. M. Yu et al., 2003). Harmine can be transformed back to harmol or be further metabolized by CYPs to 3-OH harmine, 4-OH harmine, or 6-OH harmine (A. M. Yu et al., 2003; Zhao et al., 2012).

Because HAA metabolism potentially leads to increased toxicity, decreasing CYP1A activity could potentially lead to decreased toxicity. One study found that probiotics can decrease the DNA damage formed by PhIP or IQ in human colon adenocarcinoma cells (Caco-2) (Nowak, Czyzowska, & Stanczyk, 2015). Caffeine is also known to decrease the mutagenicity of Trp-P-2 (Woziwodzka, Golunski, Wyrzykowski, Kazmierkiewicz, & Piosik, 2013). However, apiaceous vegetables increase the levels of CYP1A1 and CYP1A2 expression, which could potentially increase toxicity of HAAs (J. K. Kim, Gallaher, Chen, Yao, & Trudo, 2015). Meat eaters also are known to have more PhIP present in their hair than vegetarians, which shows that PhIP can accumulate throughout the body (Bessette et al., 2009).

1.3 HAAs: Potential Dopaminergic Neurotoxicity and Relevance to Parkinson's Disease

1.3.1 HAAs and dopaminergic neurotoxicity

Some HAAs bear significant structural similarity to MPP+, specifically the β-carbolines (Fig. 1.1). Therefore, HAAs, especially β-carbolines, have received attention as possible DAergic neurotoxins. Most epidemiological data on HAAs and neurodegeneration are focused on harmane as a risk factor for essential tremor. Essential tremor is characterized by kinetic tremors, rather than resting tremors (Lavita et al., 2016). Most commonly, the kinetic tremors are present in the arms, head, or voice (Lavita et al., 2016). An epidemiological study found that, among 56 men with essential tremor and 60 men without, the men with essential tremor ate more meat than controls (Louis et al., 2008). Increased meat consumption could imply that HAAs present in the meat could be impacting essential tremor. Furthermore, patients with essential tremor in Spain have increased levels of harmane in their blood compared to controls, with familial essential tremor

patients having more than sporadic cases (Louis, Benito-Leon, et al., 2013). To further the correlation between elevated blood harmane and essential tremor, cerebellum from patients with essential tremor had increased harmane compared to controls (Louis, Factor-Litvak, et al., 2013).

There are many similarities between PD and essential tremor, some implying that there could be a correlation between the two. Firstly, some PD patients have postural tremors, as seen in essential tremor (Dirkx, Zach, Bloem, Hallett, & Helmich, 2018). While the postural tremors in PD are usually DA-sensitive and essential tremor is not, essential tremor is still poorly understood, so it is unsure if some cases are DA-sensitive (Jankovic, 2016). Both patients with PD and essential tremor tend to have worse executive function and cognition, visual memory, language skills, and depression than healthy controls (Puertas-Martin et al., 2016; Sanchez-Ferro et al., 2017). They also both have increased sleep, mood, attention, and urinary problems (Lee, Kim, Lee, Kwon, & Koh, 2015).

Interestingly, there appears to be a familial connection between these two diseases, where they seem to run in families. Of 53 essential tremor cases, almost 2/3 had PD after ten years of essential tremor report, with the predominant side for essential tremor also being the side with worse PD symptoms (Minen & Louis, 2008). People who had PD also were more likely to have been diagnosed with essential tremor before PD (Tan, Lee, S, & Lum, 2008). The percent of people with essential tremor who also had PD was 6.1 % (Koller, Busenbark, & Miner, 1994). It is possible that it could be misdiagnosis of early PD symptoms, but the evidence to increased incidence of the two is increasing. A cohort of patients with PD were found to have more likelihood of having tremor as the main PD symptom if one of their family members had essential tremor (Hedera et al., 2009). Also, people who had a relative with earlier onset PD (<66 years old) were more likely to have essential tremor, with earlier onset being related to more risk of disease (Rocca et al., 2007). Because of the accumulating data, it appears that there is a connection between essential tremor and PD, and compounds that could cause essential tremor could also play a role in PD.

Studies have shown that the levels of harmane in the blood of PD patients is double that of controls (Kuhn, Muller, Grosse, Dierks, & Rommelspacher, 1995; Pacelli et al., 2015). Interestingly, norharmane, as well as harmane, is also increased significantly in the plasma of people with PD (Kuhn et al., 1995). Although blood levels are increased, that does not necessarily mean that levels of harmane or norharmane are increased in the brain. However, norharmane and

harmane are also increased in cerebral spinal fluid (CSF) of PD patients (Kuhn, Muller, Grosse, & Rommelspacher, 1996). This elevation in CSF is a possible indicator that these endogenous HAAs are, in fact, increased in the brains of patients with PD.

Another possibility for differential response to HAA exposure is through genetic differences that increase risk, such as the activity of enzymes affected by HAAs. Both harmane and norharmane are MAO inhibitors (May, Pawlik, & Rommelspacher, 1991). There are two forms of MAO, MAO-A and MAO-B, both of which are present in the brain and are inhibited by harmane and norharmane, respectively (Johnston, 1968; Westlund, Denney, Rose, & Abell, 1988). MAO-B is increased in the frontal cortex of PD patients compared to controls (Tong et al., 2017). Besides MAO-B, MAO-A is increased in the putamen of patients with PD (Tong et al., 2017). Furthermore, a fragmented form of MAO-A (f-MAO-A25) is increased in the SN of the same patients, which might be an indicator of MAO metabolism and that it is increased in the SN of patients with PD (Tong et al., 2017).

While there is epidemiological data implying that HAAs, specifically harmane and norharmane may impact PD, there is a surprising lack of *in vivo* studies to determine how long-term exposure to these chemicals may adversely affect the brain, and specifically the DAergic neurons in the SN. These studies would help to determine how this increase in harmane and norharmane in PD patients is affecting the brain.

1.4 HAA Effects on Dopamine

1.4.1 Dopamine formation and metabolism

The primary pathway for DA formation is through L-DOPA. Dopaminergic neurons in the brain contain tyrosine hydroxylase (TH), which converts tyrosine to L-DOPA (Nagatsu, Levitt, & Udenfriend, 1964). This conversion is the rate-limiting step in DA synthesis (Pong, Doctrow, & Baudry, 2000). From there, L-DOPA is converted to DA through L-amino acid decarboxylase (Blaschko, 1942). The other pathway to produce DA is considered to be of lesser importance with respect to neurotransmission (Hiroi, Imaoka, & Funae, 1998). This pathway is through metabolism of tyramine by CYP2D (Bromek, Haduch, Golembiowska, & Daniel, 2011; Hiroi et al., 1998).

TH activity is affected by many different pathways. One form of modulation is through regulation of mRNA production through cyclic adenosine monophosphate (cAMP) response

elements as well as other similar pathways (Nakashima, Ota, & Sabban, 2003). Direct activity of TH is modulated by phosphorylation status through a feedback loop where DA binds to TH and changes its phosphorylation status and coordination with iron (Daubner, Le, & Wang, 2011; Haavik, Martinez, & Flatmark, 1990).

After release, DA catabolism primarily occurs through two pathways. DA is metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by MAO, which is then converted to homovanillic acid (HVA) through catechol-O-methyltransferase (COMT) (Mannisto & Kaakkola, 1999; Meiser, Weindl, & Hiller, 2013). The other pathway uses COMT to produce 3-methoxytyrosine from DA, which is then metabolized to 3-methoxy-4-hydroxyphenyl acetaldehyde by MAO and produces HVA through aldehyde dehydrogenase (ALDH) (Meiser et al., 2013). As mentioned in section 2.2, both harmane and norharmane inhibit MAO through competitive binding in rat and marmoset brain (May et al., 1991).

1.4.2 HAA-induced alterations in dopamine formation

In the rat adrenal medulla pheochromocytoma cell line (PC12), harmane causes decreased mRNA expression of TH after both 6 and 12 hours (Yang, Lee, Jin, Lim, & Lee, 2008). Norharmane also decreases transcription of TH (Yang et al., 2008). This is probably through the cAMP pathway from section 1.4.1 because cAMP was decreased in the treated cells up to one hour after exposure to either harmane or norharmane (Yang et al., 2008). Furthermore, both compounds decrease the effects of L-DOPA, which usually increases DA content and rescues cells (Yang et al., 2008).

1.4.3 HAA-induced alterations in dopamine metabolism

Many derivatives of harmane and norharmane that are found in plant material have been studied for effects on DA metabolism. Tetrahydronorharmane causes decreased DA efflux in striatal slices of rats (Rommelspacher & Subramanian, 1979). Injection of 2-methyl-harmol, 2-methyl-harmaline, 2,9-dimethyl-harmane, or 2-methyl-norharmane into the SN of rats causes a lesion similar to that formed by MPP+ (Neafsey et al., 1995). In the same study, both DA and DOPAC were decreased when 2-methyl-norharmane, norharmane, 2-methyl-harmaline, 2-methyl-harmane, 2,9-dimethyl-2-harmane, and 2,9-dimethyl-2-norharmane were injected (Neafsey et al.,

1995). Furthermore, both harmane and norharmane treatment result in decreased DA after 6 to 48 hours in PC12 cells, showing that these parent compounds and derivatives are all affecting DA formation and metabolism (Yang et al., 2008). Furthermore, rats had decreased DA, DOPAC, and HVA and formed a lesion when 2-methyl-norharmane was injected into the SN (Neafsey, Drucker, Raikoff, & Collins, 1989).

Besides simply changes in DA levels, changes in DA metabolism is another factor of interest. Changes in DA turnover, calculated by dividing the sum of the amounts of DOPAC and HVA by the amount of DA, can imply various alterations in DA neurotransmission. DA is released into the synapse, and after reuptake, is generally collected in vesicles in preparation for future release and also to prevent free, cytosolic DA reacting with other chemicals in the cell (Emdadul Haque et al., 2003). Significant amounts of free DA in the cell can lead to accumulation of reactive species, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL), a highly reactive metabolite of DA (Goldstein et al., 2013). Acute exposure of rats to PhIP caused changes in DA turnover (Agim & Cannon, 2018). After 8 hours, DOPAC, HVA, and DA turn-over were significantly decreased, and there was the presence of intracellular oxidative damage (Agim & Cannon, 2018). This implies that either free cytosolic DA could be building up in the cells, or reactive DA metabolites, such as DOPAL, could be accumulating, leading to oxidative stress and potentially causing apoptosis if accumulated long enough.

1.5 HAA Behavioral Effects

1.5.1 Motor dysfunction in HAA models

One of the tests for lesions in the SN after treatment to one side of the brain is to inject apomorphine, a mixed DA agonist, and count the number of contralateral and ipsilateral turns the rat makes in a given time (Konitsiotis, Kafetzopoulos, Anastasopoulos, & Blanchet, 1998). If a lesion is present in the SN, the animal will perform more contralateral rotations than its unlesioned counterpart in response to apomorphine (Konitsiotis et al., 1998). 6-hydroxydopamine (6-OHDA) is the classic toxicant used to elicit unilateral lesions to the nigrostriatal DA system (Haghdoost-Yazdi, Hosseini, Faraji, Nahid, & Jahanihashemi, 2010).

When similar experiments were performed but with only norharmane injected into the SN, the ratio of ipsilateral to contralateral turns was significantly decreased compared to controls two

weeks after injection, indicating lesion formation (Esmaeili, Movahedi, Faraji, & Haghdoost-Yazdi, 2012). The same rats showed decreased latency to fall off the rotarod, presenting motor deficits similar to those in PD (Esmaeili et al., 2012). These results indicate that norharmane injected into the SN by itself can produce motor dysfunction. However, no histology was performed to show that lesions formed because of treatment or to determine whether there is cell-type specific toxicity, such as specificity to DAergic neurons.

Similarly, two weeks after a five-day exposure to norharmane, mice exhibited decreased spontaneous movement, locomotor activity, and rearing, indicating signs of motor dysfunction (Ostergren, Fredriksson, & Brittebo, 2006). Norharmane did not affect memory tests, however, only motor function tests, indicating specifically nigrostriatal dysfunction (Ostergren et al., 2006).

1.6 Mechanisms of Toxicity

1.6.1 Specific toxicity of HAAs in primary cultures

Cell culture is often used to determine the mechanism of action for toxins and toxicants. However, cell lines tend to be much more resistant to various toxicants, where there is a lack of the diverse population of cells relative to that found in the intact mammalian brain. Therefore, primary culture is sometimes used, where sections of brains are dissected and plated for an *in vitro* exposure, but there are the various cell types present that are found in that section of the brain (Griggs et al., 2014; Strathearn et al., 2014).

Many HAAs have been tested in rat primary midbrain cultures to determine toxicity. It was originally found that PhIP and N-OH PhIP both selectively decrease the percent of DAergic neurons in these cultures (non-DA neurons are far less sensitive) (Cruz-Hernandez et al., 2018; Griggs et al., 2014). Further research showed that IQ, MeIQ, MeIQx, and 4,8-DiMeIQx were also selectively toxic to DAergic neurons, decreasing their percentage significantly, without detectable effects on non-DAergic neurons (Cruz-Hernandez et al., 2018). MeIQ and its derivatives MeIQx and 4,8-DiMeIQx also significantly decrease the neurite length, however IQ actually increases neurite length at lower doses (Cruz-Hernandez et al., 2018). Therefore, all tested AIAs are selectively toxic to DAergic neurons in a primary midbrain culture (Cruz-Hernandez et al., 2018).

The carbolines were also studied for their specific toxicity on DAergic neurons. Both β-carbolines tested, harmane and norharmane, and the α-carboline, AαC, were also selectively toxic

to DAergic neurons, decreasing the percentage of these neurons and decreasing neurite length (Cruz-Hernandez et al., 2018). While many HAAs appear to be selectively toxic to DA neurons there are differences in the doses that are required to elicit cell loss (Cruz-Hernandez et al., 2018). Here, *in vivo* studies will be needed with single and mixture exposures to assess potential risk.

1.6.2 Potential role of neuromelanin in toxicity

In addition to inherent metabolic differences, there may be other significant weaknesses with respect to the use of rodent models to assess HAA-induced DAergic neurotoxicity. Another potential cause for concern with HAAs is their interaction with neuromelanin. Neuromelanin is a pigmented structure that is thought to be formed partially through DA oxidation and aggregation in the brain (Sulzer & Zecca, 2000). It is still unclear whether this occurs through auto-oxidation of free DA that forms quinones, or through enzymatic oxidation of DA, such as through tyrosinase, which produces DA-quinones that are found in neuromelanin (Foley & Baxter, 1958; Schroeder, Double, & Gerber, 2015; Tief, Schmidt, & Beermann, 1998; Y. M. Xu et al., 1997; Zecca, Zucca, Wilms, & Sulzer, 2003). When DA and its quinone metabolites accumulate in a cell and are not shuttled into vesicles, they can aggregate with other compounds and form neuromelanin (Sulzer & Zecca, 2000). Neuromelanin is found in primates, cats, dogs, and certain other animal species (DeMattei, Levi, & Fariello, 1986; Tribl et al., 2005). One question of importance is whether rats and mice also have neuromelanin, since they are common models of neurodegeneration. DeMattei and colleagues studied rats of various ages and found that old rats (23 months) had neuromelanin clustered in their brain, but at far lower levels than other species (DeMattei et al., 1986). This presents a pitfall for the use of rodents as models for neurodegeneration, because it is well documented that it can decrease stress on cells through aggregating reactive species but can also accumulate toxic substances and cause an immune response when neuromelanin is outside of the cell (Zecca, Zucca, Albertini, Rizzio, & Fariello, 2006).

Interestingly, certain frogs and tadpoles contain neuromelanin throughout their brains, such as in the mesencephalon, a section of the brain bearing similarity to the SN in higher order species (Kemali & Gioffre, 1985). Therefore, although frog brains are structurally very different than human brains, there is potential for their use in neurodegeneration research (Wada, Urano, & Gorbman, 1980).

Because of its potential to accumulate compounds and increase oxidative damage, neuromelanin is an important factor to consider in its reaction with PD toxicants. D'Amanto and colleagues found that MPTP and MPP+ bind to neuromelanin and retinal melanin in a cell-free environment (D'Amato, Benham, & Snyder, 1987). Supporting this study, frogs treated with MPTP had accumulation of the compound in melanin-containing cells throughout the body, including eyes, dark spots, and parts of the brain (neuromelanin) (Sokolowski, Larsson, & Lindquist, 1990). Northern leopard frogs (*Rana pipiens*) had decreased motor function and decreased quality of movement, shown by increased time for the frogs to flip onto their front when placed on the back and more slow and rigid flips and hops, after treatment with MPTP, MPP+, or paraquat (Barbeau, Dallaire, Buu, Poirier, & Rucinska, 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985; Lindquist, Larsson, & Lyden-Sokolowski, 1988). These frogs also had increased pigmentation from melanin in their skin over time of treatment (Barbeau, Dallaire, Buu, Poirier, et al., 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985). Not only was there increased pigmentation, but autoradiography showed increased accumulation of MPTP, paraquat, and diquat in the melanin-containing cells within the frogs (Lindquist et al., 1988; Ostergren, Annas, Skog, Lindquist, & Brittebo, 2004; Sokolowski et al., 1990). The decreased motor function and increased pigmentation of the treated frogs significantly improved when frogs were also treated with the MAO-B inhibitor paragyline, which decreases MPP+ formation (Barbeau, Dallaire, Buu, Poirier, et al., 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985).

Interestingly, harmane also accumulates more in black-haired C57BL/6 mice compared to albino NMRI mice (Ostergren et al., 2004). The same study showed that melanin in frogs also accumulates harmane more than tissue not containing melanin (Ostergren et al., 2004). Similarly, dogs had more melanin in their dark fur, fur that contains more melanin, than in white fur (Gu, Neuman, Modiano, & Turesky, 2012). In cell culture, DA melanin-containing PC12 cells had fewer necrotic cells after norharmane treatment and had different levels of caspase-3 activation compared to cells without DA melanin (Ostergren, Lindquist, & Brittebo, 2007). This implies that melanin could decrease the toxicity of HAAs, such as norharmane, and possibly alter the mechanism of toxicity. However, such findings are in contrast to conclusions from a number of animal model papers and would require further validation. Interestingly, mice that were injected with norharmane, which showed motor deficits as discussed in section 4.2, had increased area of glial cells in the SN (Ostergren et al., 2006). Therefore, norharmane could be causing increased

inflammation in the brain, although it is unsure if activation of glia is prior to cell death or is occurring due to the cell death from norharmane.

In general, the studies cited throughout this section suggest that in species expressing appreciable neuromelanin, toxin and toxicant accumulation is increased in neuromelanin expressing brain regions compared to species expressing far lower amounts of neuromelanin. Thus, it is apparent that neuromelanin present in the brain, specifically the SN, is an important factor for studying toxicity of compounds. Many compounds that are known neurotoxicants accumulate in tissue containing melanin, which is clear by whole body presence, but also increased accumulation in the SN. Data cited in this section indicate this to be true for HAAs. Therefore, it is important to study exposure of these compounds in animal models that contain neuromelanin or utilize other strategies to interrogate the potential role neuromelanin may have in mediated neurotoxicity.

1.6.3 Oxidative stress formation from HAAs

Oxidative stress and damage have been extensively researched in PD. Rats treated with PhIP (100 mg/kg and 200 mg/kg; doses chosen from extensive published cancer bioassays) exhibited increased nitrotyrosine (NT) in DA neurons of the SN after eight and twenty-four hours (Agim & Cannon, 2018). Here, the magnitude of increased NT was far less in non-DAergic neurons and DAergic neurons of the adjacent ventral tegmental area (Agim & Cannon, 2018), suggesting that the same neurons affected in PD are especially sensitive to systemic PhIP treatment. Ongoing research aims to elucidate mechanisms of specificity. It is worth noting that differential expression of the dopamine transporter is unlikely to underlie such differential sensitivity because PhIP is unlikely to be a substrate for the dopamine transporter (Griggs et al., 2014).

In rat primary midbrain cultures, PhIP, MeIQ and harmane increased oxidative stress, shown by increased NT, in both DAergic neurons and non-DAergic neurons (Cruz-Hernandez et al., 2018; Griggs et al., 2014). In the same model system, PhIP increased lipid peroxidation, shown by increased 4-hydroxynonenal (HNE) (Griggs et al., 2014). When cells were treated with extract from blueberries, which contained antioxidants, the percent of DAergic neurons were rescued after treatment with PhIP (Griggs et al., 2014). One of the predominant ways that oxidative stress is produced in the cell is by dysregulation of mitochondrial respiration (Horowitz et al., 2011). Given oxidative stress is a virtually ubiquitous response in PD studies, elucidating primary mechanisms that may be of specific importance to HAAs is of significant importance.

1.6.4 Cell death from HAAs

The two main forms of cell death are apoptosis, or programmed cell death, and necrosis, which is unregulated cell death (Elmore, 2007). Apoptosis is a highly regulated form of cell death which occurs after activation of caspases, with caspase 3 being a main marker for this type of cell death (Reed, 2000). Whereas, necrosis usually includes calcium flux and damage to DNA (Elmore, 2007).

When PC12 cells were treated with either harmane or norharmane, there was significantly increased apoptosis, indicated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Yang et al., 2008). Both of these HAAs also increased cell death due to L-DOPA (Yang et al., 2008). More research in PC12 cells showed that norharmane increased apoptosis through cleavage of caspase-3 and mitochondrial dysfunction, shown by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Ostergren et al., 2006). The same study also had increased trypan blue staining, indicating DNA damage, and Fluoro-jade staining, which both could be due to either apoptosis or necrosis (Ostergren et al., 2006). Not only does norharmane cause DNA damage, but harmane, Trp-P-1, and Trp-P-2 also damage DNA (Uezono et al., 2001).

1.7 Per- and Polyfluoroalkyl Substances

1.7.1 Exposure

Per- and polyfluoroalkyl substances (PFAS) are chemicals that have been used in industry since around the 1950s (Brendel, Fetter, Staude, Vierke, & Biegel-Engler, 2018; Moody, Hebert, Strauss, & Field, 2003). These compounds were used during manufacturing for common household materials, such as nonstick cookware, food packaging, antistatic agents, anti-stain agents, water repellants, firefighting foams, and hygiene products due to their surfactant properties (Moody et al., 2003; T. Stahl et al., 2012). The longer chain PFAS, with 7 or more carbons (such as perfluorooctanoic acid, PFOA, and perfluorodecanoic acid, PFDA) were first phased out of the United States in 2001 due to findings of toxicity; however, they are still prevalent in the environment due to their strong carbon–fluorine bonds, which makes them extremely stable and persistent, and because they are still used in other parts of the world and can be shipped to the United States (Figure 1.3) (Brendel et al., 2018; Moody et al., 2003). Furthermore, alternatives to

the longer chain PFAS are shorter chain PFAS (less than 7 carbons) such as perfluorobutanoic acid (PFBA) and perfluorobutane sulfonate (PFBS) because they are generally believed to bioaccumulate less (Figure 1) (Brendel et al., 2018; Gebbink & van Leeuwen, 2020; Shaw et al., 2019; Xiao, 2017). It is worth noting that there are significant caveats to this assumption. In general, far more data are needed to definitively show that shorter chain PFAS accumulate less than longer chain PFAS. This is especially highlighted by evidence of shorter chain (6 carbon) PFAS with long half lives, such as perfluorohexane sulfonate, PFHxS (>5 years) (Olsen et al., 2007). Furthermore, these compounds still are not metabolized, are prominent in water, including that for drinking, and have largely unknown toxicological effects (Brendel et al., 2018; Gebbink & van Leeuwen, 2020). Furthermore, a novel PFAS, hexafluoropropylene oxide dimer acid (GenX), has been found in both the Cape Fear River and the blood of people who live in the Lower Cape Fear River Basin along with other locations, such as in the Netherlands (Figure 1.3) (Gebbink, van Asseldonk, & van Leeuwen, 2017; Gebbink & van Leeuwen, 2020; McCord & Strynar, 2019; Sun et al., 2016). Thus far, research on the neurotoxicity of GenX is limited to the finding that rat brain capillaries had decreased transport activity of P-glycoprotein and breast cancer resistance protein, which are two enzymes that are important for the proper function of the blood–brain barrier, indicating the potential effects of transport of chemicals into and out of the brain (R. E. Cannon et al., 2020).

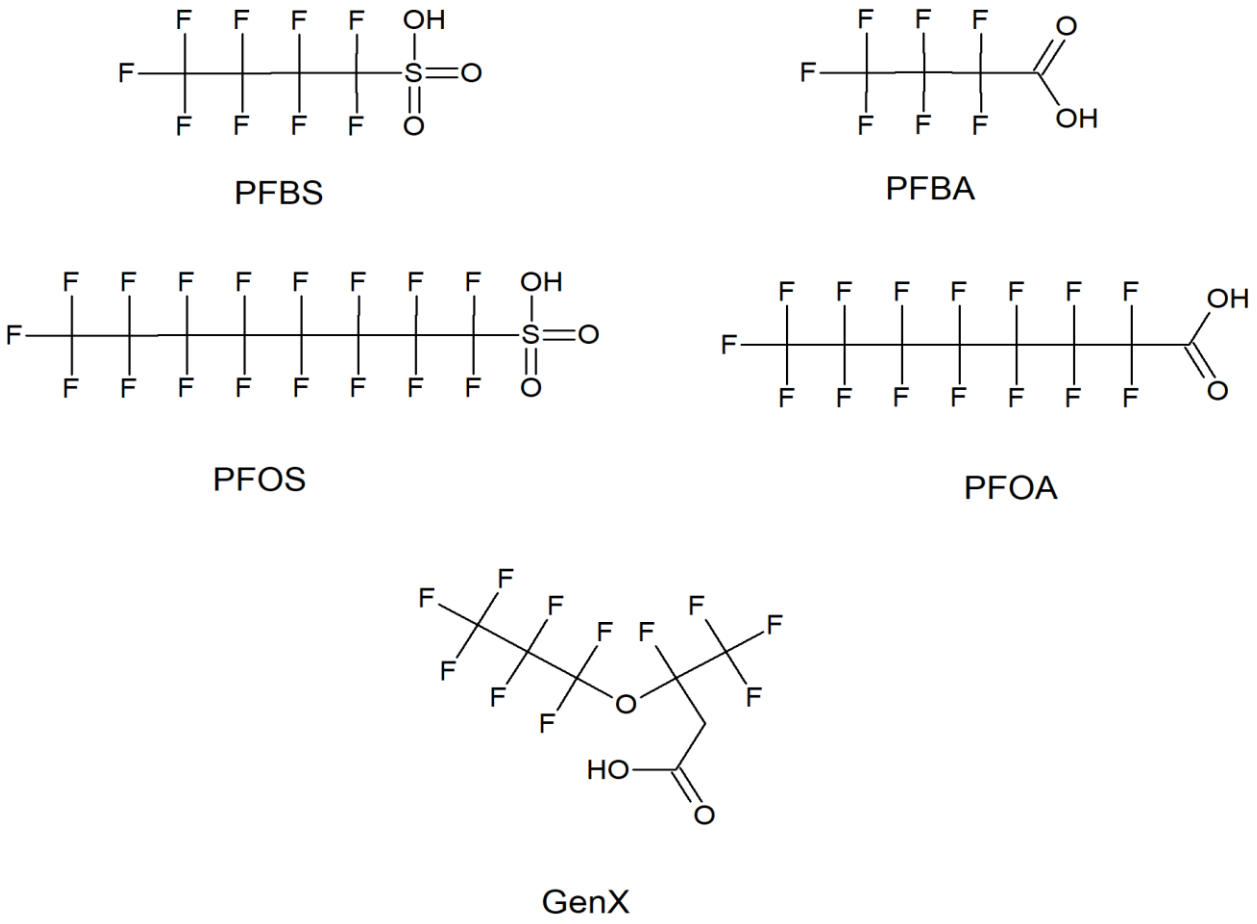

Figure 1.3 Structures of the representative per- and polyfluoroalkyl substances (PFAS) discussed in this article. Short-chain PFAS are represented by perfluorobutane sulfonate (PFBS) and perfluorobutanoic acid (PFBA). Long-chain PFAS are represented by perflourooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). Next generation PFAS are represented by GenX. For a more inclusive view of PFAS structures see Shaw et al., 2019 and Xiao, 2017 [5,6].

PFAS are now found throughout the world. There are several notable examples of major contaminated sites in the United States such as Wurtsmith Air Force Base in Michigan with water concentrations measuring around 110 µg/L perflourooctane sulfonate (PFOS), 104 µg/L PFHxS, 105 µg/L PFOA, and 5 µg/L perfluorohexanoate (PFHxA), similar to other locations throughout the world (Lien, Fujii, Tanaka, Nozoe, & Tanaka, 2008; Mak et al., 2009; Moody et al., 2003; Senthil Kumar et al., 2009). Furthermore, there are measurable levels of PFAS in humans, with the overall mean concentrations in serum from 2016 to 2018 being 2.94 ng/mL PFOS, 2.04 ng/mL PFOA, 1.10 ng/mL PFHxS, and 0.79 ng/mL perflourononanoate (PFNA) (Calafat et al., 2007; C. H. Yu, Riker, Lu, & Fan, 2020). Besides just being measured in serum, the half-life of these

compounds has been calculated from exposed fluorochemical workers to be around 5.4 years for PFOS, 8.5 years for perfluoroheptane sulfonate (PFHpS), and 3.8 years for PFOA (Olsen et al., 2007). Unfortunately, there are many more PFAS than those measured in humans thus far, so exposure is not completely understood. Exposure to these compounds is not just problematic for adults, as exposure occurs throughout life. PFAS have been quantified in breastmilk, amniotic fluid, and fetal tissue, including the liver, lung, heart, and brain, umbilical cord blood, and placenta, indicating that infants are being exposed from the very beginning of life (Aimuzi et al., 2019; Karrman et al., 2007; Mamsen et al., 2019; Olsen, Butenhoff, & Zobel, 2009; So et al., 2006; Stein, Wolff, Calafat, Kato, & Engel, 2012). Studies in model systems such as Cymologous monkeys, rats, and mice have indicated various forms of toxicity; however, the shorter half-lives of PFAS in rodents requires a large exposure to obtain internal doses similar to those in humans. It is also beneficial to determine the effects of chemicals on many different organisms because similarities can indicate toxicity that is more likely to occur in humans. One type of study that can be useful for determining toxicity is by using sentinel species, which are organisms naturally exposed to the chemicals of interest and could indicate toxicity earlier than it is seen in humans. Furthermore, certain types of sentinel species are physiologically very similar to humans, such as polar bears (*Ursus maritimus)*. The use of other model systems that are traditionally used in the laboratory, such as zebrafish (*Danio rerio*) and nematodes, could help determine effects that could be further studied in other model species more similar to humans. This article focuses on a review of the current literature that examines the neurotoxicity of PFAS on sentinel and non-traditional model species. Major areas of research on PFAS toxicity include aspects such as hepatotoxicity, cholesterol and lipid distribution, immunotoxicity, and effects on the thyroid (Thorsten Stahl, Mattern, & Brunn, 2011). One aspect of PFAS toxicity that is important to study is neurotoxicity. Research has shown that mice exposed to PFOS or PFOA at postnatal day 10 had increased activity at 2 months of age and decreased movement at 4 months of age, indicating potential effects on motor function (Johansson, Fredriksson, & Eriksson, 2008). Interestingly, mice exposed to PFOS during development had significantly decreased movement after treatment with methamphetamine, potentially indicating effects on reward-based movement (Goulding, White, McBride, Fenton, & Harry, 2017). Furthermore, rats exposed to 20 mg/kg PFOS daily for 28 days starting at 2 months had decreased myelination in the brain, also indicating neurotoxicity (Cui, Zhou, Liao, Fu, & Jiang, 2009). Unfortunately, there is still much to understand about the potential neurotoxicity of PFAS.

Exposure to these compounds is not just problematic for adults, though, because exposure occurs throughout the lifetime. Multiple studies have shown that PFAS are present in breastmilk, with PFHxS and PFOS being the most abundant, and placenta, indicating that infants are being exposed from the very beginning of life (Karrman et al., 2007; Olsen et al., 2009; So et al., 2006). While studies in model systems such as Cymologous monkeys, rats, and mice have indicated various forms of toxicity, most of these exposures are not immediately relevant to the levels humans are exposed to and don't focus on exposures to a mixture of various PFAS. Therefore, studying the effects of PFAS on sentinel and nontraditional model species could indicate potential effects to humans more relevant than those of laboratory exposures.

One of the ways to overcome potential pitfalls presented above is to use sentinel species or uncommon model systems exposed to the environmentally relevant levels of PFAS or mixtures. The following sections focus on a review of the current literature that examines the neurotoxicity of PFAS.

1.7.2 Developmental defects

Not only are PFOS and PFOA ubiquitously found in the population, they are also present in breast milk and the placenta, meaning that developmental exposure is a major issue (Jian et al., 2018; Olsen et al., 2009). Human milk has been shown to contain around 6-288 pg/mL PFOS and 18-241 pg/mL PFOA (Jian et al., 2018). A study of 256 births at Johns Hopkins found a significant effect of PFAS levels in umbilical cord serum on the birth weight-height ratio, with increased concentration leading to decreased ratio; whereas a study on 1,399 Danish births showed no correlation between maternal first trimester plasma PFAS concentrations and weight-height ratio (Olsen et al., 2009). However, in their later study, Fei et al found a significant increase in the weight-height ratio in obese mothers (Olsen et al., 2009). This discrepancy could be due to many factors, including different cohorts with variance in race, ethnicity, and life-styles of participants or the different analytic techniques to measure PFAS.

1.7.3 Neurological defects

Studies in mice exposed to PFOS and PFOA on post-natal day 10 showed that exposure increased spontaneous activity and increased proteins important for making synapses (Johansson,

Eriksson, & Viberg, 2009; Johansson et al., 2008). Also, when dames were treated with PFOA during pregnancy, male offspring had significantly shorter latency to fall on the wire-hang test when they were 5-8 weeks old (Onishchenko et al., 2011). This implies that PFAS exposure is affecting brain development, and the necessity of further research in this area is further shown because PFOS and PFOA both cross the blood-brain barrier, with PFOS accumulating 10-100x more than other PFAS, at about one fourth of the levels in the blood (Maestri et al., 2006). Interestingly, there appears to be similar accumulation of PFAS between human brains and those of red-throated divers (Mariussen, 2012).

Research to determine the mechanism of neurotoxicity is much sparser. One study using PC12 cells showed that PFOS affected differentiation of PC12 cells, which can differentiate into cholinergic or DAergic neurons (Slotkin, MacKillop, Melnick, Thayer, & Seidler, 2008). Lower concentrations of PFOS caused an increase of differentiation into cholinergic neurons and fewer DAergic neurons; however, higher concentrations shifted the preference to differentiate to DAergic neurons (Slotkin et al., 2008).

1.7.4 Non-traditional models for neurotoxicity

There are specific characteristics that make certain animals good sentinel species. Species that are more susceptible to toxicity after exposure could indicate toxicity to other species, such as humans. For a complete summary of the differences among monitors, indicators, and sentinels, refer to O'Brien, 1993 (Obrien, Kaneene, & Poppenga, 1993). Some of the characteristics to consider include body size, sensitivity, physiology, longevity, and latent period (Obrien et al., 1993). Body size is important because it could affect the accumulation of chemicals along with exposure, with larger surface areas potentially increasing exposure (Obrien et al., 1993). Sensitivity is another important aspect because a species could be exposed to the same amount of a chemical but not be as sensitive due to differences in the location of accumulation, metabolism, or pathways affected (Obrien et al., 1993; Reif, 2011). When assessing differences in sensitivity across species, it is important to consider where contaminants accumulate. For example, whales have an important amount of blubber, so they serve as good sentinels for lipophilic contaminants (Reif, 2011). To relate the effects of compounds, the physiology being studied needs to be similar between humans and the sentinel species being studied, or at least differences need to be accounted for, because differences in physiology could cause differential effects and limit extrapolation to

human relevance (Obrien et al., 1993). The longevity of a sentinel species is important because often exposure needs to occur over a long time to cause measurable effects; however, sometimes it is useful to use a species that has a shorter lifespan because it is easier to see effects that occur during aging, such as degeneration (Obrien et al., 1993). Species that have longer lives are better for studying effects such as cancer and reproductive effects (Reif, 2011). The latent period is important in a similar way to longevity because acute exposure to a chemical can cause significantly different effects than chronic exposure, such as that throughout a lifetime (Obrien et al., 1993).

There are many other qualities that should be considered when selecting sentinel species. One such quality is the location on the food chain of the species (Obrien et al., 1993; Reif, 2011). Humans are at the top of the food chain, so it is important to remember that bioaccumulation can occur, leading to increased exposure. Another quality is migratory patterns, which can affect the exposure of sentinel species, such as if birds migrate somewhere, their exposure is significantly less during half the year (Obrien et al., 1993). As touched on above, the route of exposure and distribution are important due to changes in metabolism and accumulation after exposure in different ways (Obrien et al., 1993). The last big factor to consider is the utility of captive sentinel or non-traditional model species (Obrien et al., 1993). While these qualities are important for sentinel species, they also apply to other model systems, including non-traditional model organisms. These organisms can also indicate potential aspects of toxicity due to accumulation or higher sensitivity, similar to sentinel species, and should be utilized in determining the effects of environmental exposures. This could allow for more in-depth studies than collection from the wild, including more mechanistic studies to understand what is occurring on the molecular level and possible ways to mitigate effects.

1.7.5 Neurotoxicity of PFAS in non-traditional model organisms - invertebrates

Caenorhabditis elegans

Caenorhabditis elegans are nematodes that are very useful model organisms due to their transparent bodies, fully sequenced genome, and the knowledge of the cell fate of each cell at germination. *C. elegans* are commonly found in the wild in places with rotting vegetation or soil rich in microbiota (Schulenburg & Felix, 2017). In the laboratory, *C. elegans* are studied using

both liquid and solid broth with *Escherichia coli* present as a food source. The ability to easily maintain these worms in laboratory conditions also means that they can be exposed to specific chemical insults and studied for the effects over their lifespan. They also have a relatively short lifespan, and there are many strains that contain mutations or fluorescent expression that are useful in studying development and toxicity (Sammi et al., 2019). Many of these traits have made them useful non-traditional model organisms in studying the toxicity of PFAS and have guided other studies to further determine the mechanisms of toxicity. PFAS studied in *C. elegans* neurotoxicity only focuses on PFOS thus far.

Few studies have measured the amount of PFAS absorbed and retained after exposure in *C. elegans*; however, it has been shown that *C. elegans* accumulate PFOS to 13.06 µg/mg after 1 mg/L PFOS and accumulation increases dose-dependently, while PFBS does not accumulate (Table 1.2) (F. J. Chen et al., 2018; Sammi et al., 2019). The toxicity of PFAS has been of much more interest, with the toxicity of PFOS being the most prominent due to its accumulation and high environmental levels. It has been shown many times that PFOS is toxic in a dose-dependent manner (F. J. Chen et al., 2018; T. Xu et al., 2016). The mechanisms of excretion of PFAS in *C. elegans* is currently unknown, which is a major weakeness when comparing toxicity to other systems. The lethal concentration for 50% death (LC_{50}) has been calculated to be 4.522 mg/L (9.04 µM) after 24 h, and 1.4 µM or 2.03 mM after 48 h of exposure (F. J. Chen et al., 2018; N. Chen, Li, Li, Yang, & He, 2014; H. M. Kim et al., 2020). Discrepancies between these studies could be caused by differences in age, where most papers did not note the developmental stage at exposure or differences in the amount of branching. PFOS can be produced in two different ways, one of which produces 65–79% linear PFOS, while the other produces linear PFOS almost exclusively (Greaves & Letcher, 2013). Research has indicated that the linear versus branched PFOS accumulate in tissue differently and potentially cause different toxicities (Greaves & Letcher, 2013). The LC_{50} of PFOA was found to be much higher, 22.655 mg/L (54.7 µM), which is consistent with decreased accumulation as well as decreased toxicity (H. M. Kim et al., 2020). PFBS was also found to be toxic in a dose-dependent manner, with a calculated LC_{50} of 794 µM, which was much higher than that of PFOA, possibly due to less accumulation or faster excretion due to the shorter chain length (F. J. Chen et al., 2018). This difference in accumulation is due to the shorter fluorinated carbon chain length and is also dependent on the functional group due to changes in the surfactant properties (Conder, Hoke, De Wolf, Russell, & Buck, 2008).

Table 1.2 Examples of PFOS concentrations in different sentinel and non-traditional laboratory model species.

Species	Sample Type	PFOS (µg/mg)	Exposure	Reference
C. elegans	Whole body	13.06	1 mg/L	Sammi, 2019 (Sammi et al., 2019)
Oreochromis mossambicus	Whole body	0.0000416	n/a	Bangma, 2017 (Bangma et al., 2017)
D. rerio	Whole body	0.000021.6	1 mg/L	Spulber, 2014 (Spulber et al., 2014)
R. pipiens	Whole body	0.0045	1 mg/L	Foguth, 2019 (Foguth et al., 2019)
Tursiops truncatus	Plasma	0.000571	n/a	Soloff, 2017 (Soloff et al., 2017)
Pusa hispida	Serum	57.3 ng/mL	n/a	Levin, 2016 (Levin et al., 2016)
U. maritimus	Liver	0.00002882	n/a	Biosvert, 2019 (Boisvert, Sonne, Riget, Dietz, & Letcher, 2019)
Tachycineta bicolor	Serum	137 ng/mL	n/a	Custer, 2012 (Custer, Custer, Schoenfuss, Poganski, & Solem, 2012)
Sus scrofa	Liver	0.000040 ng/g	n/a	Watanabe, 2010 (Watanabe et al., 2010)

The role of specific reactive oxygen species (ROS) in PFOS neurotoxicity has been examined. Superoxide production increases after exposure to PFOS. Mitochondria have also been shown to be decreased in neurons of worms treated with PFOS starting at 5 mg/L (10 µM) (Sammi

et al., 2019). Importantly, the synthetic antioxidant XJB-5-131, which specifically decreases mitochondrial oxidative stress, was able to rescue dopaminergic neurodegeneration and mitochondrial function after PFOS exposure (Sammi et al., 2019).

PFOS specifically causes dopaminergic neurotoxicity, starting at 75 mg/L (150 µM) at 48 h, whereas effects on γ-aminobutyric acid (GABA)ergic, serotonergic, and cholinergic neurons did not start until 100 mg/L (200 µM) (Table 1.3A) (Sammi et al., 2019). PFOS also caused behavioral defects, such as increased repulsion time, a dopamine-dependent behavior, forward movement, body bending, and thrashing, without changes in paralysis, which is controlled by the cholinergic system (Table 1.3A) (N. Chen et al., 2014; Sammi et al., 2019). Both the dopaminergic neurons and the behavioral defects were able to be rescued by daily treatment with glutathione, which further indicates ROS as part of the mechanism of toxicity (Sammi et al., 2019). PFOS has also been implicated in decreased behavioral plasticity, with 20 µM PFOS increasing the chemotaxis index and decreasing the expression of gcy5-gfp in ASE sensory neurons, although it is unclear if this protein is important for chemotaxis in *C. elegans* (Table 1.3A) (N. Chen et al., 2014). However, these changes in plasticity were not concurrent with clear changes in either cholinergic or dopaminergic neurons at 20 µM PFOS (N. Chen et al., 2014). This data indicates potential deficits in motor function after exposure, but more studies are needed, especially on other PFAS.

Table 1.3 Neurotoxicity endpoints and findings in species where neurological endpoints were tested. (A). PFOS neurotoxicity and findings in species where neurological endpoints were tested (B). PFNA neurotoxicity endpoints and findings in species where neurological endpoints were tested. NT = not tested (C). PFOA neurotoxicity endpoints and findings in species where neurological endpoints were tested. NT = not tested. Δ—indicates a change after exposure that is not consistent over dose or time, i.e., nonmonotonic dose response.

(**A**)

Organism	Concentration (μM)	Length of Exposure	Neurobehavior	Neurotransmitters	Neuropathology	Reference
C. elegans	40–400	72 h	↑Repulsion time	NT	↓Dopaminergic neurons	Sammi, 2019 (Sammi et al., 2019)
C. elegans	20	48 h	↑Forward movement and thrashing	NT	↓Dopaminergic and cholinergic neurons	Chen, 2014 (N. Chen et al., 2014)
D. japonica	1–20	5–7 d	NT	↑Dopamine Δ Serotonin Δ GABA	Δ Acetylcholinesterase activity Δ Neurodevelopmental genes	Yuan, 2018 (Yuan et al., 2018)
D. rerio	2	6 d	↓Bouts ↑Distance during bout ↑Reaction to light changes ↑Startle	NT	NT	Spulber, 2014 (Spulber et al., 2014)
D. rerio	1–8	1–114 h	Δ Speed	NT	NT	Huang, 2010 (H. Huang et al., 2010)
D. rerio	0.02–2	14 d	↑Distance Speed	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, Bugel, & Cooper, 2016)
D. rerio	2 μM	117 h, 6 m depuration	↓Hitting glass-males	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, & Cooper, 2016)

Table 1.3 continued

D. rerio	0.06–20	144 h	Δ Activity ΔTime active	NT	NT	Ulhaq, 2013 (Ulhaq, Orn, Carlsson, Morrison, & Norrgren, 2013)
R. pipiens	0.2–2	30 d	NT	↓Dopamine ↑Dopamine turnover	NT	Foguth, 2019 (Foguth et al., 2019)

(**B**)

Organism	**Concentration (μM)**	**Length of Exposure**	**Neurobehavior**	**Neurotransmitters**	**Neuropathology**	**Reference**
D. rerio	0.02–1	14 d	↓Distance ↓Speed	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, Bugel, et al., 2016)
D. rerio	2	117 h, 6 m depuration	↓Distance ↓Time in the middle ↓Time frozen ↑Speed ↑Hitting glass-males ↑Time in light-males	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, & Cooper, 2016)
D. rerio	0.06–22	144 h	Δ Activity Δ Time active	NT	NT	Ulhaq, 2013 (Ulhaq et al., 2013)

(**C**)

Organism	**Concentration (μM)**	**Length of Exposure**	**Neurobehavior**	**Neurotransmitters**	**Neuropathology**	**Reference**
D. rerio	0.2	14 d	↑Distance	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, Bugel, et al., 2016)

Table 1.3 continued

D. rerio	2	117 h, 6 m depuration	↓Time in light-females	NT	NT	Jantzen, 2016 (Jantzen, Annunziato, & Cooper, 2016)
D. rerio	7.2–2415	144 h	Δ activity Δtime active	NT	NT	Ulhaq, 2013 (Ulhaq et al., 2013)
R. pipiens	2.4	30 d	NT	↓Dopamine ↑Dopamine turnover	NT	Foguth, 2019 (Foguth et al., 2019)

Table 1.4 Other PFAS neurotoxicity endpoints and findings in species where neurological endpoints were tested. NT = not tested. Δ— indicates a change after exposure that is not consistent over dose or time, i.e., nonmonotonic dose response.

Organism	Chemical	Concentration (μM)	Length of Exposure	Neurobehavior	Neurotransmitters	Neuropathology	Reference
O. melastigma	PFBS	0.03	6 m	NT	↑Dopamine Sex-specific Δ norepinephrine Δ Serotonin over time Δ GABA over time ↑Acetylcholine	Δ Transcription factors involved in visual development	Chen, 2018 (L. Chen et al., 2018)
D. rerio	TFAA, PFBA, PFDA (perfluorodecanoate), or PFBS	48–14, 33.3–10000, 0.2–58.4, or 33.3–10000	144 h	Δ Activity ΔTime active	NT	NT	Ulhaq, 2013 (Ulhaq et al., 2013)
D. rerio	PFDoA	0.4–10	120 h	↓Speed	↓Acetylcholine ↑Dopamine	↓Acetylcholinesterase	Guo, 2018 (Guo et al., 2018)
U. maritimus	PFBS, PFHxS, PFOS, perfluorodecane sulfonate (PFDS), PFHxA, perfluoroheptanoate (PFHpA), PFOA, PFNA, PFDA, perfluoroundecanoate (PFUnDA), perfluorododecanoate (PFDoDA), perfluorotridecanoate (PFTrDA), perfluorotetradecanoate (PFTeDA), and perfluoropentadecanoate (PFPeDA) were quantified. PFAS levels were due to exposure in the wild	PFBS: 0.55 ± 0.08 PFHxS: 1.10 ± 0.10 PFOS: 22.92 ± 0.84 PFDS: 0.66 ± 0.06 PFHxA: 0.13 ± 0.03 PFHpA: not detected PFOA: 1.09 ± 0.13 PFNA: 2.59 ± 0.13 PFDA: 2.63 ± 0.15 PFUnDA: 22.30 ± 1.14 PFDoDA: 8.19 ± 0.46 PFTrDA: 37.87 ± 2.29 PFTeDA: 6.81 ± 0.40 PFPeDA: 4.71 ± 0.42 ng/g wet weight in the whole brain	Unknkown, Ages of bears at sampling were 2–10 years	NT	NT	↑Glutathione synthase in occipital lobe and frontal cortex ↓Glutathione synthase in hypothalamus ↑Monoamine oxidase activity ↓Dopamine D2 receptors in occipital lobe and cerebellum ↓ Muscarinic acetylcholine receptor activity in cerebellum ↑ GABA-A receptors ↑Muscarinic acetylcholine receptor activity in frontal cortex ↓Acetylcholinesterase activity in frontal cortex	Eggers Pederson, 2015 (Eggers Pedersen et al., 2015)

Dugesia japonica

Dugesia japonica are flatworms that live in freshwater and are exposed to PFAS mainly through contaminated water, sometimes heavily. *D. japonica* are low on the foodchain, similar to *C. elegans*, so similar precautions must be taken into consideration when extrapolating results to potential human toxicity. Research in *D. japonica* has only focused on the neurotoxicity of PFOS.

Dopamine was decreased in *D. japonica* exposed to 0.5 mg/L (1 µM) PFOS but increased at all doses between 0.5 and 10 mg/L (20 µM) after 1 day of exposure; it was significantly increased on day 4 of all exposed and continued to be increased after 10 days in all doses except 5 mg/L (10 µM) (Table 1.3A) (Yuan et al., 2018). Interestingly, this is different than that found in *C. elegans*, where dopamine was decreased after exposure to PFOS (Sammi et al., 2019). This discrepancy could be due to differences in accumulation between *D. japonica* and *C. elegans*. In *D. japonica*, serotonin was affected after one day of exposure, with 0.5 mg/L PFOS increasing serotonin levels and 1 mg/L decreasing it (Table 1.3A) (Yuan et al., 2018). Interestingly, it was decreased at both exposure levels and increased at 5 mg/L and 10 mg/L after 4 days of exposure, while only the 0.5 mg/L and 10 mg/L had significantly increased serotonin after 10 days (Table 2A) (Yuan et al., 2018).

PFOS had differing effects on GABA in *D. japonica*, with an initial decrease after 0.5 mg/L for one day and an increase after 10 mg/L (Table 1.3A) (Yuan et al., 2018). Interestingly, after four days of exposure, the 0.5 mg/L group had an increase in GABA while the 5 mg/L group had a decrease, and all exposure groups except the 10 mg/L had a decrease in GABA after 10 days of exposure (Table 1.3A) (Yuan et al., 2018).

Acetylcholinesterase (AChE) activity was as variable after exposure to PFOS as GABA levels, with activity decreasing after exposure to 0.5 mg/L and 1 mg/L PFOS and increasing at 5 mg/L and 10 mg/L after 1 day of exposure (Table 1.3A) (Yuan et al., 2018). This was followed by a decrease in activity in all exposure groups after 3 days except for 10 mg/L, which had a significant increase, with all exposure groups having increased activity after 5 days of exposure (Table 1.3A) (Yuan et al., 2018). After 7 days, all but the highest exposure group had increased activity, while the 10 mg/L had decreased activity, and the lowest exposure caused decreased activity after 10 days, while 5 mg/L caused an increase (Table 1.3A) (Yuan et al., 2018). These changes suggest different effects of PFOS on the neurotransmitter systems depending on length of exposure and PFOS concentration. Furthermore, there was a dose-dependent decrease in

synapsin+ neurons, which indicates a decrease in neurotransmitter synaptic vesicles (Table 1.3A) (Yuan et al., 2018).

Neuronal development is an area of great concern, especially because the blood–brain barrier is not fully functioning during development (Saili et al., 2017). Interestingly, in *D. japonica*, *djotxA (D. japonica* homeobox protein OTX A), a gene that is important for the development of the part of the brain necessary for vision, mRNA was significantly increased at 0.5 mg/L, 1 mg/L, and 5 mg/L PFOS but was decreased after 10 mg/L for 1 and 10 days (Table 2A) (Yuan et al., 2018). Similarly, other mRNA coding for transcription factors, such as *djotxB* (*D. japonica* homeobox protein OTX B), *djFoxD* (*D. japonica* forkhead box D), *djFoxG*, and *djnlg* (*D. japonica* neuroligin) were all affected by PFOS (Table 1.3A) (Yuan et al., 2018). *djotxB* mRNA was increased at 1 mg/L and 5 mg/L after 1 day of exposure, decreased after 10 mg/L exposure on day 4, and decreased in all exposures after 10 days (Table 1.3A) (Yuan et al., 2018). *djFoxD* mRNA was significantly decreased at 0.5, 1, and 10 mg/L and increased at 5 mg/L after 1 day of exposure but decreased at all exposures except for 0.5 mg/L after 4 days, and in all treatments after 10 days of exposure (Table 1.3A) (Yuan et al., 2018). *djFoxG* mRNA was significantly increased in all PFOS treatments after 1 day, increased at the lowest exposure and decreased with 1 and 10 mg/L after 4 days of exposure, and decreased in all exposure groups after 10 days of treatment (Table 1.3A) (Yuan et al., 2018). *djnlg* mRNA was significantly increased at 1 mg/L after the first day of exposure, but it decreased in 1 mg/L and 5 mg/L after four days, and it increased after 10 days of exposure at 0.5 mg/L and 10 mg/L (Table 1.3A) (Yuan et al., 2018). These data indicate that PFOS is significantly affecting gene expression, which is important for neurodevelopment in a dose- and time-dependent manner.

1.7.6 Neurotoxicity of PFAS in non-traditional model organisms - vertebrates

Danio rerio

Fish are exposed to the water that is contaminated by PFAS. Exposure is mostly through gills and diet. Again, this can lead to comparisons, but one must remember that there are differences when considering potential effects on humans. *D. rerio* are good models because they are relatively easy to use in laboratory settings. Development occurs outside of the parent and eggs are clear, which makes them easily manipulated, similar to *C. elegans* (Dooley & Zon, 2000).

Furthermore, the genetic knowledge we have for them makes them ideal species to study the potential effects of chemicals on gene expression (Dooley & Zon, 2000). *D. rerio* have been used abundantly in PFAS research, including studies on PFOS, PFOA, PFBS, PFNA, and more recently found PFAS including both short and long-chain PFAS.

The accumulation of PFAS in *D. rerio* has been studied for many compounds that have not been studied in other species. After 5 days of exposure that started on the day of fertilization, PFOS levels were found to be 15.2 ± 2.4 ng/embryo for 0.5 mg/L PFOS, 52.8 ± 2.1 ng/embryo for 2 mg/L, and 66.1 ± 2.4 ng/embryo for 4 mg/L (Table 1.2) (H. Huang et al., 2010). For a more acute exposure, embryos exposed for 24 h had 21.6 ± 5.4 ng/mg after 0.1 mg/L and 213.5 ± 62.7 ng/mg after 1 mg/L PFOS (Spulber et al., 2014). Unfortunately, these are not easily compared due to the measurement of PFOS being per embryo in one study. Interestingly, another study found that the co-exposure to PFOS and single-wall carbon nanotubes decreased the bioaccumulation of PFOS in internal organs, suggesting PFOS interacts with single-wall carbon nanotubes, decreasing the amount readily available for absorption (Y. Li et al., 2017). Studies have also focused on more recently produced PFAS, such as 6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB) and 6:2 fluorotelomer sulfonamide alkylamine (6:2 FTAA). Exposure to 6:2 FTAB for 180 days in fish that were 5 months old at the initiation of exposure caused an accumulation of 6:2 FTAA with no quantifiable amount of 6:2 FTAB present, implying that 6:2 FTAB is readily metabolized with 6:2 FTAA being the main metabolite (Shi et al., 2019). Interestingly, females accumulated more 6:2 FTAB in their gonads than males did, but males accumulated more in the liver; the sex difference in accumulation is thought to be due to excretion through spawning and an increased expression of ion transporters in the ovaries (Shi et al., 2019). Furthermore, when parents were exposed to 6:2 FTAB prior to spawning, the F1 generation also had dose-dependent levels present, indicating that there is transfer from parents to offspring (Shi et al., 2019). While accumulation has been studied fairly well for PFOS and some of the more recent PFAS, there is still a lack of accumulation data for the brains of *D. rerio* or for other PFAS, such as PFOA and other shorter chain PFAS.

Behavioral deficits have been a prominent area of study in *D. rerio* exposed to PFAS because behavioral changes can indicate effects on different aspects of the nervous system. In some studies, embryos exposed to 0.5 mg/L PFOS through 96 h post fertilization (hpf) or through 144 hpf at 0.03–12 mg/L swam significantly faster than controls (Table 1.3A) (H. Huang et al., 2010; Ulhaq et al., 2013). Interestingly, another study that exposed embryos through 120 hpf at

0.02 µM found that PFOS decreased swimming speed while PFOA increased it (Table 1.3A) (Jantzen, Annunziato, Bugel, et al., 2016). Therefore, the effects of PFOS on swimming speed could change based on level of exposure, as higher concentrations caused increased speed, while lower concentrations caused decreased speed. These differential effects could be common among PFAS, as PFNA had similar discrepancies, with one study showing decreased speed after exposure to 0.02–2 µM PFNA through 120 hpf and then reared to 30 days post fertilization (dpf), but another showing increased speed in males specifically after the same exposure time at 2 µM, indicating potential sex differences (Table 1.3B) (Jantzen, Annunziato, Bugel, et al., 2016; Jantzen, Annunziato, & Cooper, 2016). Perfluorododecanoate (PFDoA) also decreased the average swimming, speed starting at 0.24 mg/L PFDoA through 120 hpf (Table 1.4) (Guo et al., 2018).

Interestingly, effects on speed and the amount of movement do not tend to correlate after exposure to PFAS. PFOS (>1.5 mg/L), PFBS (>450 mg/L), PFNA (>16 mg/L), and trifluoroacetate (TFAA) (>700 mg/L) all decreased movement during developmental exposure until 144 hpf (Table 1.3) (Ulhaq et al., 2013). Furthermore, another study found that 1 mg/L PFOS through 6 dpf also decreased bouts of swimming, although it increased total movement (Table 1.3A) (Spulber et al., 2014). The same study found that co-exposure to dexamphetamine, a catecholamine reuptake inhibitor, or the dopamine receptor agonists quinipirole (D1) or SKF-81297 (D2) was able to rescue the decreased movement due to PFOS (Spulber et al., 2014). This indicates that PFOS could be affecting the motor system through dopamine, but more research is necessary to determine how PFOS is affecting it specifically. Interestingly, PFNA also decreased swimming after exposure through 120 hpf and then rearing to 30 dpf, indicating that decreased swimming could be a trend among PFAS (Table 1.3B) (Jantzen, Annunziato, & Cooper, 2016). Interestingly, PFOS, PFOA, and PFNA all increased the distance swam in the dark after exposure through 120 hpf followed by 2 weeks of depuration (Table 1.3) (Jantzen, Annunziato, Bugel, et al., 2016). The hyperactivity after PFOS exposure was corroborated in a study where *D. rerio* exposed through 6 dpf had significantly increased movement, but exposure to PFOA did not affect movement (Gaballah et al., 2020). Furthermore, 89 µM perfluoroheptanoate (PFHpA) also increased the swimming distance in the light cycle after exposure through 144 hpf (Table 1.4) (Menger, Pohl, Ahrens, Carlsson, & Orn, 2020). Both PFHxS and PFHxA caused increased activity along with perfluoropentane sulfonate (PFPeS) and PFHpS (Gaballah et al., 2020). However, the PFAS 1H-indole-2-sulfonic acid, 5-((aminocarbonyl)hydrazono)-2,3,5,6-tetrahydro-1-methyl-6-oxo salt

(ADONA), PFBS, and perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonate (PFESA1) did not have any effects on behavior (Gaballah et al., 2020). These data on swimming speed and distance indicate that various PFAS could be affecting various systems, such as motor or anxiety, which should be studied in other models as a potential effect of PFAS exposure.

Another area of neurodevelopment studied is the visual motor response, where fish generally increase activity at the beginning of the dark cycle and then slow down. Fish exposed to 1 mg/L PFOS through 6 dpf did not respond to dark stimulation, while fish exposed to 0.1 mg/L had decreased movement in general during the dark phase (Table 1.3A) (Spulber et al., 2014). Interestingly, these effects were also rescued with exposure to dexamphetamine, quinpirole, or SKF-81297 (Spulber et al., 2014). Exposure to PFDoA through 120 hpf had different effects after light phase changes, which included increased time to slow down after the lights turned on and increased time to speed up when lights were shut off, indicating potential effects on the visual motor response (Table 1.4) (Guo et al., 2018). Overall, these studies show that developmental exposure to PFOS or PFDoA affects the visual motor response, although potentially in different ways depending on the chemical and the concentration, and that this should be further studied in other systems.

There are many indicators in *D. rerio* that can be indicative of escape behavior, the fight-or-flight response, or anxiety. One of the most common studied is burst movements, which are fast, large movements for short periods of time (Kalueff et al., 2013). Interestingly, PFOA, PFNA, PFOS, and a mixture of PFHxA, PFHpA, PFOA, PFNA, PFBS, PFHxS, PFOS, and perfluoropentanoate (PFPeA) exposure through 144 hpf all increased burst movements during the dark phase, while PFHxS and PFOS were the only PFAS tested that increased burst movements during the light phase (Table 1.3) (Menger et al., 2020).

Other ways to determine anxiety behaviors are through measuring the time spent in the middle of a dish, time freezing, or the response to a startle (Kalueff et al., 2013). One study found that 2 µM PFNA exposure through 120 hpf and then 2 weeks of depuration led to increased time in the middle, indicating less anxiety, while the time did not change with PFOS or PFOA exposure (Table 1.3) (Jantzen, Annunziato, Bugel, et al., 2016). Interestingly, in another study where fish were exposed to PFAS through 120 hpf and then reared to 6 months, 2 µM PFNA exposed males spent less time in the middle of the tank, while PFOS and PFOA-exposed males did not have significant changes (Table 1.3) (Jantzen, Annunziato, & Cooper, 2016). Interestingly, in the same

study, males exposed to PFNA had decreased time of immobility, potentially further indicating decreased anxious behavior or increased reckless behavior (Table 1.3B) (Jantzen, Annunziato, & Cooper, 2016). In another study, 1 mg/L PFOS through 6 dpf caused an increased startle response and increased bouts of burst movement after startle, indicating that PFOS exposure could be increasing anxiety (Table 1.3A) (Spulber et al., 2014). Interestingly, PFNA exposure also caused males to attack their reflection more, indicating more aggression, while PFOS exposure decreased attacks (Table 1.3A, B) (Jantzen, Annunziato, & Cooper, 2016). Importantly, while the findings should be confirmed in mammalian models, it is worth noting that schizophrenia risk genes are well conserved across taxa and fish models have been highly useful in the study of chemically induced phenotypes relevant to psychiatric disorders (de Abreu et al., 2020; Gawel, Banono, Michalak, & Esguerra, 2019; Kasap, Rajani, Rajani, & Dwyer, 2018). Thus, these data indicate that PFAS could be causing neurological effects and should be studied further as potentially increasing risk of psychiatric disorders.

As for neurotransmitter levels, data are lacking on effects of exposure. It has been shown that PFOS did not affect AChE activity in the liver, intestine, or gills; however, activity was not quantified in the brain, and co-exposure with single-walled carbon nanotubes increased activity (Table 1.3A) (Y. Li et al., 2017). However, exposure to 6 mg/L PFDoA through 120 hpf decreased the total acetylcholine in the body, while both 1.2 and 6 mg/L PFDoA decreased the AChE activity and mRNA expression (Table 1.4) (Guo et al., 2018). This could indicate that PFDoA is also decreasing the formation of acetylcholine, leading to the decreased accumulation of acetylcholine and activity of AChE, although this has to be further studied with a focus on the brain. Interestingly, both 1.2 and 6 mg/L PFDoA exposure also increased dopamine levels, which is the opposite of what has been shown with PFOS in *C. elegans* (Table 1.4) *(Guo et al., 2018)*. Interestingly, PFDoA exposure decreased the expression of *syn2a* (synapsin IIa), which is a protein that plays a role in serotonin and dopamine release (Table 1.4) (Guo et al., 2018). While this could decrease the release of dopamine, PFDoA also increases *manf* (mesencephalic astrocyte-derived neurotrophic factor) expression, which plays a role in dopaminergic neuron survival (Table 1.4) (Guo et al., 2018).

Furthermore, PFAS exposure causes the differential expression of genes important for neurodevelopment and neuronal function. PFOS increases α-tubulin protein in motor neurons of the spinal cord with no changes in the brain after 96 h of exposure, but it decreases expression in

both the spinal cord and brain after 120 h (Table 1.3A) (Zhang et al., 2011). Interestingly, 6 mg/L of PFDoA exposure through 120 hpf decreased *α1-tubulin* gene expression in the brain as well, indicating that longer developmental exposures to PFAS could decrease tubulin in the brain (Table 1.4) (Guo et al., 2018). PFOS also affected DNA replication, which is indicated by proliferating cell nuclear antigen (*pcna*) gene expression decreasing after 24 h and increasing after 96 and 120 h, implying decreased DNA replication at early time points and increased DNA replication at later time points (Table 1.3A) (Zhang et al., 2011). The neurogenesis-regulating gene *cdk5* (cyclin-dependent protein kinase 5) was also increased at the later time points after PFOS exposure, corroborating the idea that neurogenesis occurs later in PFOS-exposed *D. rerio* (Table 1.3A) (Zhang et al., 2011). However, PFDoA exposure through 120 hpf shows opposite results, such as decreased *gap43* (growth-associated protein 43), *shha* (sonic hedgehog protein A), and *Elavl3* (ELAV like RNA binding protein 3), which are all important for neurogenesis, and decreased fluorescence of GFP expressed through the promoter for *elavl3*, which is commonly used as a neuronal marker (Table 1.4) (Guo et al., 2018). Interestingly, it also decreases *gfap* (glial fibrillary acidic protein), potentially indicating decreased astrocytes, and *mbp* (myelin basic protein) (Table 1.4) (Guo et al., 2018).

Oryzias melastigma

The main reasons for using fish as sentinel species is discussed in Section 1.7.6.1, although most fish are not used in the laboratory setting, such as *D. rerio*. However, when studying other types of fish, you must consider their place in the food chain. Marine medaka (*Oryzias melastigma*) are relatively low on the food chain, eating zooplankton and phytoplankton. These animals still have similar systems to humans, but their accumulation and effects could be different than humans and should be taken into consideration. Unfortunately, the only neurotoxicology studies in *O. melastigma* is on PFBS.

Both norepinephrine and epinephrine were increased in female *O. melastigma* exposed to 9.5 µg/L PFBS, but males had significantly decreased epinephrine after the same exposure (Table 1.4) (L. Chen et al., 2018). There were no changes in serotonin after exposure to PFBS, though, indicating that toxicity is specific to certain neurotransmitters (Table 1.4) (L. Chen et al., 2018).

Acetylcholine levels were increased in male *O. melastigma* exposed to 1 µg/L (3.33 nM) PFBS, although there were not any changes in AChE activity or choline, which are the enzymes

that metabolize acetylcholine to choline and acetylcholine's major metabolite, respectively (Table 1.4) (L. Chen et al., 2018). Interestingly, acetylcholine levels were increased in both 1 and 9.5 µg/L (31 nM) PFBS in female *O. melastigma*, while choline was significantly increased at the higher exposure without changes in AChE activity (Table 3) (L. Chen et al., 2018). This further indicates that the cholinergic system function should be studied as potential areas of concern with exposure to PFBS.

Again, neuronal development is an area of great concern, especially because the blood–brain barrier is not fully functioning during development (Saili et al., 2017). Studies in *O. melastigma* found that both males and females exposed during development had decreased ratios of eye-to-body weight and increased water in the eye after 1 µg/L PFBS for males and 9.5 µg/L for females, and that PFBS accumulated in the eye in a dose-dependent manner (L. Chen et al., 2018). Both β- and γ-crystallin, proteins important for many functions within the eye, were decreased in male *O. melastigma* exposed to 9.5 µg/L PFBS, while in females, only γ-crystallin was significantly down-regulated (Table 1.4) (L. Chen et al., 2018). Male *O. melastigma* also had decreased arrestin, which is a G-protein coupled receptor that is important for the visual rhodopsin system, and increased lumican, which is a proteoglycan that is important for maintaining transparency of the eye, after exposure to PFBS (Table 1.4) (L. Chen et al., 2018). These data indicate that PFBS is affecting the eye, potentially indicating toxicity in other organisms in a similar manner.

Frogs

Frogs are common species used for studying toxicology, especially chemicals that are found in the environment, such as PFAS. The types of frogs used for studying toxicology vary greatly. *Xenopus* are commonly used in laboratories because of their fully known genetic makeup and ease of use in the laboratory, including breeding (Cannatella & Sa, 1993). However, *Xenopus* are not very closely related to other frogs, so extrapolation of data is difficult (Cannatella & Sa, 1993). One of the advantages to using frogs as model systems is that, while their anatomy is not as closely related as mammals, they have similar systems to humans, and certain frogs, specifically Northern leopard frogs (*Rana pipiens*), contain neuromelanin, which is a conglomeration of lipids, proteins, and oxidized dopamine, similar to humans, whereas lower order mammals, such as rats

and mice, do not produce this to as large an extent as humans (Lindquist et al., 1988). The neurotoxicity of PFOA and PFOS has been studied in *R. pipiens*.

Exposure to PFOS or PFOA caused an accumulation of 4500 ppb (ng/g) PFOS and 169 ppb (ng/g) PFOA in *R. pipiens* exposed to 1000 µg/L PFOS or PFOA, with PFOS increasing in a dose-dependent manner while PFOA did not change based on exposure level (Table 1.2) (Foguth et al., 2019). American bullfrogs (*Lithobates catesbeianus*) have a calculated LC_{50} of 144 mg/L (288 µM) PFOS and 1004 mg/L (2.42 mM) PFOA, which is much higher than for *C. elegans* (Flynn et al., 2019).

Little has been studied on the effects of PFAS on the brain of frogs and how it affects neurotransmitter systems. We previously showed that 100 and 1000 µg/L PFOS and 1000 µg/L PFOA were able to significantly decrease dopamine levels in whole brain of *R. pipiens*, with a significant increase in dopamine turnover at 1000 µg/L for both chemicals (Table 1.3A, C) (Foguth et al., 2019). Dopamine was the only neurotransmitter affected in this study, indicating that exposures of PFOS or PFOA could lead to specifically dopaminergic degeneration, similar to *C. elegans*, although more studies need to be performed to determine effects at more environmentally relevant levels and to determine the specific systems being affected.

Ursus maritimus

Aquatic and semiaquatic mammals such as *Ursus maritimus* have been proposed as leading sentinel species (Bossart, 2011). This is partially due to their similar physiology to humans, being mammals. Other factors are that they have long lives, allowing for lifetime exposure similar to humans, and that they are normally high on their food chain (Bossart, 2011). They also are thought to accumulate chemicals in a manner more similar to humans, and they are proposed to have similar effects with chemical exposure through routes such as lactation, as humans do (Bossart, 2011). It is also thought that they might show signs of toxicity from chemicals more strongly than other sentinel species, making them better to make clear the necessity for further research and working toward solutions (Bossart, 2011). Research on *U. maritimus* has been performed on bears that were in the wild, so exposure was to all PFAS in the environment.

U. maritimus in East Greenland had liver levels of PFAS averaging 3546 ng/g wet weight while male *U. maritimus* had an average of 22.92 ng/g wet weight PFOS, 28.82 ng/g total perfluorosulfonates, 1.09 ng/g PFOA, and 99.40 ng/g total perfluorocarboxylates in the brain

(Table 1.2) (Boisvert et al., 2019; Eggers Pedersen et al., 2015). Another study on *U. maritimus* mothers found plasma levels of total PFAS to be 539.0 ± 20.8 ng/g wet weight, with PFOS and PFOA levels equaling 431.9 ± 17.0 ng/g wet weight and 6.4 ± 0.6 ng/g wet weight (Bytingsvik et al., 2012).

Another study on *U. maritimus* from East Greenland studied the effects on various parts of the brain (Eggers Pedersen et al., 2015). Glutathione synthase activity was positively correlated with levels of PFOS, total perfluorosulfonates, and perfluoroundecanoate (PFUnDA) and borderline correlated to levels of perfluorododecanoate (PFDoDA), perfluorotetradecanoate (PFTrDA), and total perfluorocarboxylates in the occipital lobe, and positively correlated with PFOS and total perfluorosulfonates in the frontal cortex while being negatively correlated with total perfluorosulfonates in the hypothalamus (Table 1.4) (Eggers Pedersen et al., 2015). This indicates that there is generally an upregulation of oxidative stress mechanisms with increased levels of PFAS in the brain. This study also found that monoamine oxidase activity, one of the main enzymes that metabolizes catacholamines such as dopamine, serotonin, and norepinephrine, was positively correlated with levels of PFTrDA, total perflurocarboxylates, and trended to correlate with PFDoDA in the occipital lobe and with levels of PFOS, total perfluorosulfonates, PFUnDA, PFDoDA, and total perfluorocarboxylates in the whole brain (Table 1.4) (Eggers Pedersen et al., 2015). There was also a negative correlation between the density of dopamine D2 receptors and PFUnDA, PFDoDA, PFTrDA, and total perfluorocarboxylates in the temporal cortex and a borderline negative correlation with PFTrDA and total perfluorocarboxylates in the cerebellum (Table 1.4) (Eggers Pedersen et al., 2015).

The density of GABA-A receptors was also positively correlated with PFOS and PFDoDA levels and borderline correlated with total perfluorosulfonate levels, PFUnDA, and total perfluorocarboxylate levels across the brain (Table 1.4) (Eggers Pedersen et al., 2015). Furthermore, there was a negative correlation between muscarinic acetylcholine receptor density and PFUnDA, PFTrDA, and total perfluorocarboxylates that was also borderline for PFOS and total perfluorosulfonates in the cerebellum (Table 1.4) (Eggers Pedersen et al., 2015). This was accompanied by a negative correlation between AChE activity and PFDoDA and PFUnDA in the cerebellum (Table 1.4) (Eggers Pedersen et al., 2015). Therefore, PFAS are affecting cerebellar cholinergic transmission, which is similar to that seen in fish and *C. elegans*. There was also a negative association between AChE activity and PFDoDA in the thalamus and with PFTrDA and

total perfluorocarboxylates in the frontal cortex (Table 1.4) (Eggers Pedersen et al., 2015). Muscarinic acetylcholine receptor density was also positively correlated with PFOS and total perfluorosulfonate levels in the frontal cortex (Table 1.4) (Eggers Pedersen et al., 2015). The difference in correlations with muscarinic acetylcholine receptors and PFOS between the cerebellum and the frontal cortex implies that there might be different reactions to PFOS and other perfluorosulfonates in different parts of the brain.

1.8 Relationship Between HAAs and PFAS

While HAAs and PFAS are classes of chemicals that are structurally disparate, there are similarities between them that are important to take into account when studying them for neurotoxicity.

Both types of compounds are similar exposures for humans. HAAs are found in the diet, in food that is consumed throughout one's life, including meat, dairy products, certain grains, and alcoholic beverages (Herraiz, 2004). PFAS are also largely consumed, although mostly through contaminated water, with small levels known to be from contaminated food sources (Domingo et al., 2012; Gao et al., 2019; Jogsten et al., 2009). Therefore, the majority of the exposure to both of these sets of compounds is through oral exposure in the diet. Furthermore, the exposure to both HAAs and PFAS are chronic, either during the entire lifespan, as with PFAS, or during the majority of life, for HAAs.

Interestingly, some of the findings in toxicity of these compounds is related. *C. elegans* exposed to harmane and PFOS each had selective loss of DAergic neurons along with increased repulsion time, an indicator of loss of DAergic motor function, without a loss of the cholinergic neurons (Sammi, Agim, & Cannon, 2018; Sammi et al., 2019). Both harmane and PFOS treatment also decreased viable mitochondria in *C. elegans*, indicating potential similarities in their loss of mitochondrial function (Sammi et al., 2018; Sammi et al., 2019). Of note, harmane and harmine are both MAO inhibitors, as discussed previously (May et al., 1991). Interestingly, there was a correlation between increased MAO activity with increased PFAS levels in polar bear brains (Eggers Pedersen et al., 2015). This could indicate that both classes of compounds are affecting catecholamine metabolism, but in different ways. However, further research needs to be performed to determine if the mechanisms of toxicity are similar.

Therefore, although on initial reading, HAAs and PFAS appear to be very different, it is important to study the potential neurotoxicity of each of them and compare to potentially find a common mechanism of toxicity.

1.9 Conclusions

PD is a common neurodegenerative disease with no known cure or prevention. With less than 10% of cases known to be caused by genetic mutations, most cases of PD are idiopathic. Therefore, it is imperative to find environmental causes or risk factors for the disease. Research has shown that various pesticides, including rotenone and paraquat, and drug contaminants, such as MPTP, cause PD (J. R. Cannon & Greenamyre, 2010; J. R. Cannon et al., 2009). However, there is not enough exposure to these compounds for them to be big risk factors for most people.

In this chapter, we have discussed a class of compounds, HAAs, that are found within the diet and are structurally very similar to MPP+, which has led to research on their potential role in the onset of neurodegenerative diseases like PD. Unfortunately, the currently published studies are not enough to show a causative effect, and further research needs to be done both *in vitro* and *in vivo* to determine their toxicity and the mechanism of action.

Current epidemiological studies show an increase in the HAA harmane in patients with essential tremor (Louis, Benito-Leon, et al., 2013; Louis, Factor-Litvak, et al., 2013). The relationship between essential tremor and PD has been an area of interest for many years; however, further research needs to be done following patients with essential tremor and PD to determine if these two diseases have increased comorbidity. The best way to do this would be through longitudinal studies, which take a long time to obtain results, but are more accurate than questionnaires or recollection. Both harmane and norharmane are also increased in the blood and CSF of patients with PD (Kuhn et al., 1995; Kuhn et al., 1996). However, it is unclear if the accumulation of these compounds is a cause of PD or an effect that occurs after the disease has started progressing.

Research has shown that many HAAs cause changes in DA formation or metabolism. Many of these studies have been performed *in vitro*, which is beneficial for mechanistic studies, but more *in vivo* studies would show whether these changes are specific to the DAergic neurons in the SN and whether these changes cause oxidative stress that could lead to cell death. This also includes determining the effects on motor function of these compounds, and possibly other

symptoms of PD that are not as frequently studied, such as olfactory function and gastrointestinal function. Few studies have performed behavioral tests on animals treated with HAAs alone, and fewer at doses that are relevant to human exposure and subchronic or chronic exposures. Because these compounds are in food that would be consumed throughout a person's life, the cumulative exposure could be leading to the toxicity of these compounds.

Studies in rat primary midbrain show that HAAs are specifically toxic to DAergic neurons (Cruz-Hernandez et al., 2018; Griggs et al., 2014). Most work besides primary cultures has been in a single cell type, such as PC12 cells. Co-cultures would be more similar to the exposures that occur in the brain because of the presence of other cell types, such as microglia and astrocytes. It is unclear as to whether these cells would increase the toxicity of HAAs to DAergic neurons.

Neuromelanin is another potential factor in toxicity to DAergic neurons. The human SN contains neuromelanin, which can bind to HAAs and potentially cause increased accumulation and specific toxicity in neuromelanin-containing cells (Barbeau, Dallaire, Buu, Poirier, et al., 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985; Lindquist et al., 1988; Ostergren et al., 2004; Sokolowski et al., 1990). However, most *in vivo* neurotoxicology research is performed in rodent models, such as rats and mice, which do not have neuromelanin in the brain. Interestingly, certain frogs have neuromelanin present within their brains, and have previously been used as models of PD (Barbeau, Dallaire, Buu, Poirier, et al., 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985; Lindquist et al., 1988; Ostergren et al., 2004; Sokolowski et al., 1990). While their neuroanatomy varies from human much more than rat and mouse do, the presence of neuromelanin makes them valuable model organisms for accumulation and toxicity in neuromelanic cells.

HAAs cause oxidative stress and cell death in many reports. However, the exact mechanism of cell death is unknown for these compounds. Further research on this should include formation of oxidative stress through mitochondrial function and accumulation of DA. Toxicity could also be due to accumulated effects of the many HAAs that are found in the diet, and co-exposure studies are important to look at synergistic effects.

We also discussed emerging research on the toxicity from PFAS exposure. PFAS are present in blood of >99% of people worldwide and is transported to fetuses and infants through the placenta and breast milk (Calafat et al., 2007; Jian et al., 2018). This means there is developmental exposure, during which time the blood-brain barrier is not completely functional, potentially leading to neurodevelopmental defects. Furthermore, these compounds have been

measured in the brains of wildlife such as polar bears and were correlated with changes in activity of enzymes such as acetylcholinesterase and MAO, indicating that these compounds affect neurotransmitter metabolism (Eggers Pedersen et al., 2015). Unfortunately, further research is lacking to determine the effects of these compounds on the brain, especially during neurodevelopment.

Cardiac function is regulated by sympathetic and parasympathetic innervation. Interestingly, effects of PFAS on cardiac function is varied, with one study finding increased angina, myocardial infarction, and stroke in workers exposed to PFOA, but another study finding a decreased risk of dying from ischemic heart disease, and yet another finding no differences (Anderson-Mahoney, Kotlerman, Takhar, Gray, & Dahlgren, 2008; Lundin, Alexander, Olsen, & Church, 2009; Sakr, Symons, Kreckmann, & Leonard, 2009). Furthermore, there is currently no research to our knowledge that has determined potential effects of PFAS exposure on heart innervation.

1.10 Central Hypothesis

Although it is known that certain environmental contaminants can cause PD, exposure to these compounds is not frequent or high enough to cause a majority of cases of PD or other neurological disorders. Due to the accumulating literature that implicate chemicals found in the diet in affecting the DA systems, specifically the motor system, we hypothesize that exposure to specific dietary exposures such as HAAs or PFAS cause neurotoxicity leading to altered brain function.

1.11 Specific Aims

Specific Aim 1. Harmane and harmine decrease mitochondrial function through metabolism and inhibition of mitochondrial function.

Rationale: *In vitro* research on the effects of HAAs on mitochondrial function will develop the mechanism of toxicity of these compounds as well as potentially indicate processes that are affected in cases of PD caused by other exposures. Therefore, this could indicate potential mechanisms through which therapies can be produced for PD.

The human neuroblastoma cell line SH-SY5Y supplemented with galactose media was treated with individual HAAs and mitochondrial function was compared to control cells using the Seahorse Extracellular Flux Analyzer. Mitochondria from galactose-supplemented SH-SY5Y cells were treated with harmane or harmine to determine the specific mitochondrial complex or function that is being affected.

Impact: The exact mechanism of toxicity in DAergic neurons is unknown for most of PD. Therefore, this research will elaborate on the cellular mechanisms that are affected in PD and could implicate a pathway that could be targeted for therapeutic use in PD.

Specific Aim 2. PhIP and harmane cause decreased motor function and alter the nigrostriatal system.

Rationale: It is unknown whether oral exposure to HAAs causes neurodegeneration and motor dysfunction like that seen in PD, although research has shown that it specifically targets DAergic neurons *in vitro*. Therefore, this study will determine the effects on motor function and pathology in the nigrostriatal system after exposure to PhIP or harmane.

Rats were treated via oral gavage with harmane for 16 weeks, with motor function testing weekly during exposure. Treated rats were compared to control rats to determine motor dysfunction, and striatal and SN changes in immunohistochemistry and neurochemistry to determine pathological effects.

Mice were treated with PhIP via oral gavage for acute, subacute, or subchronic exposures (8 hours, 4 weeks, or 16 weeks, respectively) with motor function analyzed weekly for subacute and subchronic exposures. Motor function was compared to control animals, and the striatum was analyzed through immunohistochemistry and neurochemistry to determine affects.

Impact: These studies will determine the effects of various lengths of exposure to PhIP or harmane *in vivo*, which would indicate whether it is potentially a neurotoxin that could cause PD in humans.

Specific Aim 3. Developmental exposure of PFAS in vivo affect neurotransmitters in the brain and innervation of the heart.

Rationale: There is very little research on the effects of developmental exposure to PFAS on the brain or innervation of the heart; however, it is known that PFAS enter the brain. *In vitro* studies indicate that PFAS exposure, especially exposure to PFOS, can decrease the number of

DAergic neurons during differentiation, and some *in vivo* studies have found developmental exposure to decrease motor function in mice. However, the current research does not determine mechanism of toxicity or directly indicate PFAS as developmental neurotoxicants.

Northern leopard frog (*Rana pipiens*) larvae were exposed to PFAS through spiked sediment or water for 30 days or through metamorphosis. Whole brains were analyzed for neurotransmitter levels to determine effects on different neurotransmitter systems and compared to controls. Heart innervation was also studied by measuring neurotransmitter levels in hearts of frogs exposed to PFAS through metamorphosis.

Impact: This research will determine developmental toxicity of PFAS in Northern leopard frogs, which are potentially an important model system for neurotoxicity due to the expression of neuromelanin. Changes in neurotransmitter levels in the heart will also be related to whole brain changes, which could indicate denervation of the heart through the autonomic nervous system, leading to risk of cardiovascular problems.

1.12 Format of the Dissertation

This dissertation contains seven chapters. The first chapter is an introduction to the background for this research, the overarching hypothesis of this dissertation, and the specific aims.

The second through sixth chapters are the experimental methods and results of the specific aims. Chapter 2 contains *in vitro* data on the effects of HAAs on mitochondrial function which is imperative to specific aim 1. Chapters 3 and 4 are *in vivo* data obtained after exposure of mice to PhIP and rats to harmane. These are the focus of specific aim 2. Chapters 5 and 6 contain data from PFAS exposure in Northern leopard frogs (*Rana pipiens*) to determine effects on neurotransmitters. Both chapters 5 and 6 are the focus of specific aim 3. Each of these chapters are written in journal article format, with a brief introduction on the rationale for the research presented in that chapter, the methods used, results, and a brief discussion as to how the data fits into the published literature.

The seventh chapter is a brief summary of the data obtained through this dissertation and future work that should be done to further the field and how this relates to the current understanding of the effects of toxins and toxicants in the diet that could increase risk of PD or other neurological diseases.

CHAPTER 2. HARMANE AND HARMINE DECREASE MITOCHONDRIAL FUNCTION *IN VITRO*

2.1 Rationale

Parkinson's disease (PD) is a neurodegenerative disease that affects dopaminergic (DAergic) neurons in the substantia nigra (SN). Various genetic mutations cause PD, such as mutations or overexpression of alpha-synuclein, PINK, PARKIN, DJ-1, and LRRK2 (Hernandez, Reed, & Singleton, 2016; Karuppagounder et al., 2016; Volta et al., 2015). However, these known mutations are the cause of less than 10% of PD cases, the rest being idiosyncratic. Therefore, research has been focusing on finding environmental factors that can lead to PD. One such chemical found is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which was found as a contaminant of street drugs (Heikkila, Hess, & Duvoisin, 1985; Heikkila, Nicklas, Vyas, & Duvoisin, 1985). MPTP is metabolized to its cation, 1-methl-4-phenylpyridinium (MPP+) and transported into DAergic neurons through the dopamine transporter (Heikkila, Hess, et al., 1985; Heikkila, Nicklas, et al., 1985). MPP+ then inhibits mitochondrial complex I, leading to oxidative stress and DAergic cell death (Heikkila, Hess, et al., 1985; Heikkila, Nicklas, et al., 1985).

MPTP is not a common contaminant that would lead to many cases of PD. However, there are many heterocyclic aromatic amines (HAAs) found in the diet that are structurally similar to MPP+ (Figure 2.1). These compounds are generally formed through Maillard reactions involving amino acids and sugars (Skog, Johansson, & Jagerstad, 1998). HAAs are, therefore, found in foods such as various forms of alcohol, soy sauce, seasoning, cereals, well done meats, chocolate, coffee, and tobacco (Herraiz, 2004). Although amount consumed varies based on diet, it is estimated that the average person consumed about 160 ng of the main HAAs (MeIQx, PhIP, norharmane, and harmane) per day (Gibis & Weiss, 2015). This exposure would be a chronic exposure lasting a person's entire life, which could lead to accumulation and toxicity.

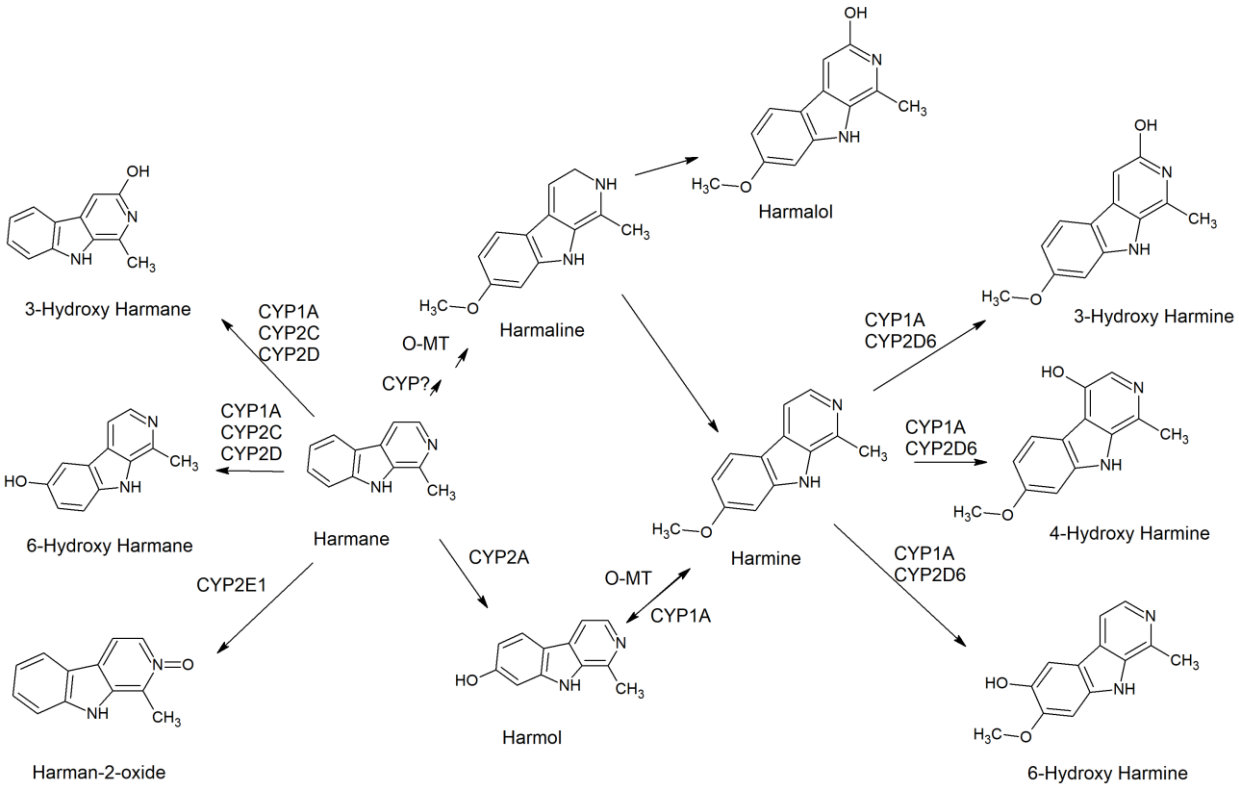

Figure 2.1 Proposed mechanism of Phase I metabolism of harmane and harmine. The majority of Phase I metabolism is proposed to be performed by cytochrome P450s; however some metabolic enzymes are as yet unknown.

Many derivatives of harmane and norharmane, especially the methylated derivatives, cause changes in DA metabolism, including decreasing DA efflux, decreasing levels of DA and its metabolites, and decreasing oxygen consumption (Agim & Cannon, 2018; Albores, Neafsey, Drucker, Fields, & Collins, 1990; Neafsey et al., 1995; Neafsey, Drucker, Raikoff, & Collins, 1989; Rommelspacher & Subramanian, 1979; Yang, Lee, Jin, Lim, & Lee, 2008). However, these compounds are not commonly found in the body nor are common compounds people would be exposed to. Harmane and norharmane, however, have not been studied for their effects on mitochondrial function, but are HAAs that humans are exposed to daily through their whole life time.

Louis and colleagues found that patients with PD had double the amount of harmane in blood compared to controls (Louis, Michalec, Jiang, Factor-Litvak, & Zheng, 2014). Further research showed that PD patients also had significantly increased levels of norharmane and higher levels of harmane (though not significant) in blood (Kuhn, Muller, Grosse, Dierks, &

Rommelspacher, 1995). They also found that norharmane and harmane were increased in the cerebral spinal fluid of patients with PD (Kuhn, Muller, Grosse, & Rommelspacher, 1996). Interestingly, in mice, norharmane and harmane also cause decreased motor function (Esmaeili, Movahedi, Faraji, & Haghdoost-Yazdi, 2012; Ostergren, Fredriksson, & Brittebo, 2006).

Because of these links, research has started to determine whether HAAs cause DAergic neuron toxicity. Norharmane, harmane, and 2-methylnorharmane accumulate more in the SN of human brains compared to cortex, potentially showing increased accumulation in neurons susceptible to oxidative stress (Matsubara et al., 1993). The main HAAs found in the diet, including PhIP, MeIQ, harmane, and norharmane, are selectively toxic to DAergic neurons in rat primary midbrain cultures (Cruz-Hernandez et al., 2018; Griggs et al., 2014). Interestingly, many of the derivatives that affect dopamine and mitochondrial function are metabolites of harmane or norharmane (Figure 2.1). Therefore, it is possible that harmane is causing similar toxicity to compounds not as prominently present in the diet due to metabolism to the toxic derivative. However, the mechanism is yet unclear. Therefore, we hypothesized that harmane and one of its metabolites, harmine, would cause mitochondrial dysfunction, as seen in MPP+ toxicity.

2.2 Methods

2.2.1 Reagents

RPMI, sodium pyruvate, sodium bicarbonate, harmane (98%), norharmane, and 1-methyl-4-phenylpyridinium (MPP+) iodide, oligomycin, equine cytochrome c, ATP, quinidine, Hoechst 33342, and Coenzyme Q_{10} were purchased from Sigma-Aldrich. L-glutamine was purchased from Gibco, 20% galactose was from Teknova, 2',7' –dichlorofluorescin diacetate and Mitotracker Red CMH2Xros were purchased from Fisher Scientific, and N-acetyl-L-cysteine was from Acros. Base media, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone, and antimycin A used for extracellular flux analyses were purchased from Agilent. 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-amino-3,4-dimethyl-3H-imidazo[4,5-f]quinoline (MeIQ), and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) were purchased from Toronto Research Chemicals. PhIP metabolites N-OH PhIP and 4'-OH PhIP were received as gifts from the Turesky laboratory.

2.2.2 Time course assay

SH-SY5Y cells supplemented with galactose (RMPI, 1 mM sodium pyruvate, 44 mM sodium bicarbonate, 1x L-glutamine, 10 mM galactose, 10 mM HEPES, 1x pen/strep, 15% nu-serum) were split to 35,000 cells/well in wells B-G of an Agilent XFp miniplate. One day after plating, media was changed to basic media (Agilent base media containing 0.18% galactose, 3.76 mM L-glutamine, 1 mM sodium pyruvate) one hour before extracellular flux analysis using the Seahorse XFp Extracellular Flux Analyzer. After baseline measurements were obtained, the compound of interest was injected into the well and measurements continued for three hours. Each plate was normalized to control, and each experiment was run 3-6 times.

2.2.3 Mitochondrial stress test

SH-SY5Y cells supplemented with galactose (RMPI, 1 mM sodium pyruvate, 44 mM sodium bicarbonate, 1x L-glutamine, 10 mM galactose, 10 mM HEPES, 1x pen/strep, 15% nu-serum) were split to 35,000 cells/well in wells B-G of an Agilent XFp miniplate. After the cells adhered to the plate overnight, media was changed to fresh media containing the compounds of interest for 24 hours. Mitochondrial stress tests were run according to manufacturer protocol. In brief, media was changed to basic media (Agilent base media containing 0.18% galactose, 3.76 mM L-glutamine, 1mM sodium pyruvate) one hour before baseline measurements were obtained. Oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and Rotenone and Antimycin A were injected sequentially to obtain concentrations of 1 µM, 0.5 µM, and 0.5 µM, respectively. Each plate was normalized to control, and each experiment was run 3-6 times.

2.2.4 Oxidative stress

Oxidative stress was measured using 2',7' –dichlorofluorescin diacetate (DCFDA). Briefly, cells were plated on black 96 well plates with clear bottoms. When cells reached 80% confluency, they were treated with the concentrations of harmane or harmine as in the mitochondrial viability test for 24, 12, 8, 4, or 1 hour. After exposure, cells were washed with HBSS three times and then exposed to 10 µM DCFDA in HBSS for 30 minutes. Cells were then washed with PBS once and imaged in a plate reader with excitation/emission of 495 nm/525 nm. Plates were run in quadruplet with four individual experimental plates.

2.2.5 Mitochondrial membrane potential

Mitochondrial membrane potential was measured using the Mitotracker Red CM-H2Xros with Hoescht 33342 as a nuclear counterstain. Briefly, cells were plated in 24 well plates with coverslips and treated with 18.75 µM, 37.5 µM, 75 µM harmane or harmine, or a DMSO equivalent to the highest dose for 24 hours after they reached about 70% confluency. After treatment, cells were rinsed in PBS and incubated in 1:4000 Mitotracker Red CM-H2Xros in HBSS for 30 minutes. After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde, pH 7.4, for 15 minutes, rinsed in PBS again and incubated in ice-cold acetone for 10 minutes to permeabilize. Cells were then incubated in 1:1000 Hoescht 33342 in water for 5 minutes, rinsed in PBS, and put on slides using DPX mountant (VWR). Cells were imaged on a Nikon A1R confocal microscope and analyzed using Image J and the plug-in Mitochondrial Network Analysis (MiNA) to quantify mitochondria (Valente, Maddalena, Robb, Moradi, & Stuart, 2017).

2.2.6 Specific mitochondrial complex activities

Mitochondria were isolated from liver of male Sprague Dawley rats according to the protocol of Graham et al. (Graham, 2001). Briefly, rats were decapitated and liver was isolated and rinsed in liver homogenization medium (LHM) (0.2M mannitol, 50mM sucrose, 10mM KCl, 1mM EDTA, 10mM HEPES, pH 7.4), chopped, washed in LHM, and homogenized with a Potter-Elvejem homogenizer. The suspension was centrifuged for 10 minutes at 1,000xg, 4oC, the supernatant was transferred to a new tube and centrifuged for 10 minutes at 3,000xg, 4oC. Supernatant was aspirated and the pellet was washed with LHM two more times and the mitochondrial pellet was suspended in water.

Individual mitochondrial complex activities were measured in the same way as Spinazzi et al. (Spinazzi, Casarin, Pertegato, Salviati, & Angelini, 2012). Briefly, complex I was measured through incubation of 10 µg mitochondria, 50 µM potassium phosphate buffer (pH 7.5), 3 µg bovine serum albumen (BSA), 0.3 mM KCN, 0.1 mM NADH, and 10 µM rotenone, 2.5 µM harmane, or 2.5 µM harmine for 30 minutes and baseline absorbance was measured at 340 nm for 2 minutes prior to addition of 60 µM ubiquinone and reading of absorbance for another 2 minutes.

Complex II activity was measured through 30 minute incubation of 25 mM potassium phosphate (pH 7.4), 1 µg BSA, 0.3 mM KCN, 20 mM succinate, 0.002175% 2,6-

Dichlorophenolindophenol (DCPIP), 10 µg mitochondria, and 10 mM malonate, 2.5 µM harmane, or 2.5 µM harmine for 30 minutes and baseline absorbance was measured at 600 nm for 3 minutes prior to addition of 50 µM decylubiquinone and reading the absorbance for 3 minutes.

Complex III activity was measured through 30 minute incubation of 25mM potassium phosphate (pH 7.5), 75 µM oxidized cytochrome c, 0.5mM KCN, 0.1mM EDTA, 0.025% Tween 20, 3 µg mitochondria, and 0.1 µg antimycin A, 2.5 µM harmane, or 2.5 µM harmine for 30 minutes and baseline absorbance was measured at 550 nm for 1 minutes prior to addition of 0.1 mM decylubiquinol and reading the absorbance for another minute.

Complex IV activity was measured through 30 minute incubation of 50 mM potassium phosphate (pH 7.0), 60 µM reduced cytochrome c, and 0.3 mM KCN, 2.5 µM harmane, or 2.5 µM harmine for 30 minutes and baseline absorbance was measured at 550 nm for 3 minutes prior to addition of 1 µg mitochondria and reading for another 3 minutes.

The interaction between complexes I and III was measured through 30 minute incubation of 6 µg mitochondria, 50 mM potassium phosphate (pH 7.5), 1 µg BSA, 0.3 mM KCN, 50 µM oxidized cytochrome c, and 10 µM rotenone, 2.5 µM harmane, or 2.5 µM harmine for 30 minutes and baseline absorbance was measured at 550 nm for 2 minutes prior to addition of 0.2 mM NADH and reading of absorbance for another 2 minutes.

The interaction between complexes II and III was measured through 30 minute incubation of 1 µg mitochondria, 20 mM potassium phosphate (pH 7.5), 10 mM succinate, 0.3 mM KCN, and 10 mM malonate, 2.5 µM harmane, or 2.5 µM harmine before addition of 50 µM oxidized cytochrome c and reading the absorbance at 550 nm for 3 minutes.

All complex activities were quantified by the following calculation:

$$Enzyme\ activity\ \left(\frac{nmol}{min*mg}\right) = \frac{{}^{\Delta absorbance}/_{min}*1{,}000}{extinction\ coefficient*mL\ sample*sample\ protein\ concentrations\ (\frac{mg}{mL})}.$$

After calculation of activity, the inhibited control activity was subtracted from control, harmane, or harmine treated activities to get specific activity.

2.2.7 Statistical analysis

Statistics were performed in Graphpad Prism 8. A t-test or one-way ANOVA was performed for all experiments with a Sidak's post hoc test to compare control and each treatment group where appropriate. $p<0.05$ was considered statistically significant.

2.3 Results

2.3.1 No HAAs studied caused decreased mitochondrial respiration after 3 hours

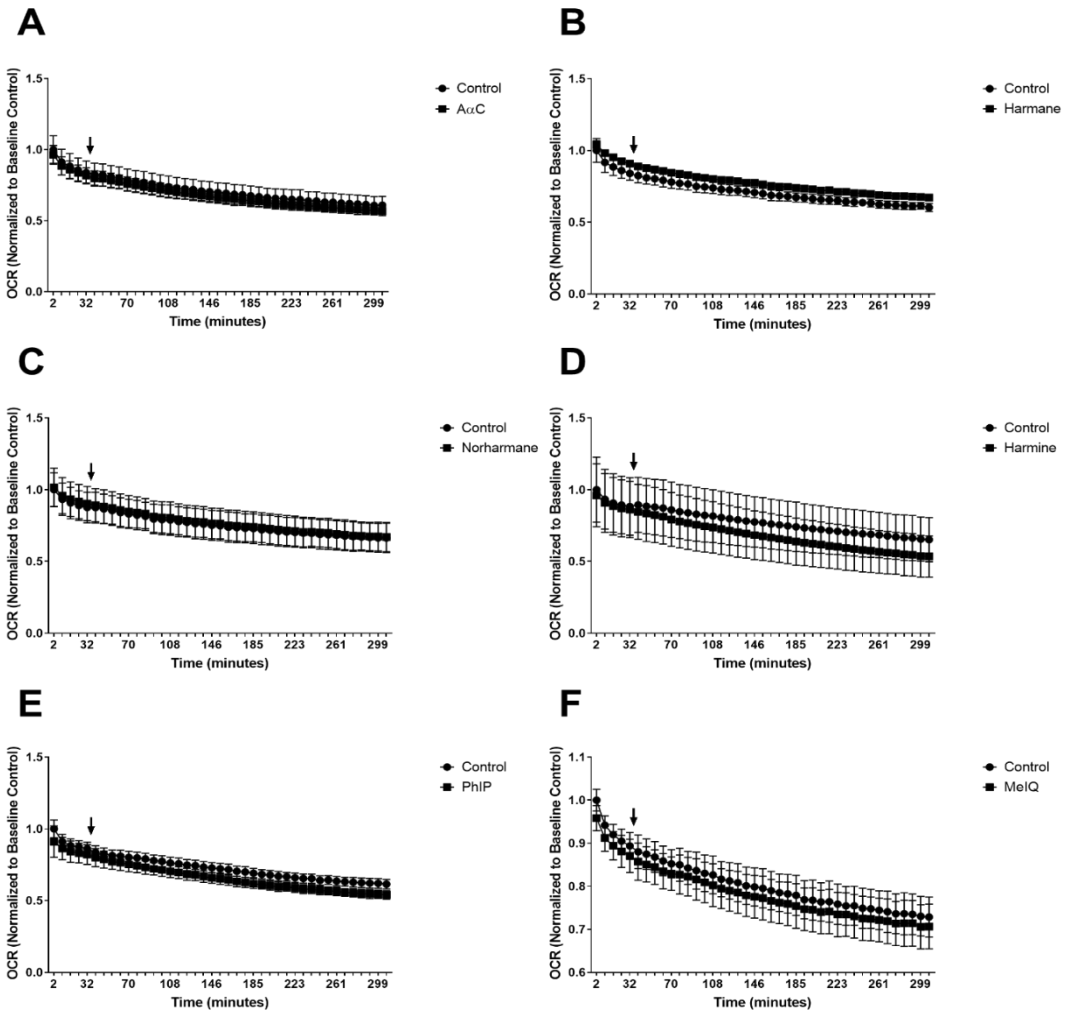

Figure 2.2 No HAA tested significantly decreased mitochondrial function after 3 hours. Oxygen consumption rate was measured using a Seahorse Extracelluar Flux Analyzer with addition of A) AαC, B) harmane, C) norharmane, D) harmine, E) PhIP, or F) MeIQ. Arrows indicate where treatment was added.

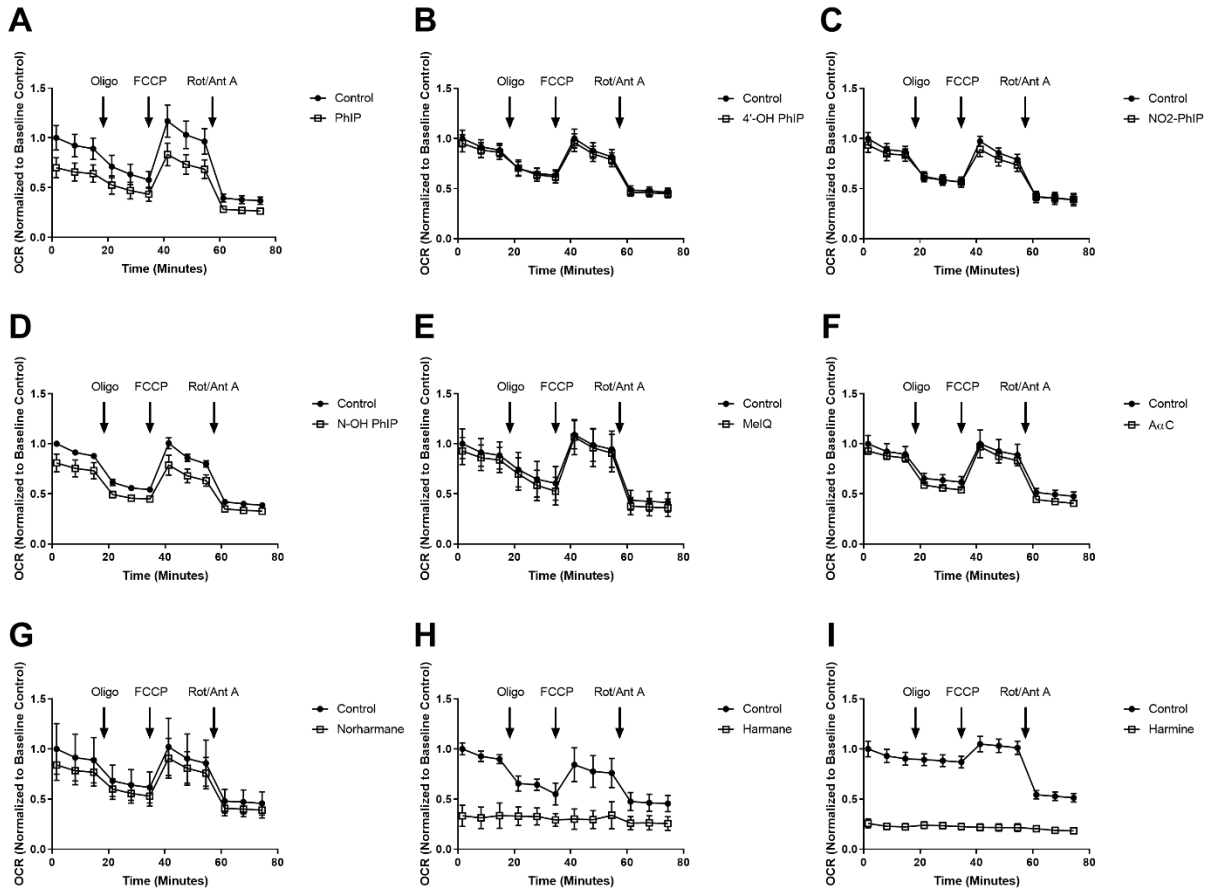

Figure 2.3 Harmane and harmine, but not other HAAs, significantly decrease mitochondrial respiration after 24 hours of exposure. Galactose SH-SY5Y cells were treated with A) PhIP, B) 4'-OH PhIP, C) NO_2-PhIP, D) N-OH PhIP, E) MeIQ, F) AαC, G) norharmane, H) harmane, or I) harmine for 24 hours. A mitochondrial stress test was then performed on cells.

Time course assays run on galactose-supplemented SH-SY5Y cells were used to determine whether acute exposure to HAAs can cause decreased mitochondrial function. Prior to extracellular flux analysis, cell viability assays were performed to determine a concentration that would not cause cell death after 24 hours (data not shown). Extracellular flux analysis showed no decreased mitochondrial function after three hours of exposure to all HAAs except harmine, which caused a decrease over time that did not reach significance at 3 hours.

2.3.2 Mitochondrial stress test

Mitochondrial stress tests were performed after 24 hours of exposure to concentrations of HAAs that did not cause cell death. The mitochondrial stress test determines changes in basal respiration, ATP production, proton leak, maximal respiration, and spare capacity through injection of chemicals known to affect different aspects of mitochondrial respiration.

None of the AIAs studied (MeIQ, PhIP, and the PhIP metabolites 4'-OH PhIP, N-OH PhIP, and NO_2-PhIP) caused significant changes in any of the mitochondrial functions tested (Fig. 2.3A-E). The α-carboline, AαC, and norharmane did not cause significant differences in mitochondrial function, either (Fig. 2.3F and G). Interestingly, both harmane and harmine caused significantly decreased mitochondrial function (Fig. 2.3H and I).

Both 75 μM harmane and 18.75 μM harmine significantly decreased basal respiration (control vs. harmane: 0.966 ± 0.079 vs. 0.199 ± 0.124, p = 0.0024; control vs. harmine: 1.00 ± 0.05 vs. 0.468 ± 0.074, p = 0.0001) (Fig. 2.4A and B). ATP production was significantly decreased after exposure to 75 μM harmane and decreased, although not significantly, after exposure to harmine (control vs. harmane: 0.958 ± 0.078 vs. 0.141 ± 0.121, p = 0.0009; control vs. harmine: 1.00 ± 0.22 vs. 0.539 ± 0.109, p = 0.2694) (Fig. 2.3C and D). Harmine caused significantly decreased proton leak (control vs. harmine: 1.00 ± 0.05 vs. 0.792 ± 0.105, p = 0.0137), while 75 μM harmane nonsignificantly decreased proton leak (control vs. harmane: 0.989 ± 0.112 vs. 0.352 ± 0.139, p = 0.0801) (Fig. 2.3E and F). Harmine also caused significantly decreased maximal capacity (control vs. harmine: 1.00 ± 0.07 vs. 0.435 ± 0.107, p = 0.0008) while harmane trended to decreased maximal capacity (control vs. harmane: 0.945 ± 0.133 vs. 0.202 ± 0.074, p = 0.0507) (Fig. 2.3G and H). Neither harmane nor harmine caused significant differences in spare capacity (data not shown).

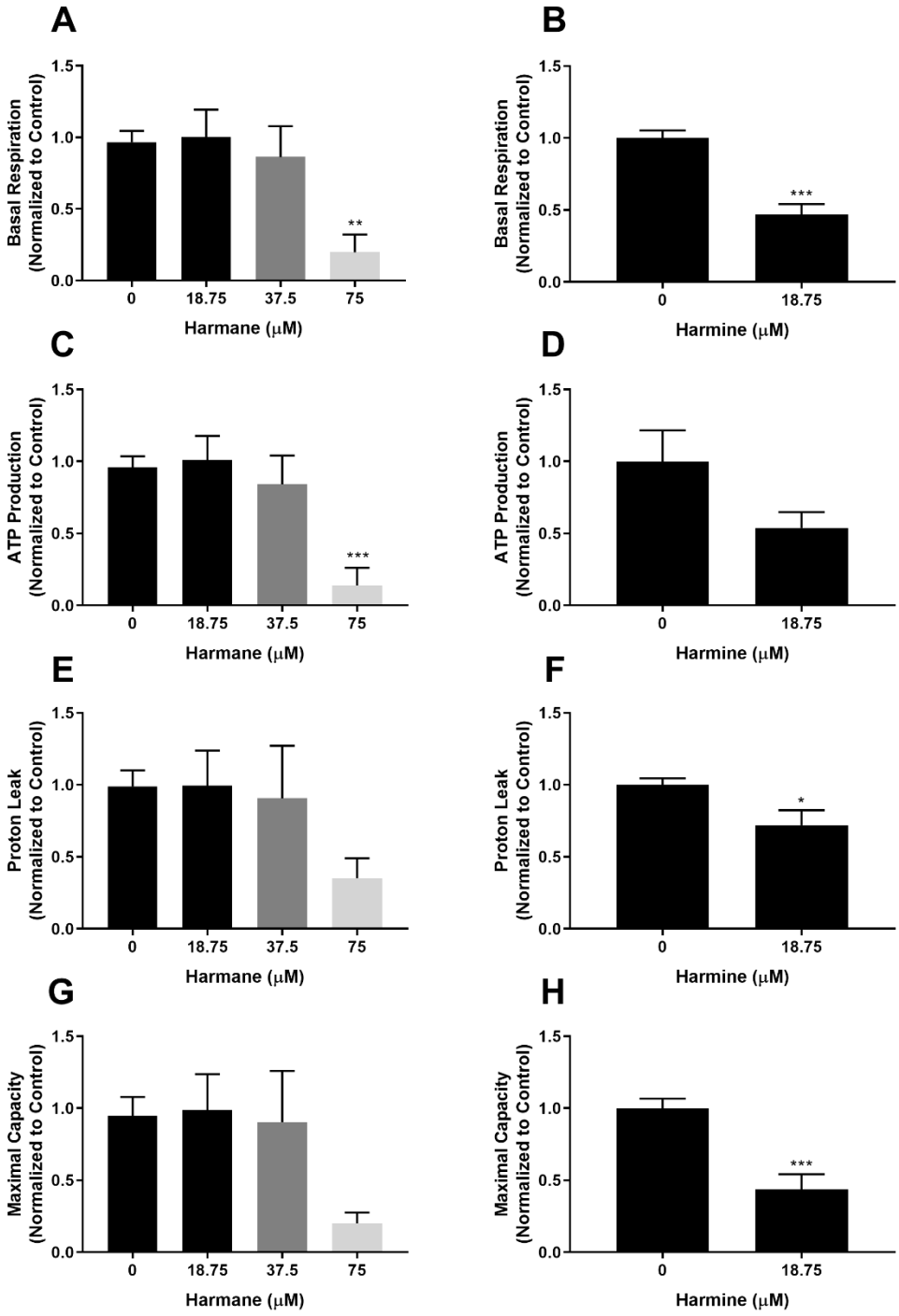

Figure 2.4 Harmane significantly decreased ATP production while harmine significantly decreased proton leak and maximal capacity. Mitochondrial function of galactose SH-SY5Y cells treated with harmane (A, C, E, and G) or harmine (B, D, F, and H) for 24 hours was tested through mitochondrial stress tests. Basal respiration (A and B), ATP production (C and D), proton leak (E and F), and maximal capacity (G and H) were calculated from the mitochondrial stress tests to determine aspects of decreased respiration.

2.3.3 Harmane and harmine acutely increased oxidative stress

Oxidative stress was measured at 18.75 µM, 37.5 µM, 75 µM for both harmane and harmine at 24, 12, 8, 4, and 1 hour after exposure using DCFDA. Oxidative stress was significantly increased at 1 hour for all treatments and decreased over time in a manner where oxidative stress was not significantly increased at any later time points (Fig. 2.5A and B).

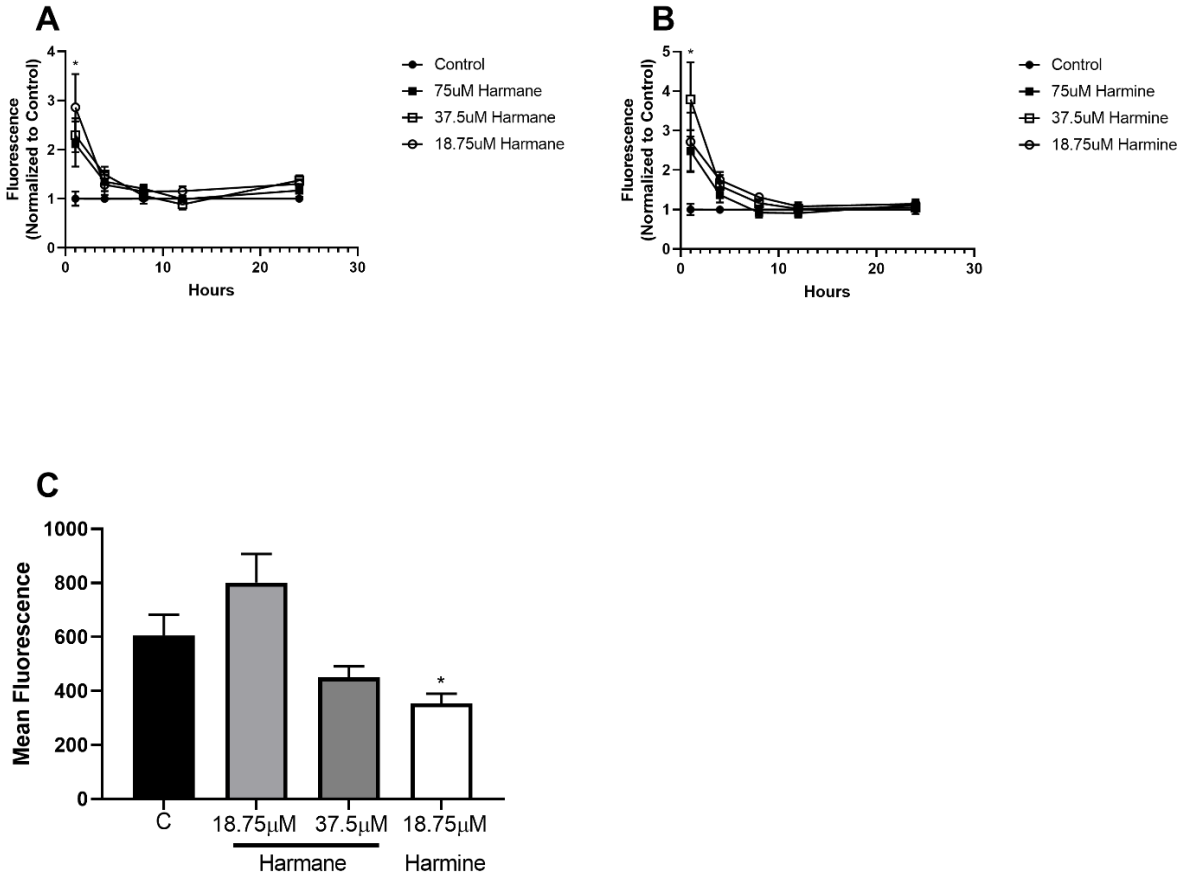

Figure 2.5 Oxidative stress is acutely increased and mitochondrial membrane potential is decreased after 24 h harmane or harmine exposure. Cells were exposed to DCFDA after 1, 4, 8, 12, or 24 hours of exposure to A) harmane or B) harmine to measure oxidative stress. Cells exposed to harmane or harmine for 24 hours were treated with Mitotracker Red CM-H2XROS, imaged, and quantified using ImageJ.

2.3.4 Harmine significantly decreases mitochondrial membrane potential

One potential mechanism of decreased mitochondrial activity is a decrease in viable mitochondria or loss of mitochondrial membrane potential. Therefore, we measured mitochondrial membrane potential and viability through treatment with a Mitotracker dye that enters into the mitochondria through the membrane potential and then is metabolized so that it fluoresces only in viable mitochondria. Cells treated with 75μM harmane were too stressed after 24 hours of treatment and then exposure to Mitotracker, so the majority of cells washed off and the rest were too stressed to quantify mitochondrial fluorescence. After 24 hours of treatment with harmane or harmine, cells treated with 18.75 μM harmine had significantly decreased mitochondrial fluorescence and cells treated with 37.5 μM harmane had decreased fluorescence, although it was not significantly different than controls (control: 605.7 ± 76.4, 37.5μM harmane: 450.7 ± 40.2, 18.75μM harmine: 353.9 ± 35.4, control vs. harmane $p = 0.1530$, control vs. harmine $p = 0.0374$) (Fig. 2.5C). Interestingly, cells treated with 18.75 μM harmane had a slight increase in mitochondrial fluorescence compared to control (control vs. 18.75 μM harmane: 605.7 ± 76.4 vs. 801.0 ± 106.1, $p = 0.1669$).

2.3.5 Pre-treatment with antioxidants did not change mitochondrial respiration

Due to the increased oxidative stress, we tested whether pretreatment with the antioxidants coenzyme Q_{10} (CoQ_{10}) or N-acetylcysteine (NAC) could rescue mitochondrial function. Interestingly, neither CoQ_{10} nor NAC rescued any aspect of mitochondrial respiration (data not shown).

2.3.6 Harmane and harmine don't acutely affect individual mitochondrial complexes after acute exposure to isolated mitochondria

We wanted to determine the mechanism of toxicity leading to decreased mitochondrial function. We therefore tested individual mitochondrial complex activities in isolated mitochondria treated with harmane or harmine. There were no significant differences in activity of complexes I, II, III, IV, or interaction between complexes I and III or II and III after treatment with harmane or harmine.

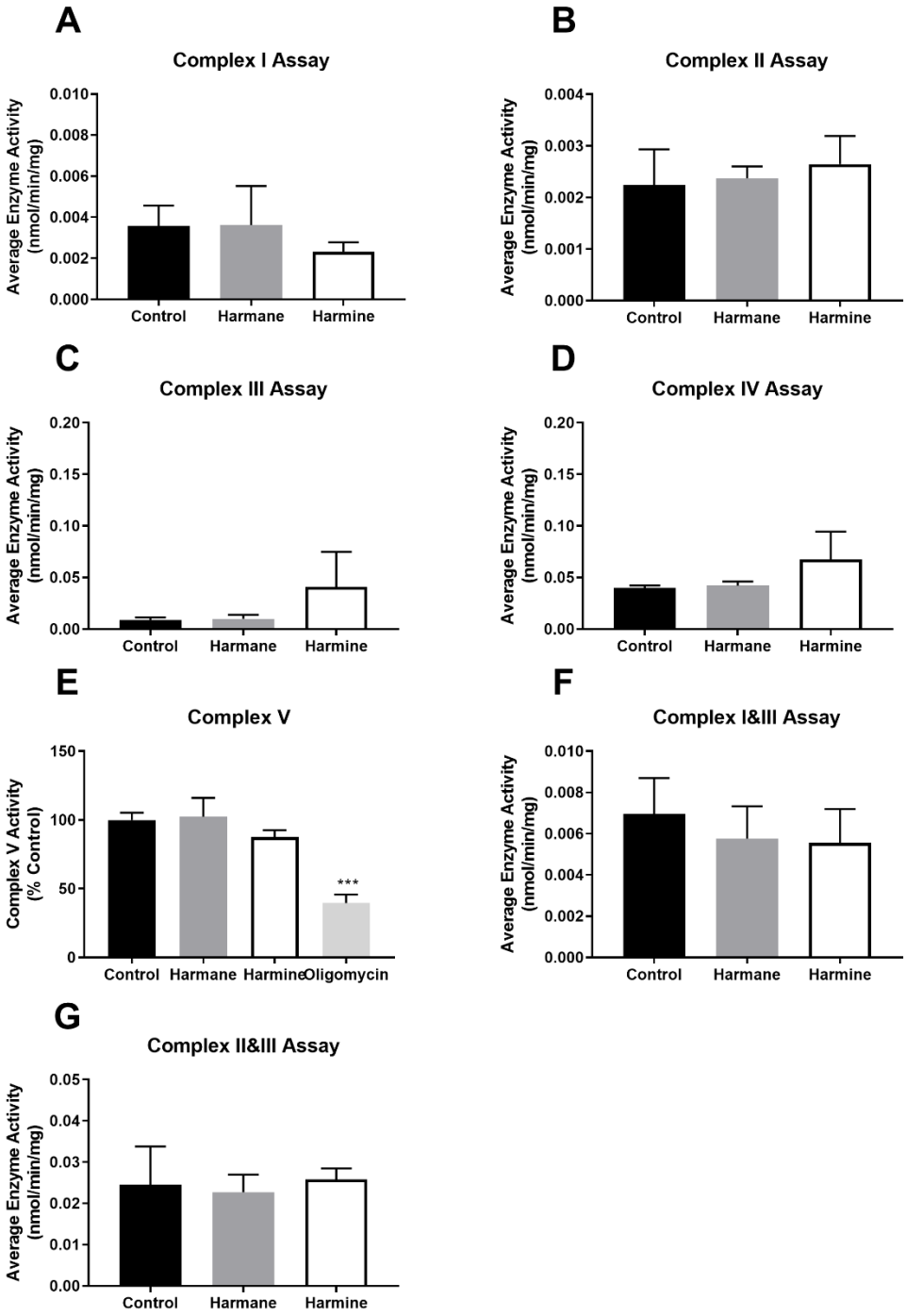

Figure 2.6 Neither harmane nor harmine decrease specific mitochondrial complexes after 1 hour. Isolated rat liver mitochondria were incubated with harmane or harmine prior to quantification of A) complex I, B) complex II, C) complex III, D) complex IV, or the interaction between E) complexes I and III, or F) complexes II and III.

2.3.7 Inhibition of CYP2D or monoamine oxidase A does not rescue mitochondrial function

Because neither harmane nor harmine decreased individual mitochondrial complex assays after approximately 1 hour of treatment, but mitochondrial function is decreased after 24 hours of treatment to cells, we hypothesized that metabolism of harmane and harmine is necessary to cause mitochondrial dysfunction. Because one of the main enzymes that metabolizes harmane and harmine is CYP2D6, we tested whether pretreatment with the CYP2D6 inhibitor, quinidine, could rescue mitochondrial function. We also hypothesized that harmane and harmine could be metabolized through monoamine oxidase (MAO), as it can metabolize the structurally similar MPTP, so we also tested whether pretreatment with the MAO-A inhibitor, clorgyline, could rescue mitochondrial function. Similar to the findings of pretreatment with antioxidants, neither quinidine nor clorgyline increased mitochondrial function compared to harmane or harmine treatment alone (data not shown).

2.4 Discussion

It is known that harmane is increased in blood and CSF of patients with PD (Kuhn et al., 1995; Kuhn et al., 1996; Louis, Michalec, Jiang, Factor-Litvak, & Zheng, 2014). Previous research has also shown that derivatives of harmane and norharmane cause mitochondrial dysfunction (Albores, Neafsey, Drucker, Fields, & Collins, 1990; Neafsey et al., 1995; Neafsey et al., 1989). We showed here that harmane and harmine also decrease mitochondrial respiration in galactose-supplemented SH-SY5Y cells. Interestingly, this appeared to take time, as respiration was decreased after 24 hours of treatment in cells, but no effects were found after 3 hours or after isolated mitochondria were treated for 1 hour. Therefore, we proposed that harmane and harmine must be metabolized to produce the chemical that is toxic to mitochondria. The proposed mechanism of metabolism shown in Figure 2.1 led us to study the effects of inhibiting specific enzymes important for formation of different metabolites (Fig. 2.7). We pre-treated cells with quinidine, a CYP2D6 inhibitor, which would decrease production of hydroxylated metabolites. This pretreatment did not significantly affect mitochondrial function. MPTP has been shown to be metabolized by MAO, so inhibition of MAO activity decreased toxicity (Heikkila et al., 1985). Therefore, we proposed that MAO could metabolize harmane to a toxic metabolite in a similar

manner. Pre-treatment with clorgyline, an inhibition of MAO-A, did not significantly affect mitochondrial function, either.

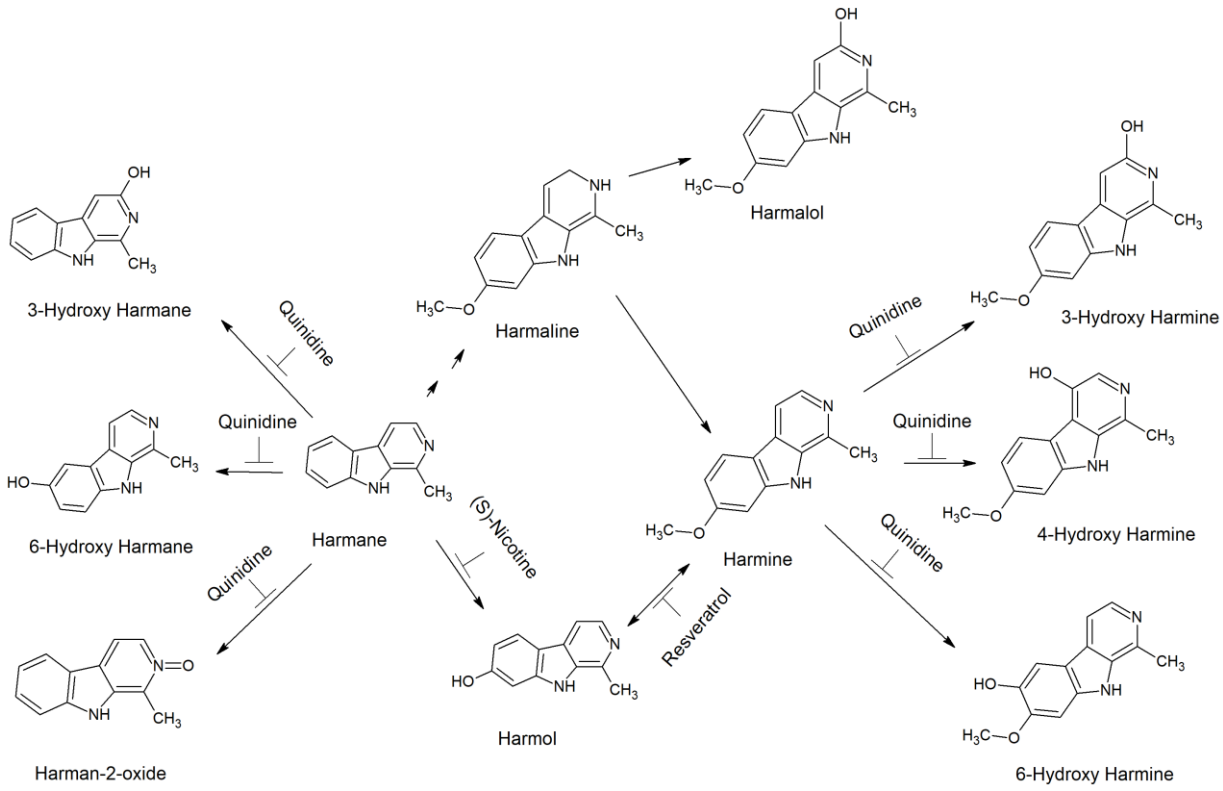

Figure 2.7 Proposed metabolism of harmane and harmine with inhibitors of the known enzymes that produce metabolites**.**

It is imperative that research be performed to more fully elucidate the metabolism of harmane and harmine, and which enzymes are important for metabolism. Enzymes not yet known to metabolize harmane and harmine could be important for the production of toxic metabolites. It is also imperative to determine the effects of inhibition of the other enzymes known to metabolize harmane and harmine, such as CYP2A, which is inhibited by (S)-nicotine. These studies could help determine toxic chemicals that are produced from harmane, which could help find a mechanism that could be targeted for therapeutic use to fight PD.

CHAPTER 3. HARMANE POTENTIALLY AFFECTS DOPAMINE IN SUBSTANTIA NIGRA OF RATS TREATED VIA ORAL GAVAGE

3.1 Rationale

PD is the second most common neurodegenerative disease. It is characterized by motor dysfunction such as a short shuffled gait, postural instability, rigidity, and resting tremors (Hernandez et al., 2016; Lavita et al., 2016; Ross et al., 2004). Unfortunately, less than 10% of cases of PD are known to be caused by genetic mutations, so environmental factors must play a role in disease onset.

One of the chemicals known to cause PD is MPTP, a contaminant that was a byproduct of synthetic heroin that lead to onset of PD for a young group of people who used the tainted batch (Langston, 2017). MPTP is metabolized to ultimately form MPP+, which is taken up by DAergic neurons through the dopamine transporter and inhibits mitochondrial complex I (Heikkila et al., 1985; Heikkila et al., 1984; Ramsay et al., 1986; Singer & Ramsay, 1990). However, exposure to MPTP is not a common occurrence, so structurally similar chemicals found in the diet which could be increasing risk of PD are of interest.

Interestingly, HAAs found in the diet are structurally very similar to MPTP and MPP+. Harmane and norharmane are two of the most abundant HAAs and are found in many different types of food, such as charred or overcooked meat, coffee, tobacco, and certain types of plants (Gibis & Weiss, 2015; Herraiz, 2004). Interestingly, harmane and norharmane are also increased in both blood and CSF of patients with both essential tremor and PD (Kuhn et al., 1995; Kuhn et al., 1996; Pacelli et al., 2015).

There are many derivatives of harmane and norharmane that have been studied for their effects on the brain. Harmine and harmaline were both shown to cause kinetic tremor, spontaneous activity, and sometimes clonic convulsions in rats and rabbits (Nasehi, Piri, Abdollahian, & Zarrindast, 2013). Interestingly, neither harmane nor norharmane caused increased activity like harmine and harmaline, but decreased movement and caused a catatonic-type state (Nasehi et al., 2013). At higher doses, 20 mg/kg intravenous (iv), both harmane and norharmane caused clonic and clonic-tonic seizures, but not tremors (Nasehi et al., 2013). Other research showed that harmane can cause seizures and decreased body temperature with treatment at 3.6 mg/kg causing

half the rats to have seizures and 5 mg/kg causing a significant decrease in body temperature (Adell, Biggs, & Myers, 1996; Rommelspacher et al., 1981).

Harmane and norharmane both have been shown to cause decreased motor function multiple times. 0.5 mg/kg intraperitoneal (ip) injection of harmane or norharmane for 29 days caused decreased activity that lasted 27 days after exposure ended and decreased latency to fall on a rotarod after 14-15 days of exposure, although it did not affect rearing (Goodwin et al., 2015). Harmane exposure also causes decreased reaction time when rats were put on a hot plate, indicating delayed response (Rommelspacher et al., 1981). Rats that had norharmane injected into the SN had biased swings to the ipsilateral side as well as decreased latency to fall on the rotarod after eight weeks, but no significant changes in apomorphine-induced rotation (Esmaeili et al., 2012). This indicates a lesion in the side of the brain injected, which is similar to that seen in 6-OHDA or MPP+ lesions. Subcutaneous injections of norharmane also caused decreased rearing and movement two weeks after treatment ended and increased the motor deficits in mice treated with norharmane and 6-OHDA four weeks after treatment (Ostergren et al., 2006).

There are mixed results when neurotransmitters are studied after harmane and norharmane treatment. After ip exposure to norharmane for 29 days, mice did not have any changes in neurotransmitter levels in the striatum (Goodwin et al., 2015). Injection of 3 µg/h harmane into the hippocampus for 14 days caused increases in serotonin and norepinephrine in the midbrain and striatum of rats (Adell & Myers, 1995). Nigral injection of 2-methyl-norharmane or 2-methyl-harmane both caused decreased DA and DOPAC in the striatum of rats three weeks after injection (Neafsey et al., 1989). Interestingly, norharmane along with many other derivatives of harmane and norharmane caused lesions in the SN after they were injected into the SN of rats, although harmane was not tested in this study, and norharmane produced nigral degeneration after subcutaneous treatment as well (Neafsey et al., 1995; Ostergren et al., 2006).

While much research has been performed on the toxicity of norharmane and derivatives of both norharmane and harmane to show that they have deleterious effects on the motor system, harmane has been studied much less. Furthermore, most studies performed have been injection straight into the brain, which is not very relevant for human exposure, who consume HAAs and, therefore, have first pass metabolism in the liver prior to chemicals entering into the bloodstream. Therefore, we proposed that subchronic oral exposure to harmane would cause decreased motor function along with neurotoxicity affecting the nigrostriatal system.

3.2 Methods

3.2.1 Animals

Animal studies were approved by the Purdue Animal Care and Use Committee. Sprague Dawley rats were housed two per cage and treated starting at 6 weeks of age (4 males and 2 females per treatment) or 9 weeks of age (four females per treatment). Rats were treated with 10 mg/kg harmane dissolved in corn oil or an equivalent volume of corn oil via oral gavage three times per week for sixteen weeks. Prior to the first treatment, baseline behavior was recorded, and behavior tasks were performed weekly during treatment to measure motor function. Behavior consisted of the cylinder test, where the amount of rears was counted, differentiating the rears based on limbs that touched the cylinder (ie. left, right, both, or neither), postural instability, and gait analysis using the DigiGait system, which was performed for the cohort that was treated starting at 6 weeks using the DigiGait Imager 16A5 for recording, and the DigiGait Analysis 15 software for analysis.

After the treatment paradigm, brains were harvested through decapitation and sliced in half in the sagittal plane. Half the brain was fixed in 4% paraformaldehyde in phosphate buffered saline, pH 7.4 while the striatum and SN were collected from the other half using a brain mold from Braintree Scientific and flash frozen for neurotransmitter quantification.

3.2.2 Justification for dose

Humans are exposed to harmane daily, from meat, plants, coffee, tobacco, etc. We wanted to expose animals through oral gavage because this is the most relevant route of exposure, and harmane will undergo first pass metabolism similar to that in humans. Previous studies have determined the bioavailability of harmane to be 19.2-19.41 % in rats (Guan et al., 2001; S. Li et al., 2016). The c_{max} was determined to be 1059.56 ng / mL after oral gavage and 583.19 ng / mL after iv injection (Guan et al., 2001; S. Li et al., 2016). Previous studies have determined that iv exposure to 10 mg/kg harmane caused a clonic state in rats, and 20 mg/kg caused clonic and clonic-tonic seizures (Nasehi et al., 2013). Rats treated with 0.5 mg/kg harmane iv two times per day starting at postnatal day 27 for 29 days did not have convulsions but had decreased motor function and decreased DA in the striatum (Goodwin et al., 2015).

From the above studies, we determined that exposure to 10 mg/kg harmane (equivalent to about 14.25 m/kg intravenously administrated) three times per week would allow for accumulation

of harmane without a high enough exposure to cause concern for hypothermia or convulsions. Because humans are exposed to harmane throughout their lives, we decided to perform a subchronic exposure of 16 weeks unless the rats showed signs of extreme stress or sickness, such as losing 20% of original bodyweight.

3.2.3 Neurotransmitter quantification

Neurotransmitters were quantified through high performance liquid chromatography (HPLC) using an electrochemical detector (ECD) by measuring the area under the curve and comparing areas to a standard curve as previously described (Agim & Cannon, 2018). The mobile phase for measurement of catecholamines was 2 mM 1-octanesulfonic acid, 25 µM ethylenediaminetetraacetic acid, 80 µM sodium phosphate, 200 µM triethylamine, and 10 % ethanol at a pH of 2.4. To quantify GABA and glutamate, the mobile phase was 0.1 M sodium phosphate, 22 % methanol, and 4 % acetonitrile at pH 6.75. Derivatization of samples for GABA and glutamate quantification was performed on line with 0.2 M o-phthalaldehyde (OPA) with 0.05 % 2-mercaptoethanol in OPA diluent.

3.2.4 Immunohistochemistry

Striatal terminal density and oxidative stress in the striatum were measured using immunohistochemistry. Briefly, striatal sections 35 µm thick were selected, blocked in 10 % normal donkey serum (NDS) in PBS with 0.3 % Triton X for one hour, incubated in 1:2000 sheep anti-TH (Pel-Freez) and 1:500 rabbit anti-NT (Millipore) with 1 % NDS and 0.3 % Triton X for 48 hours. Secondary antibodies were Donkey IR800 anti-sheep (Rockland) or Donkey IR800 anti-rabbit (Li-COR) and donkey dylight680 anti-sheep (Novus) in 1 % NDS with 0.3 % Triton X for 2 hours. Tissue was dehydrated in increasing concentrations of ethanol (75 %, 90 %, and 100 %) and Histoclear II (National Diagnostic HS200) and coverslipped with DPX mountant (VWR) before imaging on a Li-COR Odyssey scanner. Regions of interest were drawn around the striatum and mean fluorescent intensity was normalized to control levels.

Striatal terminals were stained using 3,3’-diaminobenzidine (DAB) staining for TH. Briefly, striatal sections 35 µm thick were selected, washed in a 3 % hydrogen peroxide solution to quench peroxidases before blocking in 10 % NDS and 0.3 % Triton X for one hour. Tissue was

then incubated in 1:2000 mouse anti-TH (Millipore) for 48 hours and then biotinylated donkey anti-mouse secondary antibody (1:200, Jackson Immuno) for ninety minutes. Tissues were put on slides and dried overnight before being dehydrated in increasing concentrations of ethanol (70 %, 95 %, and 100 %) and then Histoclear II (National Diagnostics) and coverslipped with DPX mountant (VWR). Tissue was imaged on an Olypmus DP72 light microscope with a Prior H31XYZE-US stage and Hamamatsu C10600 camera controller with the CellSens 2 software.

3.2.5 Statistical analysis

Weight and motor tests were analyzed using a two-way ANOVA with Sidak's post hoc test. Neurotransmitters, striatal terminal density, and striatal nitrotyrosine were analyzed through a student's t-test.

3.3 Results

3.3.1 Harmane did not cause significant changes in weight or motor function

Harmane exposure did not significantly affect weight for either cohort tested (Fig. 3.1A-D). Rearing and postural instability are common behavioral tests to determine effects on motor function. If an animal has decreased motor function, this will show as decreased number of rears in five minutes, and if it has decreased ability to determine stability and center of balance, its body will be displaced further before it realizes it is off balance and takes a catch step to get back on balance. Neither rearing nor postural instability were significantly changed during exposure to harmane for either the young cohort (starting at 6 weeks of age) or the old cohort (starting at 9 weeks of age) (Fig. 1A-D). Stride length was also measured in the rats treated with harmane starting at 6 weeks of age. A decrease in stride length would indicate PD-like symptoms, but there was no significant difference in stride length between rats treated with harmane and the control rats (Fig. 3.2E).

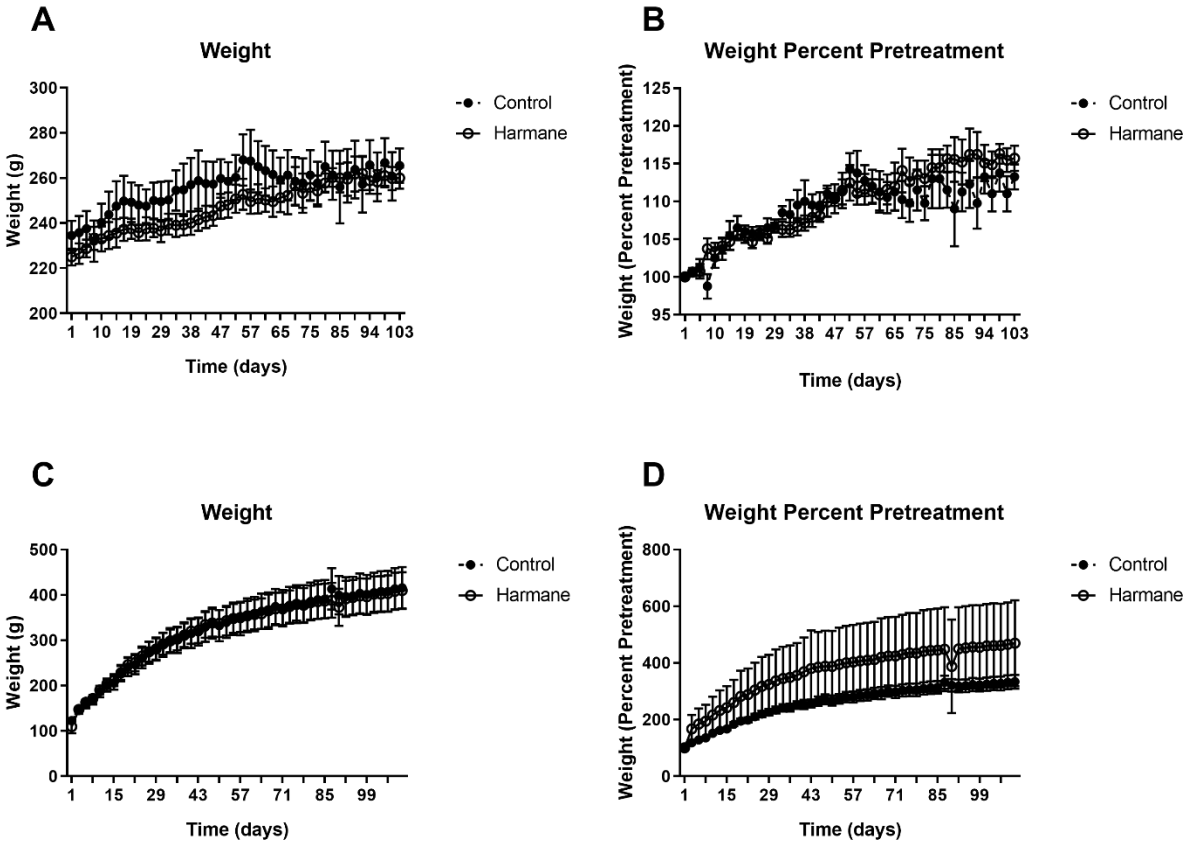

Figure 3.1 Rats exposed to harmane via oral gavage for 16 weeks did not have significant changes in weight. Rats treated with harmane starting at 9 weeks (A and B) or 6 weeks (C and D) were weighed three times per week and weights were compared between treatments (A and C) and change in weight from pretreatment (B and D) was calculated.

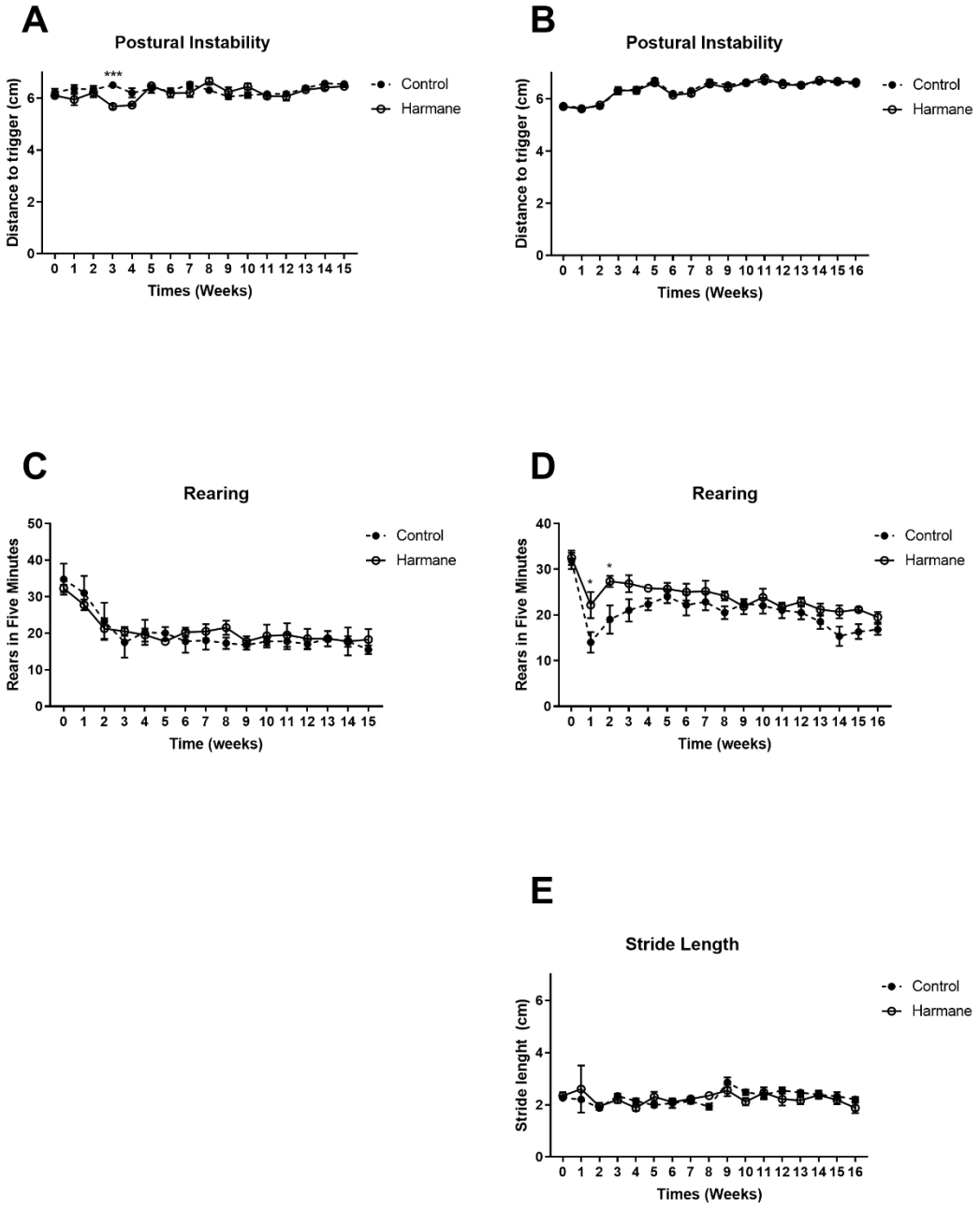

Figure 3.2 Rats exposed to harmane via oral gavage for 16 weeks did not have significant changes in motor function. Rats treated with harmane starting at 9 weeks (A and C) or 6 weeks (B, D, and E) performed the postural instability test (A and B), rearing test (C and D), and gait analysis (E) (only for the rats exposed from 6 weeks of age) weekly to determine changes in motor function.

3.3.2 Harmane significantly increased dopamine in the substantia nigra of older rats

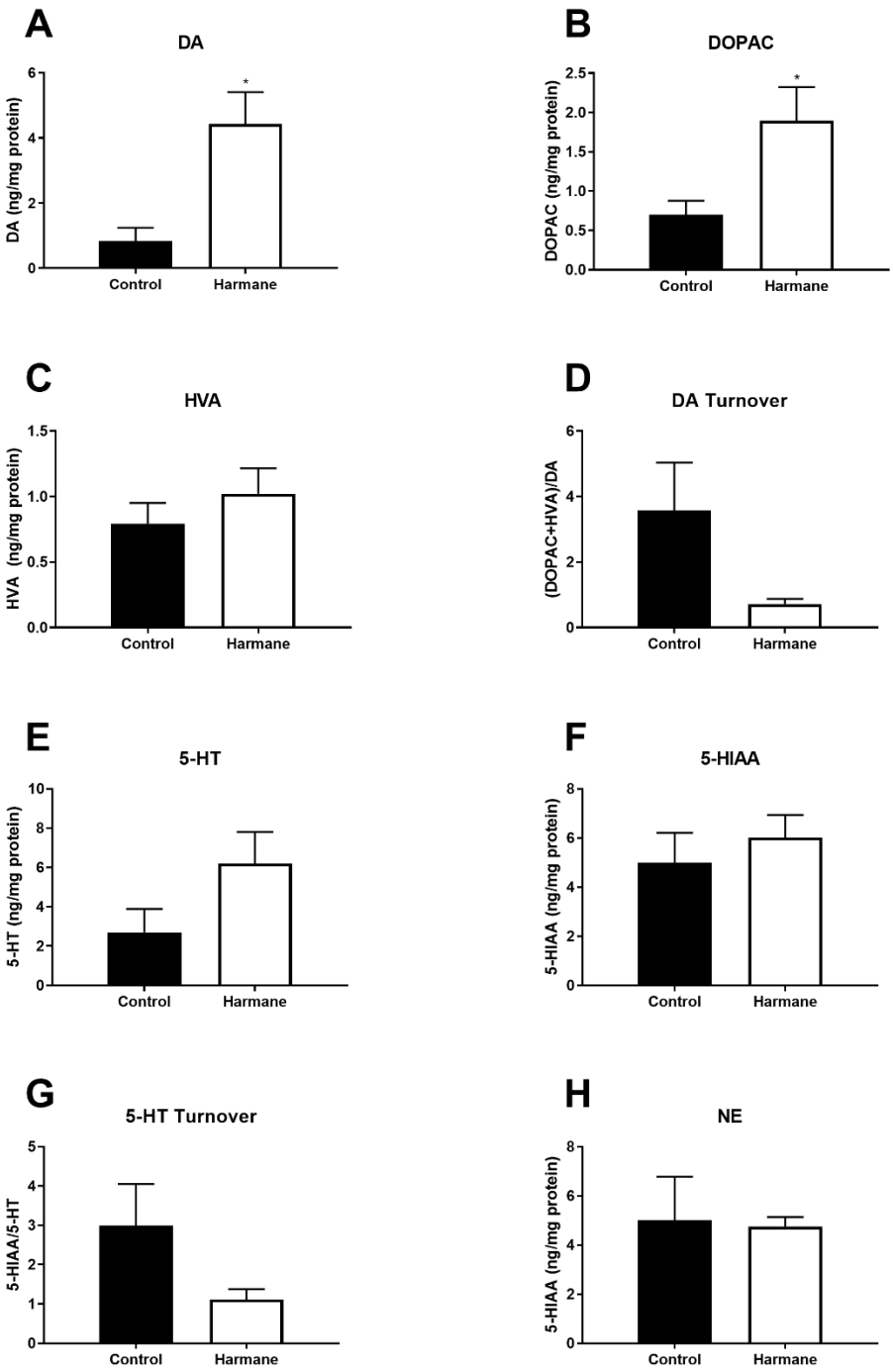

Figure 3.3 DA and DOPAC were significantly increased in the SN of rats treated with harmane starting at 9 weeks of age. A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) 5-HT turnover, and H) NE were measured via HPLC in the SN of rats treated with harmane for 16 weeks.

Animals treated with harmane starting at nine weeks of age had significantly increased levels of DA and DOPAC in the SN, with controls having 0.8369 ± 0.4064 ng/mg protein and harmane-treated animals having 4.426 ± 0.981 ng/mg protein for DA ($p = 0.0148$) and DOPAC being 0.6992 ng/mg protein for controls and 1.896 ng/mg protein for harmane-treated ($p = 0.0416$) (Fig. 3.3A-B). No other neurotransmitters (HVA, 5-HT, 5-HIAA, NE, glutamate, or GABA) were significantly different in the SN, and no neurotransmitters were affected in the striatum (Fig. 3.3-3.6).

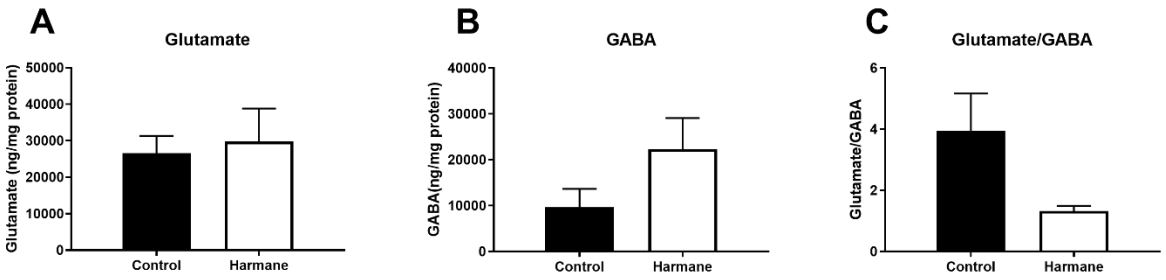

Figure 3.4 Glutamate and GABA levels were not changed in the SN of rats treated with harmane starting at 9 weeks of age. A) Glutamate and B) GABA were measured via HPLC in the SN of rats treated with harmane. C) The ratio of glutamate to GABA was calculated.

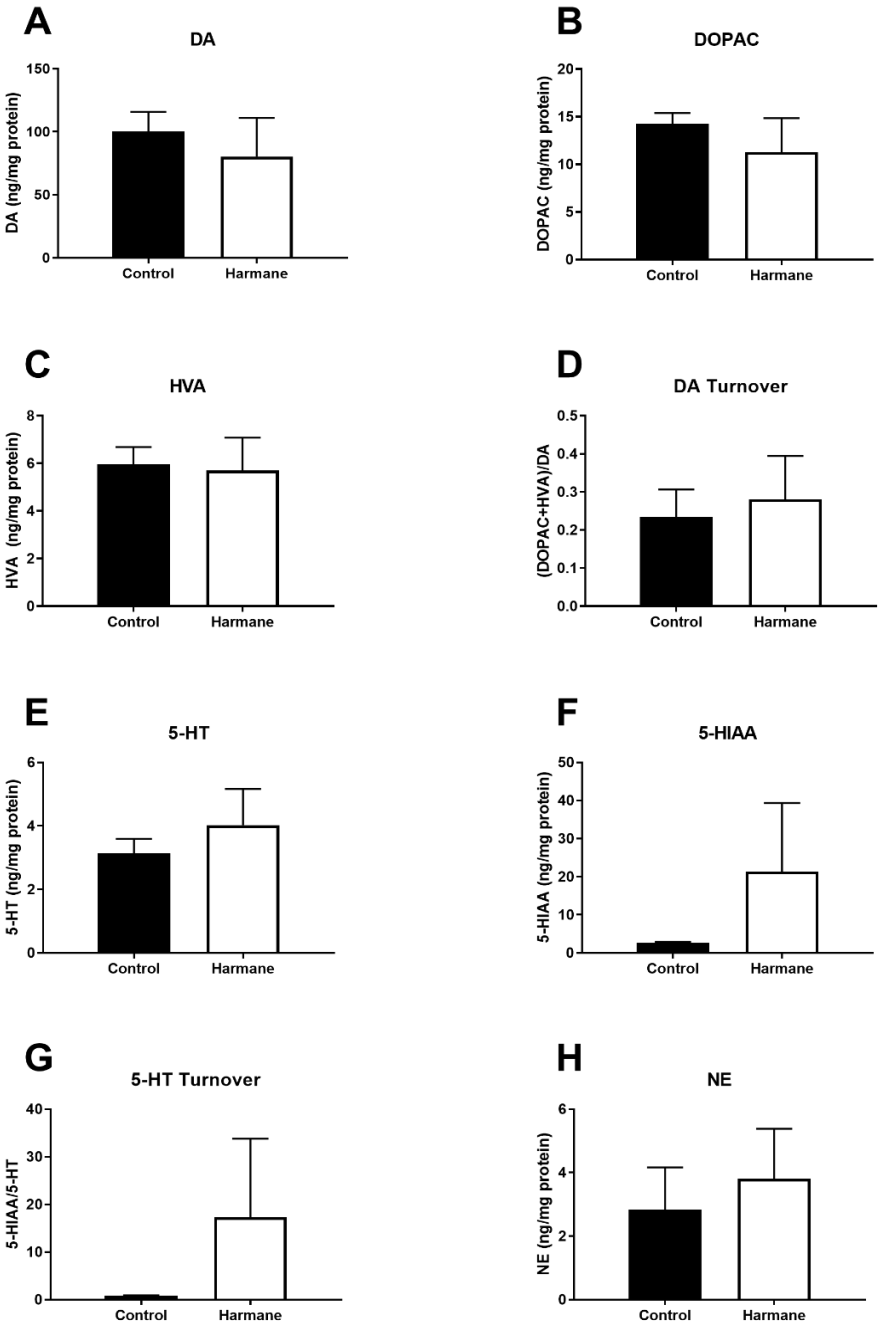

Figure 3.5 No neurotransmitter levels were changed in the striatum of rats treated with harmane starting at 9 weeks of age. A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) 5-HT turnover, and H) NE were measured via HPLC in the striatum of rats treated with harmane starting at 9 weeks of age.

Interestingly, the animals treated with harmane starting at six weeks of age did not have any significant changes in any neurotransmitter levels in the SN or the striatum (Fig. 3.7-3.10).

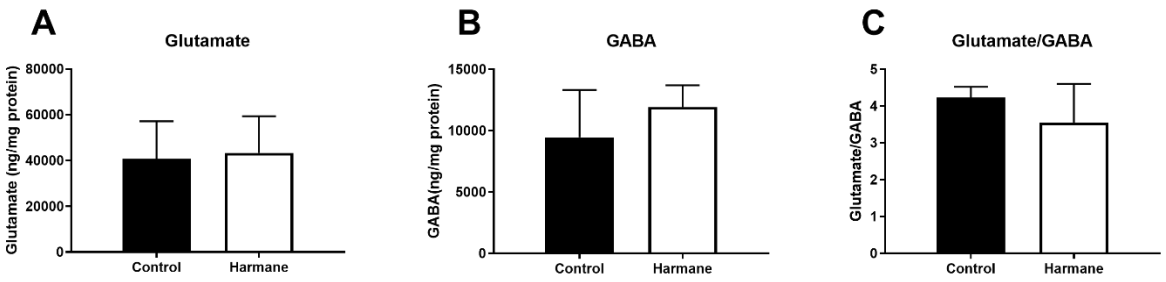

Figure 3.6 Glutamate and GABA were not changed in the striatum of rats treated with harmane starting at 9 weeks of age. A) Glutamate and B) GABA were measured via HPLC in the striatum of rats treated with harmane. C) The ratio of glutamate to GABA was calculated.

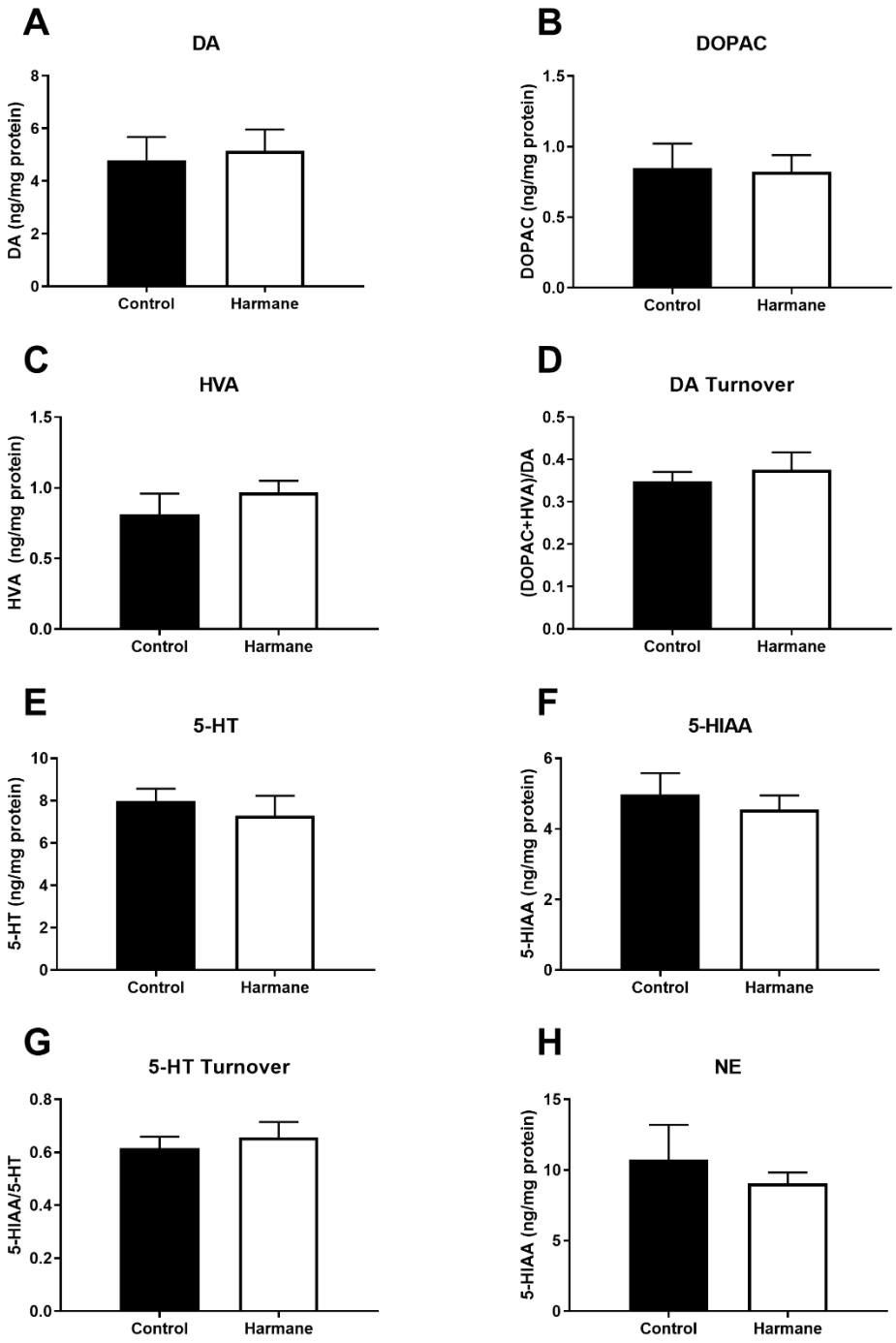

Figure 3.7 No neurotransmitter levels were changed in the SN of rats treated with harmane starting at 6 weeks of age. A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) serotonin turnover, and H) norepinephrine were measured in the SN of rats treated with harmane via HPLC.

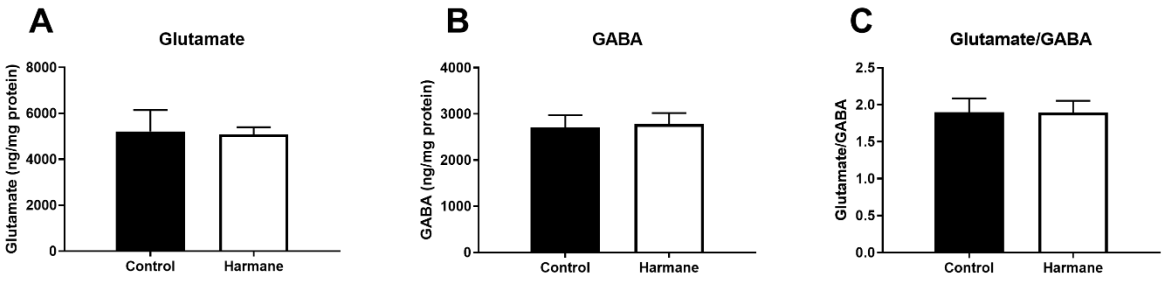

Figure 3.8 Glutamate and GABA levels were not changed in the SN of rats treated with harmane starting at 6 weeks of age. A) Glutamate and B) GABA were measured in the SN of rats treated with harmane via HPLC. The C) ratio of glutamate to GABA was calculated.

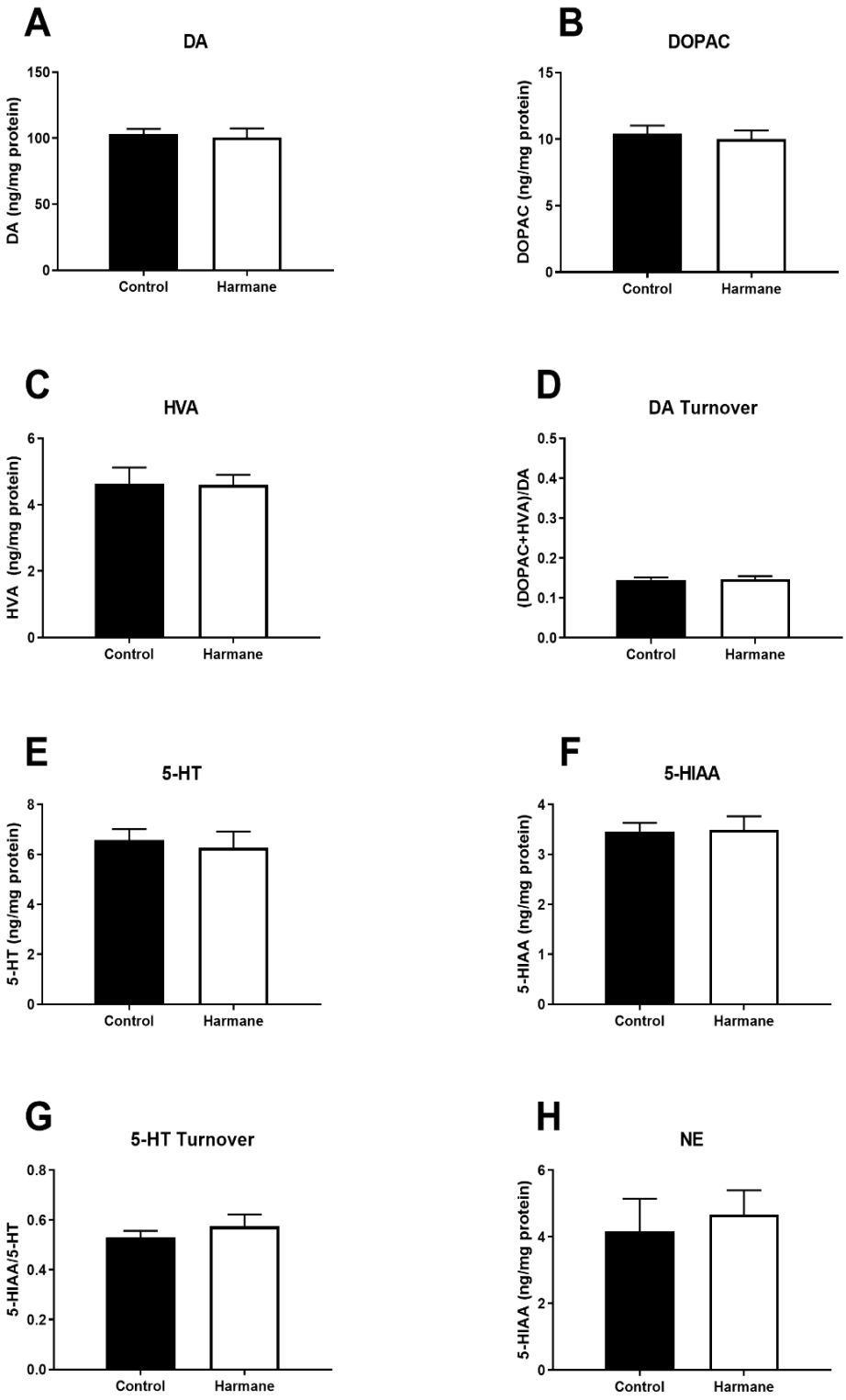

Figure 3.9 There were no significant effects on neurotransmitters in the striatum of rats treated with harmane starting at 6 weeks of **age.** A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) serotonin turnover, and H) norepinephrine were measured via HPLC in the striatum of rats treated with harmane.

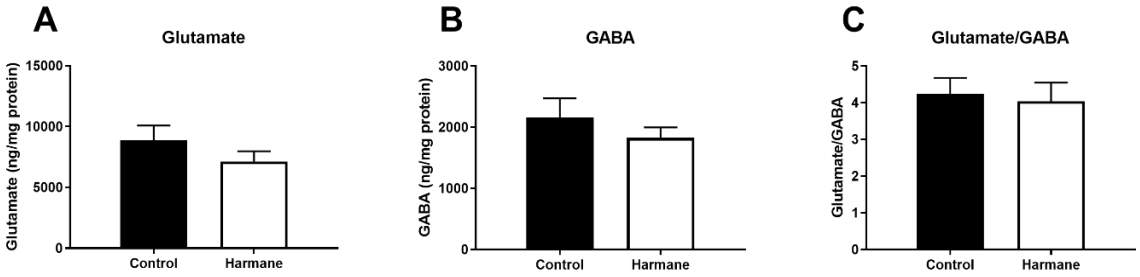

Figure 3.10 Glutamate and GABA levels were not changed in the striatum of rats treated with harmane starting at 6 weeks of age. A) Glutamate and B) GABA were measured in the striatum of rats treated with harmane via HPLC. The C) ratio of glutamate to GABA was calculated.

3.3.3 Harmane decreased striatal terminal density in rats starting at 9 weeks of age

Rats treated with harmane starting at 9 weeks of age also had significantly decreased levels of tyrosine hydroxylase in the striatum (195.2 ± 10.20 and 164.1 ± 10.29 for control and harmane treated, respectively, $p = 0.0396$) (Fig. 3.11A). Interestingly, this decrease was not present in rats exposed starting at 6 weeks of age (Fig. 3.11C). There was no significant change in nitrosylated tyrosine residues in either cohort, which is an indicator of oxidative stress (Fig. 3.11B and D). DAB was performed on striatum to corroborate the increased TH levels in rats treated with harmane starting at 9 weeks of age (Fig. 3.12).

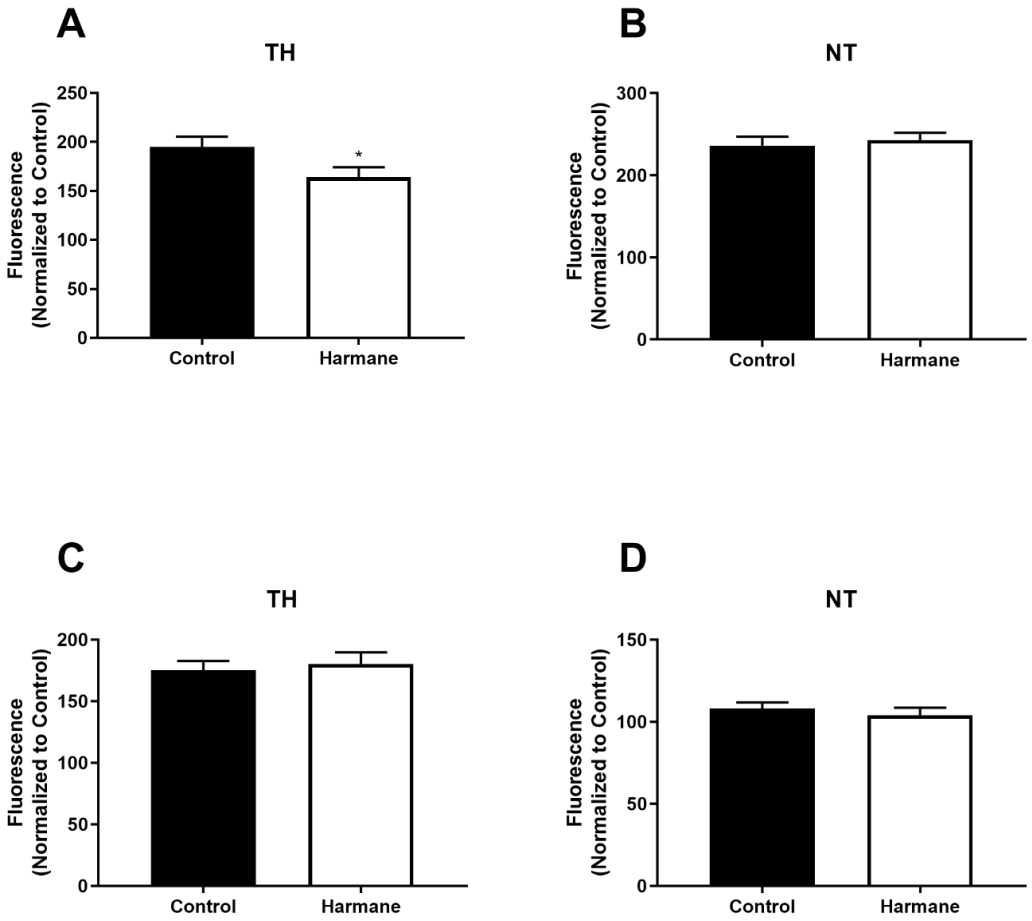

Figure 3.11 Striatal terminal density was significantly decreased in rats treated with harmane starting at 9 weeks of age, but not starting at 6 weeks. Striatum of rats treated with harmane starting at 9 weeks (A and B) or 6 weeks (C and D) of age were stained for TH (A and C) or NT (B and D) and regions of interest were drawn around the dorsal striatum and the mean fluorescence was used to determine the striatal terminal density and the striatal oxidative stress.

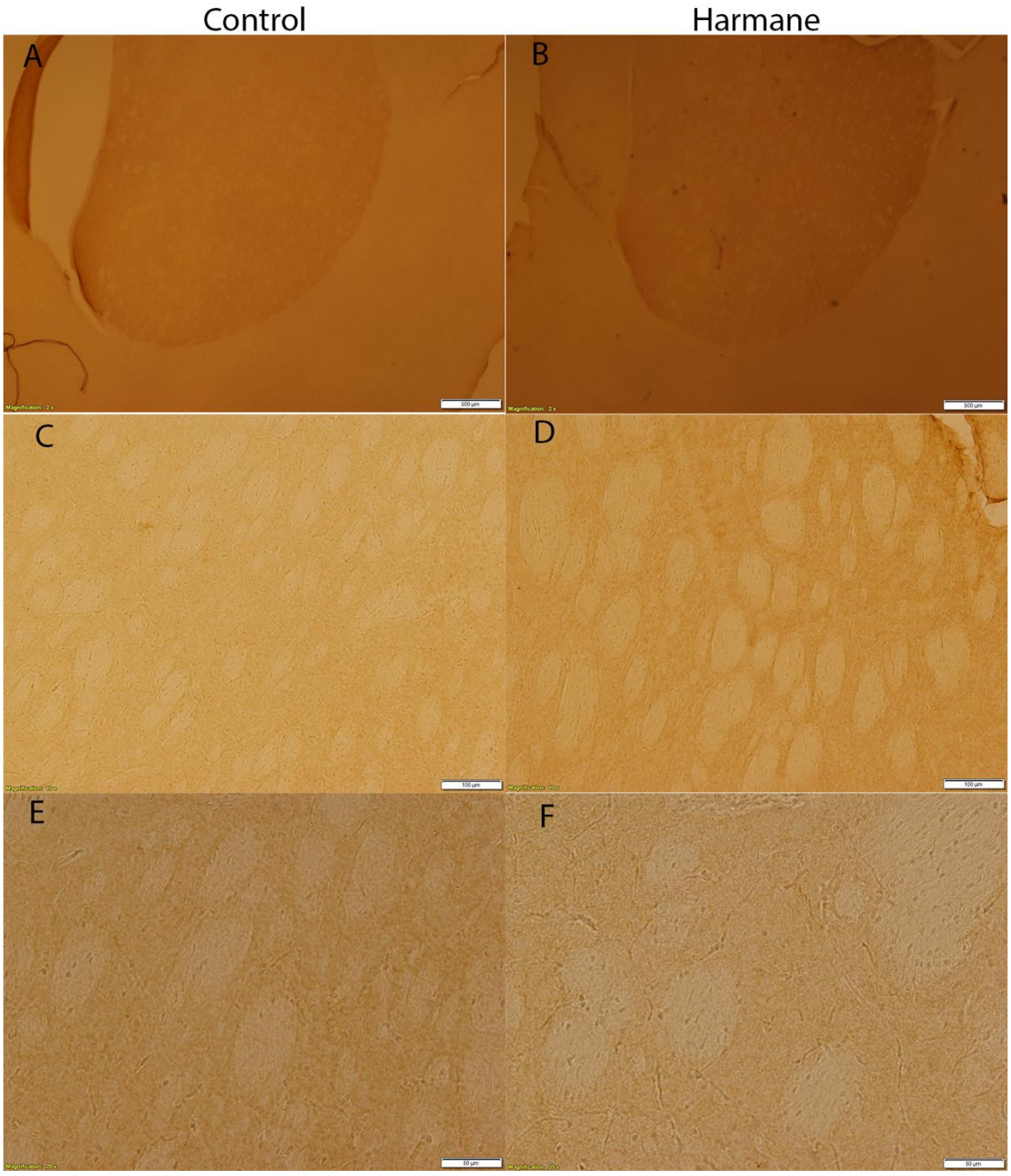

Figure 3.12 Striatal DAergic Terminals Aren't Changed. DA striatal terminals were stained with DAB for TH in control (A, C, E) and harmane treated (B, D, F) at 2x (A and B), 10x (C and D), and 20x (E and F).

3.4 Discussion

Few studies have been performed to determine effects of harmane on motor function and the nigrostriatal system. This research is the first of its kind to determine that harmane does not significantly affect motor function after subchronic oral exposure. Previous research showed that injection of norharmane into the SN caused decreased motor function; however, there was no histology to determine if the injection was specifically targeting DAergic neurons or just caused a loss of total neurons in the region (Esmaeili et al., 2012).

Our studies found that there were no significant changes in neurotransmitter levels in the striatum of rats treated with harmane starting at either 6 or 9 weeks. However, the rats treated with harmane starting at 9 weeks had significantly more DA and DOPAC in the SN and significantly decreased striatal terminal density, which was not seen in the rats that were treated starting at 6 weeks of age. Harmane is known to be a reversible MAO-A inhibitor (Herraiz, 2007; Herraiz & Chaparro, 2006). MAOs are important for the metabolism of DA to DOPAC and HVA, so the inhibition of MAO by harmane would cause an increased accumulation of DA, as found in the SN of rats treated starting at an older age. Interestingly, this does not explain the increase in DOPAC, which is a metabolite of DA through MAO. However, MAO-B, which is only inhibited by harmane at higher concentrations, could be metabolizing DA to DOPAC in a compensatory manner. Furthermore, MAO is necessary to form HVA through main metabolic pathway for DA, so it is plausible that MAO-A inhibition is decreasing metabolism to HVA compared to the amount of DA present, leading to no significant differences compared to control animals.

One of the interesting findings of this research is that the increase in DA and DOPAC was only seen in the rats that were treated when they were older. This difference could be a sex difference, as the first study was performed with only females while the second cohort included both males and females. It is also possible that these findings are an artifact due to the small number of animals used in each cohort. These studies were used as preliminary trials to determine if harmane could cause effects on the nigrostriatal system.

Overall, we have shown that subchronic oral exposure to harmane does not affect motor function. We also showed that dopamine metabolism might be affected. However, the small number of animals and variety in sex distribution among cohorts could be decreasing the power of analyses. Further research should be performed to determine whether harmane is affecting DA metabolism and accumulation and if this is sex- dependent. There was no obvious lesion that

occurred after exposure, but histology could indicate whether exposure is increasing oxidative stress in DAergic neurons in the SN, accumulation of protein aggregates, or dysfunction of mitochondria.

CHAPTER 4. ACUTE AND SUBCHRONIC PHIP EXPOSURE AFFECTS DOPAMINE LEVELS IN THE STRIATUM OF MICE AFTER ORAL EXPOSURE

4.1 Rationale

PD is a prominent neurodegenerative disease that affects over 10 million people worldwide and is predicted to increase due to increased life expectancy. Less than 10 % of PD cases are known to be caused by genetic mutations, so research has started to find environmental exposures that could cause or increase risk of PD. Some of the compounds known to cause PD include MPTP, a chemical contaminant of synthetic heroin, and rotenone, a pesticide (Langston, 2017). MPTP is metabolized to MPP+ and taken up through the dopamine transporter before inhibiting mitochondrial complex I activity (Heikkila et al., 1985; Heikkila et al., 1984; Ramsay et al., 1986; Singer & Ramsay, 1990). However, there is not a large risk of exposure to MPTP, so chemicals that are structurally similar that are present in our diet are important to study for potential neurotoxicity.

Heterocyclic aromatic amines (HAAs) are compounds formed through a Malliard reaction between amino acids and sugar (Skog et al., 1998). They are present in many types of food, such as meat, coffee, and tobacco. One of the most abundant HAAs in cooked meat is 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), measuring up to 304.71 µg/g chicken (Gibis & Weiss, 2015; Ni et al., 2008). HAAs, including PhIP, are structurally very similar to MPTP and MPP+, so it is hypothesized that they could be causing similar DAergic toxicity through chronic low levels of exposure throughout a lifetime.

In support of this hypothesis, PhIP has been shown to cause specific DAergic toxicity at 1 µM in a rat primary midbrain culture (Griggs et al., 2014). Furthermore, studies in rats found that exposure to PhIP at 200 mg/kg bodyweight caused significant decreases in the DA metabolites DOPAC and HVA and that both 100 mg/kg and 200 mg/kg PhIP significantly decreased DA turnover after 8 hours (Agim & Cannon, 2018). Because of these findings, we hypothesized that exposure to PhIP would affect the nigrostriatal system, leading to PD-like behavior and pathology in mice. Because exposure is over a lifetime and chemicals cause different toxic profiles over time, we wanted to determine what happened over time, so exposures included an 8 hour exposure for

acute effects, a 4 week exposure for subacute effects, and a 16 week exposure for subchronic effects.

4.2 Methods

4.2.1 Animals

Animal studies were approved by the Purdue Animal Care and Use Committee. C57BL/6 mice were group housed and treated starting at 6 to 8 weeks of age for acute, subacute, or subchronic exposures. Mice were treated with 100 or 200 mg/kg PhIP for acute exposure (approximately eight hours) and 75 mg/kg PhIP for subacute (4 weeks, 3 times per week) and subchronic (16 weeks, 3 times per week) exposure via oral gavage. Prior to the first treatment for subacute and subchronic exposures, baseline behavior was recorded, and behavior tasks were performed weekly during treatment to determine effects on motor function. Behavior consisted of the cylinder test, where the amount of rears was counted, differentiating the rears based on limbs that touched the cylinder (ie. left, right, both, or neither), and the pole test for the subchronic exposure. For the pole test, mice that fell during the experimental run were considered to take the maximal amount of time allotted for one descent, 60 seconds.

After the treatment paradigm, brains were harvested through decapitation and sliced in half in the sagittal plane. Half the brain was fixed in 4% paraformaldehyde in phosphate buffered saline, pH 7.4 while the striatum and SN were collected from the other half using a brain mold from BrainTree Scientific and flash frozen for neurotransmitter quantification.

4.2.2 Neurotransmitter quantification

Neurotransmitters were quantified through HPLC using an ECD through measuring the area under the curve and comparing areas to a standard curve. The mobile phase for measurement of catecholamines was 2 mM 1-octanesulfonic acid, 25 µM ethylenediaminetetraacetic acid, 80 µM sodium phosphate, 200 µM triethylamine, and 10 % ethanol at a pH of 2.4. To quantify GABA and glutamate, the mobile phase was 0.1 M sodium phosphate, 22% methanol, and 4% acetonitrile at pH 6.75. Derivatization of samples for GABA and glutamate quantification was performed on line with 0.2 M OPA with 0.05 % 2-mercaptoethanol in OPA diluent.

4.2.3 Immunohistochemistry

Striatal terminal density and oxidative stress in the striatum were measured using immunohistochemistry. Briefly, striatal sections 35 µm thick were selected, blocked in 10 % NDS in PBS for one hour, incubated in 1:2000 sheep anti-TH (Pel-Freez) and 1:500 rabbit anti-NT (Millipore) for 48 hours. Secondary antibodies were Donkey IR800 anti-sheep (Rockland) or Donkey IR800 anti-rabbit (Li-COR) and donkey dylight680 anti-sheep (Novus) for 2 hours. Tissue was dehydrated in increasing concentrations of ethanol (75 %, 90 %, and 100 %) and Histoclear II (National Diagnostic HS200) and coverslipped with DPX mountant (VWR) before imaging on a Li-COR Odyssey scanner. Regions of interest were drawn around the striatum and mean fluorescence intensity was normalized to control levels.

4.2.4 Statistical analysis

Weight and motor tests were analyzed using a two-way ANOVA with Sidak's post hoc test. Neurotransmitter levels for the 8 hour exposure group were normalized to controls for each cohort prior to further analysis. Neurotransmitters, striatal terminal density, and striatal NT were analyzed through one-way ANOVA with Sidak's post hoc test for the 8 hour exposure group and through student's t-test for the subacute and subchronic exposures.

4.3 4.3 Results

4.3.1 PhIP caused significant motor dysfunction starting at eleven weeks of exposure.

Exposure to PhIP caused significantly decreased weight compared to controls starting at 15 days for the subacute exposure and starting at 31 days for the subchronic exposure cohort (Fig. 4.1).

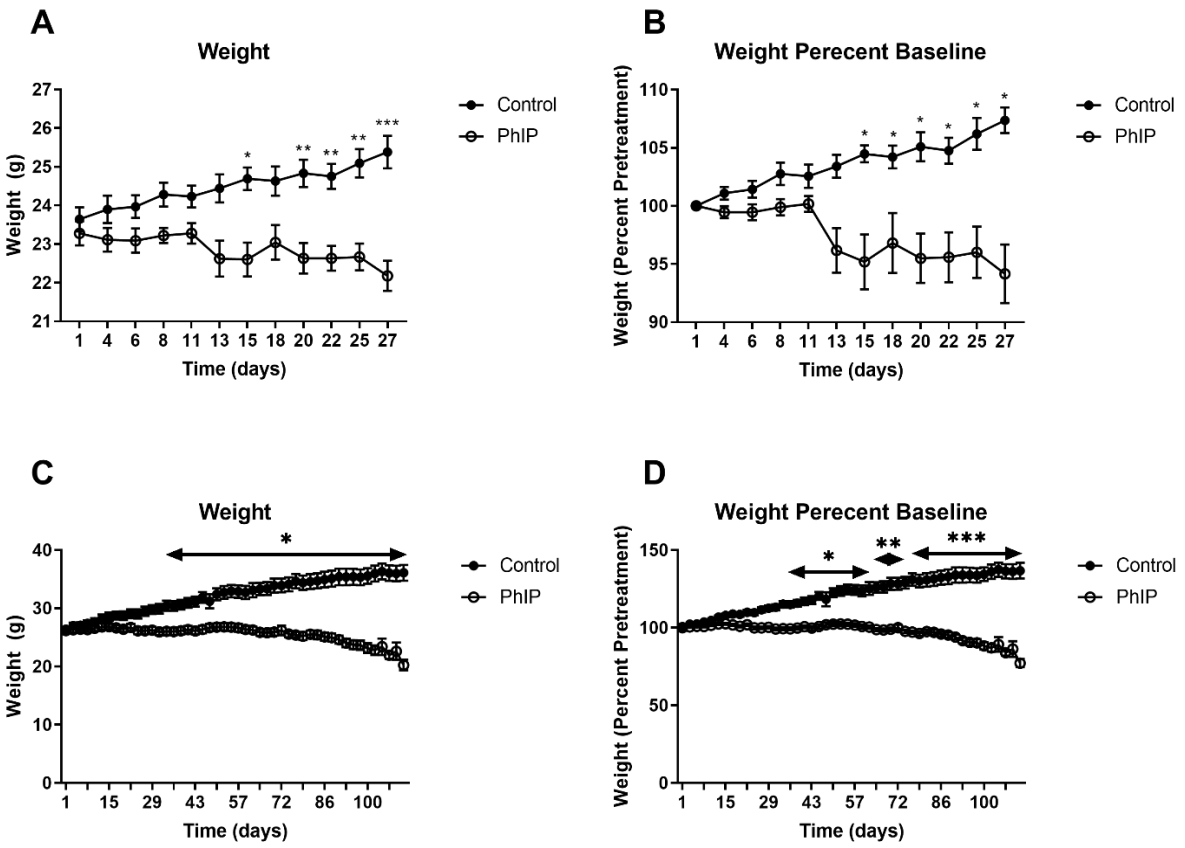

Figure 4.1 Mice exposed to PhIP via oral gavage for 4 or 16 weeks had significant decreases in weight. Mice treated with PhIP for 4 weeks (A and B) or 16 weeks (C and D) were weighed three times per week and weights were compared between treatments (A and C) and change in weight from pretreatment (B and D) was calculated.

Motor function was tested weekly during the exposures by testing rearing, with a decrease in number of rears indicating decreased motor function, and the pole test, where animals having decreased motor function will take more time to descend. Subacute exposure did not cause significant changes in motor function, as measured by the rearing test. Starting at 11 weeks, the mice treated with PhIP had significantly decreased total number of rears (15.8 ± 2.0 rears and 5.10 ± 0.87 rears for control and PhIP-treated, respectively) and starting at 13 weeks had significantly increased time to descend the pole (6.920 ± 1.073 seconds and 25.58 ± 4.39 seconds for control and PhIP-treated, respectively), indicating decreased motor function (Fig. 4.2).

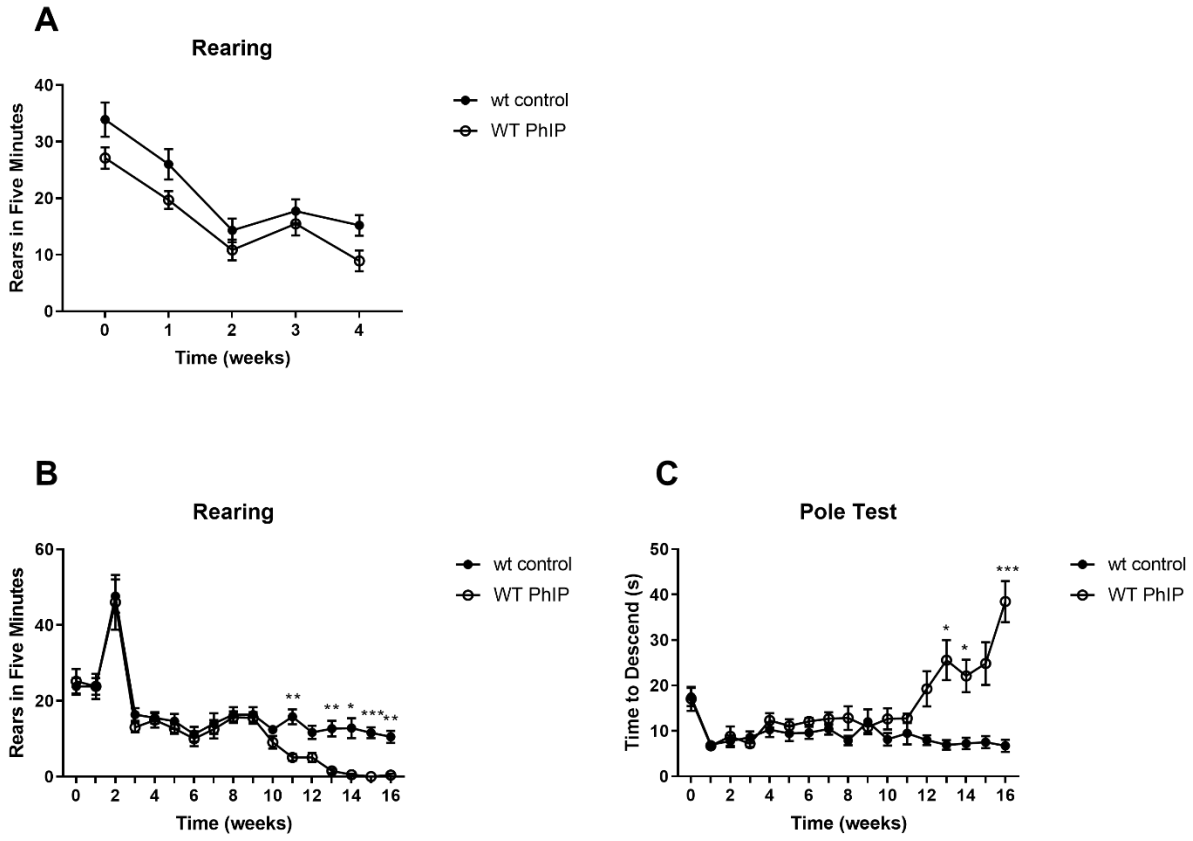

Figure 4.2 Mice exposed to PhIP via oral gavage had significantly decreased motor function starting at 11 weeks. Mice treated with PhIP for 4 weeks (A) or 16 weeks (C and D) performed the rearing test (A and B) or the pole test (C) weekly to determine changes in motor function.

4.3.2 PhIP significantly decreases dopamine and its metabolites in the striatum after acute exposure, but significantly increases dopamine after subchronic exposure.

Neurotransmitters were quantified in the striatum of mice treated with PhIP for acute, subacute, and chronic exposures (Fig. 4.3-4.8). DA and its two main metabolites DOPAC and HVA were significantly decreased after eight hours of exposure to PhIP (Table 4.1, Fig. 4.3A-C), which was not see in the subacute treatment group (Fig. 4.5A-C). Interestingly, mice treated with PhIP for 16 weeks had significantly higher DA levels, with no changes in DOPAC or HVA (Table 4.1, Fig. 4.7A-C). Serotonin and its metabolite, glutamate, and norepinephrine were not affected in the striatum due to exposure at any time point (Fig. 4.3-4.8).

Interestingly, mice exposed to PhIP for 4 weeks had a significant increase in the ratio of glutamate to GABA (3.555 ± 0.346 ng/mg protein for control and 5.037 ± 0.501 ng/mg protein for

PhIP treated, p = 0.0255) (Figure 4.6C). Glutamate is a precursor of GABA, so this could indicate that GABA is not being produced by the metabolism of glutamate to the same extent as in control animals. Interestingly, this change was not present in either the acute or subchronic exposure groups (Fig. 4.4 and 4.4.8).

Table 4.1 DA, DOPAC, and HVA levels in striatum of mice treated with PhIP. Levels were normalized to control for the 8 hour exposure. Levels are reported as mean ± SEM.

	DA (ng / mg protein)	**DOPAC (ng / mg protein)**	**HVA (ng / mg protein)**
Control 8 h	100.00 ± 12.01	100.00 ± 14.35	100.00 ± 11.85
100 mg / kg PhIP 8 h	62.97 ± 7.710	65.35 ± 8.483	60.44 ± 6.606
200 mg / kg PhIP 8 h	58.29 ± 6.755	60.79 ± 7.751	61.94 ± 5.904
Control 4 wks	80.76 ± 16.85	7.124 ± 0.9789	10.21 ± 1.904
75 mg / kg PhIP 4 wks	75.50 ± 18.37	5.750 ± 1.152	7.141 ± 1.237
Control 16 wks	133.2 ± 13.86	12.26 ± 2.224	9.973 ± 1.154
75 mg / kg PhIP 16 wks	199.3 ± 15.65	13.62 ± 1.286	12.09 ± 1.092

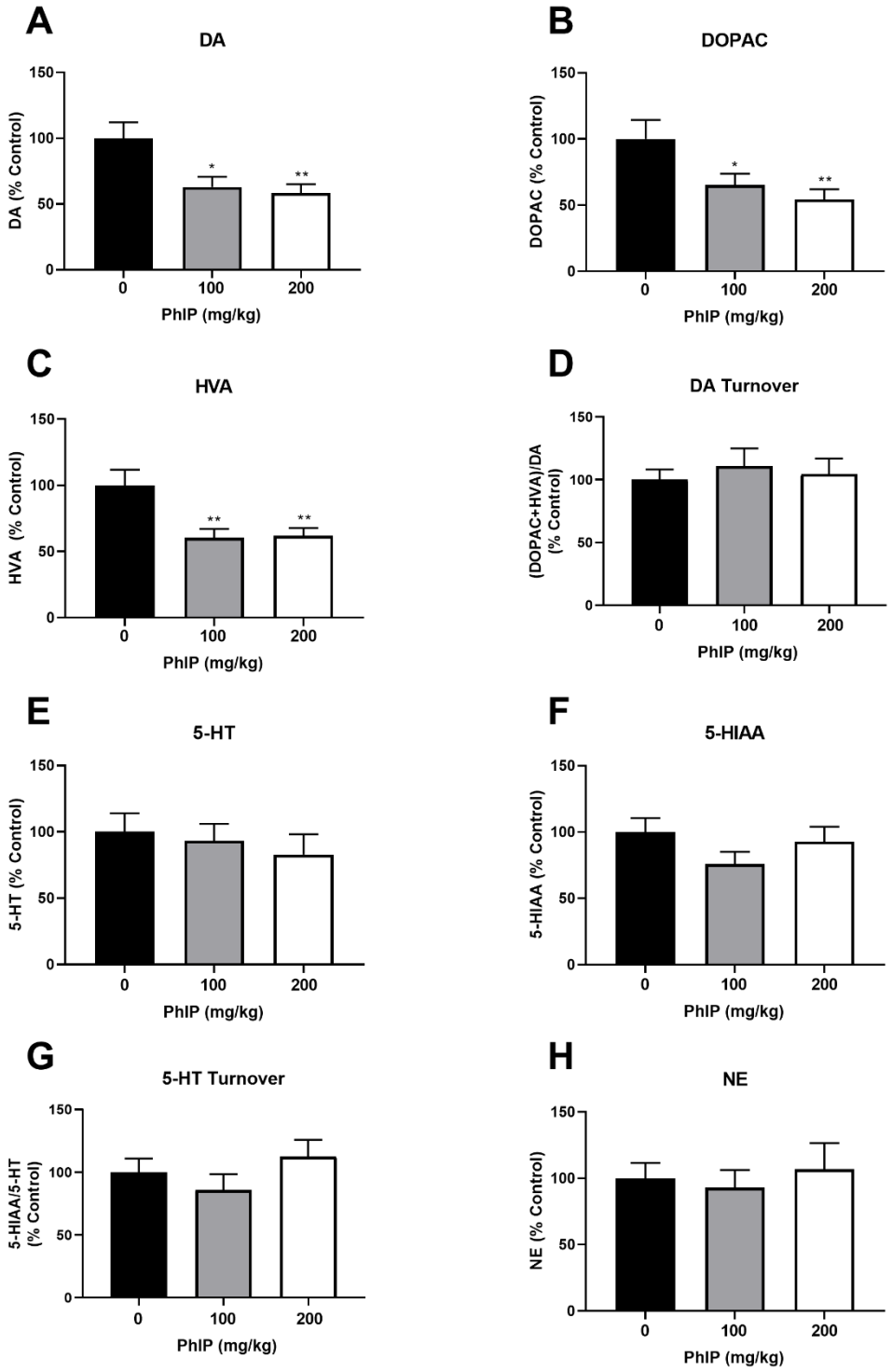

Figure 4.3 DA and its metabolites DOPAC and HVA were decreased in the striatum of mice treated with PhIP for 8 hours. A) DA, B) DOPAC, C) HVA, D) dopamine turnover, E) 5-HT, F) 5-HIAA, G) serotonin turnover, and H) norepinephrine were measured via HPLC in the striatum of mice treated with PhIP.

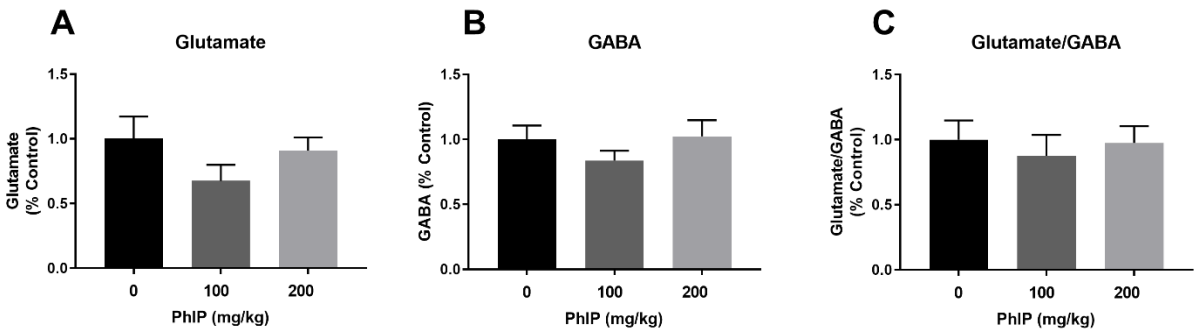

Figure 4.4 Neither glutamate nor GABA were changed in the striatum of mice treated with PhIP for 8 hours. A) Glutamate and B) GABA were measured in the striatum of mice treated with PhIP. C) The ratio of glutamate to GABA was calculated.

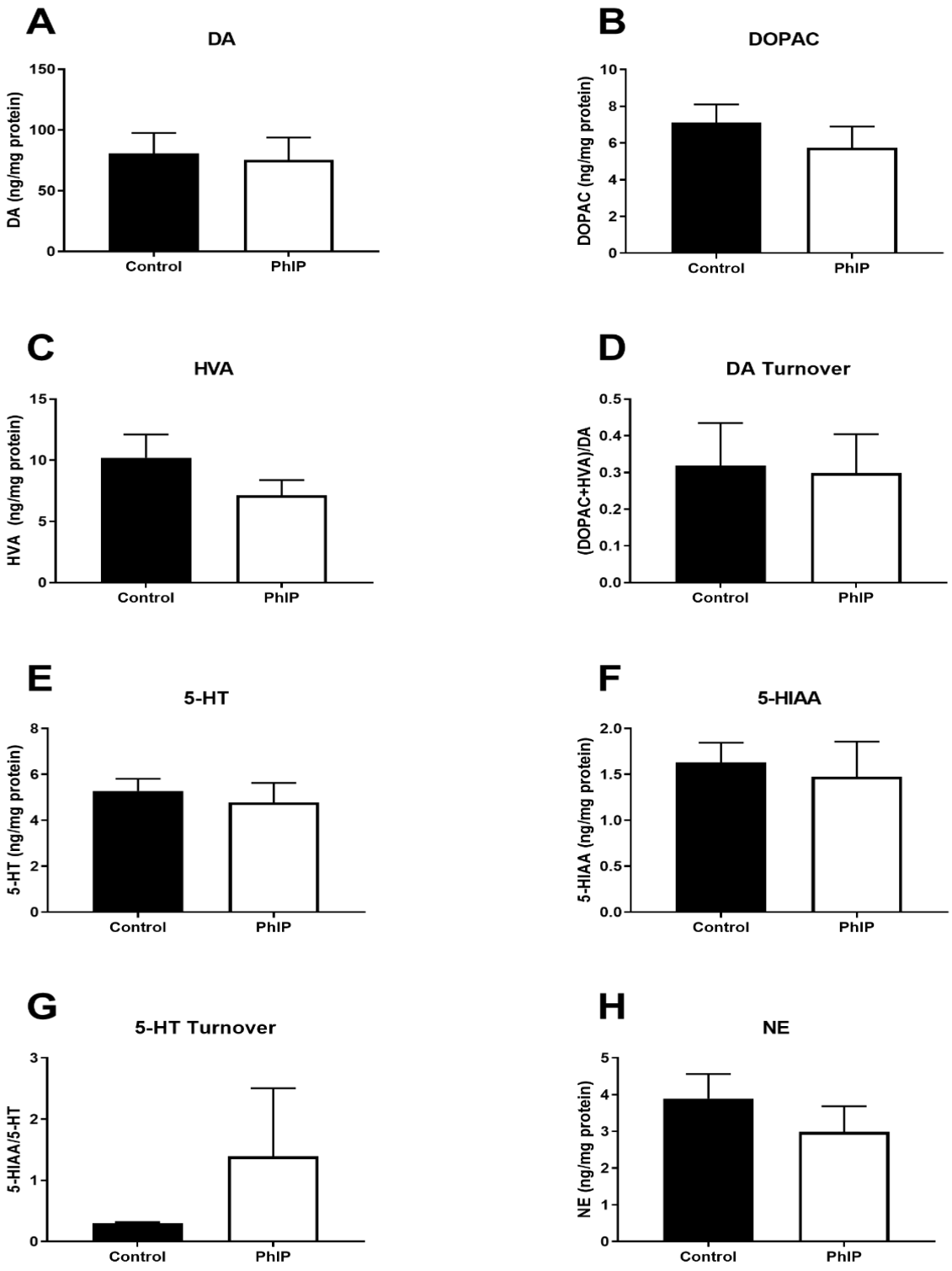

Figure 4.5 No neurotransmitters were affected in the striatum of mice treated with PhIP for 4 weeks. A) DA, B) DOPAC, C) HVA, D) dopamine turnover, E) 5-HT, F) 5-HIAA, G) serotonin turnover, and H) norepinephrine were measured in the striatum of mice treated with PhIP.

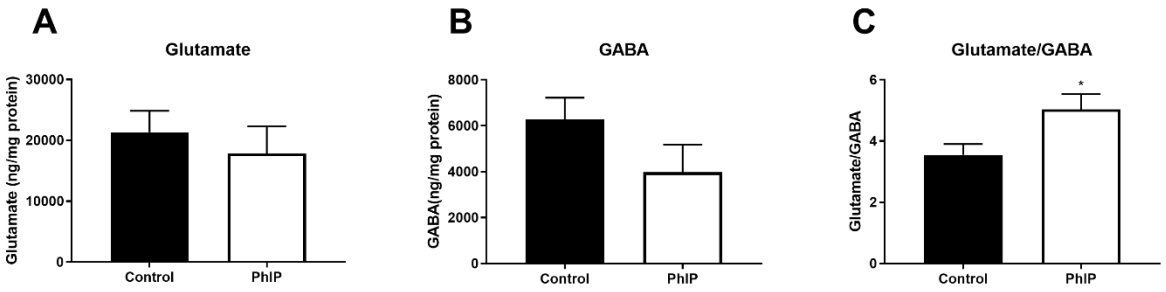

Figure 4.6 Neither glutamate nor GABA were changed in the striatum of mice treated with PhIP for 4 weeks. A) Glutamate and B) GABA were measured in the striatum of mice treated with PhIP. C) The ratio of glutamate to GABA was calculated.

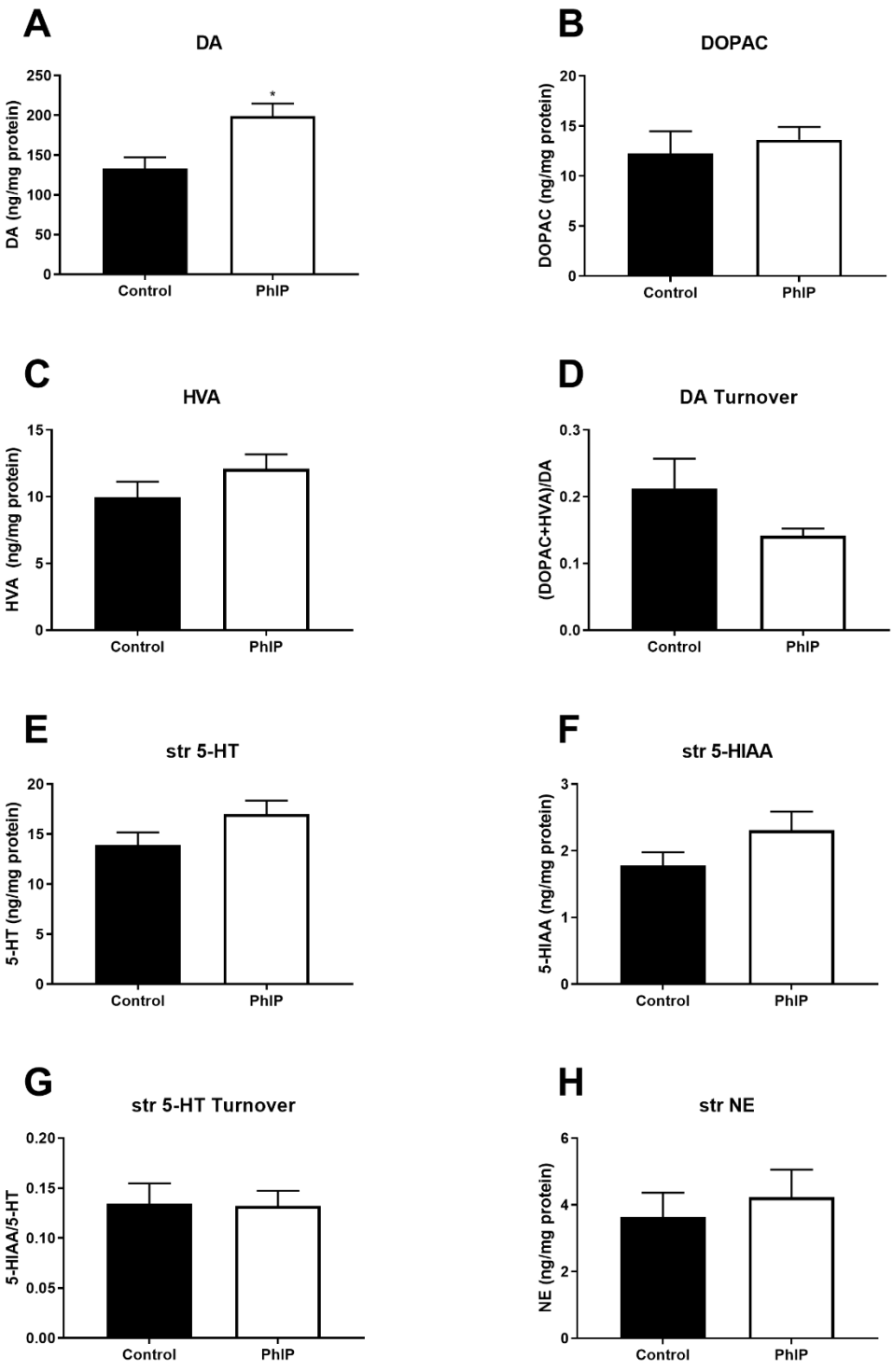

Figure 4.7 Dopamine was significantly increased in the striatum of mice treated with PhIP for 16 weeks. A) Dopamine, B) DOPAC, C) HVA, D) dopamine turnover, E) 5-HT, F) 5-HIAA, G) serotonin turnover, and H) norepinephrine were measured in the striatum of mice treated with PhIP.

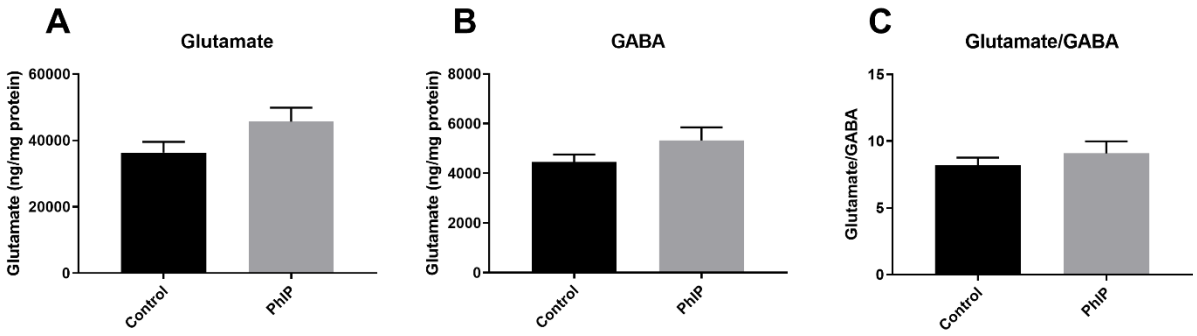

Figure 4.8 Neither glutamate nor GABA were changed in the striatum of mice treated with PhIP for 4 weeks. A) Glutamate and B) GABA were measured in the striatum of mice treated with PhIP. C) The ratio of glutamate to GABA was calculated.

4.3.3 PhIP significantly decreases striatal terminal density in acute exposure, but not in subacute or subchronic exposures while oxidative stress is not affected at any time point.

Striatal terminal density and striatal oxidative stress were measured by staining for TH and NT, respectively. Acute exposure led to significantly decreased striatal terminal density after 100 mg/kg PhIP exposure, while no other treatments affected striatal terminal density (Fig. 4.7A,C,E). Oxidative stress in the striatum was not significantly affected at any time point or any dose (Fig. 4.7B,D,F).

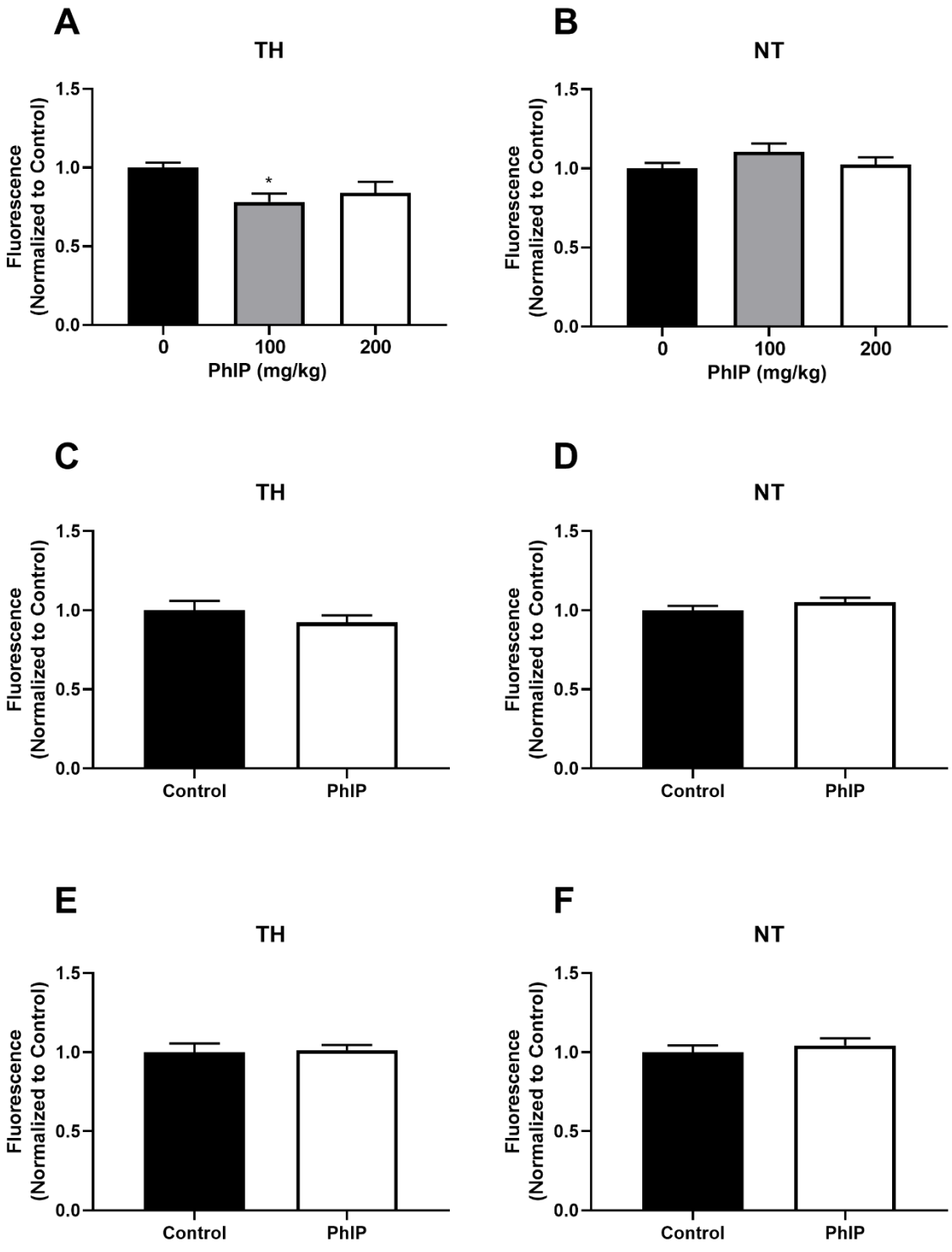

Figure 4.9 Striatal terminal density was significantly decreased after 100 mg / kg PhIP exposure for 8 hours. Striatal terminal density was quantified after exposure to PhIP for A) 8 hours, C) 4 weeks, and E) 16 weeks. Striatal NT was also quantified after exposure to PhIP for B) 8 hours, D) 4 weeks, and F) 16 weeks.

4.4 Discussion

In this study, we show that acute exposure to PhIP causes decreases in DA and its metabolites without affecting other neurotransmitters in the striatum. There is also a decrease in striatal terminal density after 8 hour exposure to 100 mg/kg PhIP, which could be indicating a decrease in DA formation, and, therefore, a decrease in its metabolites. Interestingly, this decrease is not present in the 200 mg/kg PhIP animals, indicating that this high of an exposure could be leading to compensatory mechanisms that keep striatal terminal density equivalent to control animals.

Interestingly, this appears to only be an acute affect, because after 4 weeks of exposure, there are no effects on neurotransmitter levels, and after 16 weeks, DA is significantly increased in the striatum. It is possible that between 8 hours and 4 weeks, neurons are compensating for the initial decrease in TH and DA, bringing it to a level similar to controls. However, there is an increase in DA in the striatum of mice exposed to 75 mg/kg PhIP for 16 weeks, which could indicate that the compensation that occurs is not through a feedback loop, leading to increased DA.

PhIP is mainly metabolized through CYP1A1 and CYP1A2 to 4'-OH PhIP and N-OH PhIP (Lin et al., 1995). These enzymes are homologous through humans and classical model systems; however, the ratio of metabolites formed differs (Lin et al., 1995). Mice and rats form significantly less N-OH PhIP and more 4'-OH PhIP than humans (Lin et al., 1995). Because of this, we wanted to determine whether mice expressing human CYP1A1/2 would be more susceptible to PhIP than wild-type mice. We treated CYP1A1/2 knockout and humanized CYP1A1/2 mice at the same time as the acute exposure animals. However, when we quantified neurotransmitter levels in the striatum, there was a significant decrease in DOPAC both knockout and humanized mice treated with corn oil compared to wild-type corn oil treated mice, along with a significant decrease in HVA in the humanized mice and a trend to decreased DA in both groups which did not reach significance (data not shown). To our knowledge, there are no reports that CYP1A1/2 play roles in DA metabolism. However, CYP2D6 is known to play a role, so it could be that CYP1A1 or CYP1A2 can help metabolize DA or it could be that there were off-target effects of the genetic manipulation of these mice. Due to these changes in control treated animals, though, no further experiments were performed including knockout or humanized CYP1A1/2 mice.

CHAPTER 5. EFFECTS OF INDIVIDUAL PFAS ON FROG NEURODEVELOPMENT

*This chapter was published in *Toxicology and Applied Pharmacology* on 6/10/2019 under the title "Developmental exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) selectively decreases brain dopamine levels in Northern leopard frogs" PMID: 31195004. doi: 10.1016/j.taap.2019.114623 It has been adapted for this dissertation.

5.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are compounds that have been extensively used in manufacturing of surfactants, paper and packaging treatments, anti-static agents, corrosion inhibitors, insecticides, shampoos, and firefighting foams (Posner, 2012; Stahl et al., 2011). PFAS are of significant concern due in part to the stable carbon-fluorine bond and, therefore, long half-life and potential for accumulation (Moody et al., 2003). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have been extensively investigated for adverse health effects. While PFOS and PFOA have largely been phased out in favor of shorter chain length PFAS, they are ubiquitously detected in the global population (Jian et al., 2018; Olsen et al., 2009).

PFOS and PFOA both cross the blood-brain barrier, with PFOS generally accumulating 10-100x more in mammals than many other major PFAS (Maestri et al., 2006). Studies in large mammals suggest that PFAS accumulate in the brain and are potentially neurotoxic. In polar bears, brain PFAS levels (including PFOS) were found to correlate with neurotransmitter alterations (Dassuncao et al., 2019). Further, PFAS in North Atlantic pilot whales (Globicephala melas) were found to accumulate in brain, where only the liver had higher levels. Here, PFAS were strongly correlated with the high phospholipid content in the brain (Dassuncao et al., 2019). Laboratory studies in rodents on PFAS neurotoxicity have suggested neurobehavioral alterations. For example, mice exposed to various PFAS on post-natal day 10 showed that exposure increased spontaneous activity and increased proteins important for making synapses (Johansson et al., 2009; Lee and Viberg, 2013). After pregnant dams were treated with PFOA, male offspring had significantly shorter latency to fall on the wire-hang test when they were 5-8 weeks old, suggesting a potential motor deficit (Onishchenko et al., 2011). This implies that PFAS exposure may affect brain development. Exposures to invertebrates have shown that C. elegans have decreased motor function after 48 hour exposure to PFOS (Chen et al., 2014). Finally, in vitro exposures suggest

that PFAS may influence neuronal differentiation, where, in PC12 cells, PFOS promoted differentiation to cholinergic neurons at the expense of differentiation to dopaminergic (DA)rgic neurons, whereas other tested PFAS suppressed or enhanced overall differentiation (Slotkin et al., 2008). This research suggests that developmental exposure could lead to a long-term deficit in the number of DA neurons.

In general, there is a lack of data on PFAS neurotoxicity and potential relevance to neurological diseases. Neurobehavioral alterations have been examined in a number of developmental and adult studies, with some behavioral endpoints suggesting the cholinergic system as a target (Johansson et al., 2008). In vitro studies have suggested glutamatergic signaling as a target (Liao et al., 2009). However, environmental relevance of the tested doses is generally lacking and to our knowledge no studies measured neurotransmitter levels from multiple systems in response to PFAS. The primary goal of this report was to determine the effects of developmental PFAS exposure at doses which include those detected in environmental samples in a sentinel species. The long-term goal is to prompt specific research into how exposure to PFAS during development may affect specific neurotransmitter systems and influence neurological disease.

5.2 Methods

5.2.1 Animals

Animals, nominal exposure, and measured water and sediment PFAS levels.

All animal studies were approved by Purdue Animal Care and Use Committee standards. The Northern leopard frog (Lithobates pipiens) was chosen as a relevant sentinel species (Hoover et al., 2017). Eggs were collected and reared to Gosner stage 25 in outdoor tanks prior to the start of the experiment. Treatment tanks were prepared with 75 L of aged well water (containing algae and zooplankton) and 10 kg of sediment spiked with PFOS or PFOA to achieve nominal sediment concentrations of 0, 10, 100, or 1000 ppb. We chose to spike sediments, instead of simple addition of chemicals to the water to represent a more ecologically relevant exposure scenario. Tadpoles unintentionally consume sediment while grazing for algae and detritus. Thus, spiking sediments provided more realism in incorporating all pertinent routes of exposure amphibian larvae would likely experience in the environment. The doses were selected to include concentrations detected at contaminated sites (Moody et al., 2003). Measured water and sediment concentrations, along

with body burdens were determined by tandem mass spectrometry from representative samples as previously reported (Hoover et al., 2017). Exposure lasted 30 days and tadpoles were sacrificed immediately using tricaine (MS-222). For this short initial report on neurotoxicity, 6-14 frogs/treatment (PFOA or PFOS at 0 to1000 ppb) were randomly selected from 32 treatment tanks (~20 tadpoles housed/tank) for detailed neurotransmitter analysis (81 total samples analyzed for each neurotransmitter), where the majority of the animals were devoted to a long-term, non-neuronal study on systemic ecotoxicity. Brains were removed, frozen in liquid nitrogen, and stored at -80oC until processed.

5.2.2 Neurotransmitter quantification

Six neurotransmitter systems were assessed. The majority of neurotransmitters were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection as we have previously reported (Agim & Cannon, 2018; Cannon et al., 2009; Horowitz et al., 2011; Wirbisky, Weber, Lee, Cannon, & Freeman, 2014; Wirbisky et al., 2015). Neurotransmitter levels in the samples were quantified by measuring the area under the curve and comparing it to a standard curve. Neurotransmitter levels were then normalized to protein levels in the sample (ng neurotransmitter/mg protein). DA turnover was calculated as [(DOPAC + HVA)/DA where DOPAC and HVA refer to DA metabolites 4-dihydroxyphenylacetic acid and homovanillic acid, respectively] (Agim and Cannon, 2018).

Acetylcholine (ACh) was quantified using the Invitrogen Molecular Probes Amplex ACh/Acetylcholinesterase Assay Kit (A12217). Briefly, samples were diluted 1:1 in the reaction buffer. Samples and standards were incubated in 200 µM amplex red, 1 U/mL horseradish peroxidase, 0.1 U/mL choline oxidase and 0.5 U/mL acetylcholinesterase 1:1 reaction buffer. Concentrations were determined with fluorescence spectroscopy (530 nm excitation and 590 nm emission). Acetylcholine levels were normalized to protein levels in the sample (ng neurotransmitter/mg protein).

5.2.3 Statistical analysis

Neurotransmitters were analyzed by one-way ANOVA with Sidak's post-hoc test to compare all treatment groups within an exposure (PFOA or PFOS) and to controls. Whole brain

analyses of lower order species (i.e., zebrafish) produce considerably more variable neurochemistry data than micro-dissected brain analysis in rodents (Dukes et al., 2016; Lee et al., 2015). Thus, outlier analysis was conducted. Statistical analyses were conducted both before and after outlier analysis. Importantly, outlier analyses for raw neurotransmitter data had no effect on statistical significance, where no new significant comparisons were revealed, nor were any significant comparisons eliminated after outlier analysis. Outliers were identified using stringent ROUT analysis (Q = 0.1%) (Motulsky and Brown, 2006). Outlier analyses resulted in no more than 3 samples/group being excluded, with the following total exclusions/endpoint: DA (0/81 data points omitted), DOPAC (7/81 data points omitted), HVA (0/81 data points omitted), 5-HT (0/81 data points omitted), 5-HIAA (0/81 data points omitted), NE (1/81 data points omitted), DA turnover (9/81), 5-HT turnover (1/81 data points omitted), glutamate (0/81 data points omitted), GABA (11/81 data points omitted), and acetylcholine (0/81 data points omitted). All data were expressed as mean ± standard error of the mean (SEM) and $p < 0.05$ used to identify significant differences.

5.3 Results

5.3.1 Neurotransmitter levels

Quantification of catecholamine neurotransmitters showed a significant decrease in DA in frogs treated with 1000 ppb PFOA and 100 or 1000 ppb PFOS, (3.61 ± 0.61 ng/mg protein, 1.62 ± 0.18 ng/mg protein, 2.41 ± 0.15 ng/mg protein, 2.09 ± 0.24 ng/mg protein for the control, 1000 ppb PFOA, 100 ppb PFOS, and 1000 ppb PFOS, respectively) (Figure 5.1A), but no significant changes in the DA metabolites DOPAC or HVA (Figure 5.1B, C). DA decreases, relative to metabolite levels led to a significant increase in DA turnover [(DOPAC + HVA)/DA] for both 1000 ppb PFOS and PFOA (0.332 ± 0.051, 1.22 ± 0.20, 0.693 ± 0.085, 1.02 ± 0.10 for the control, 1000 ppb PFOA, 100 ppb PFOS, and 1000 ppb PFOS, respectively) (Figure 5.1D). Neither PFOS nor PFOA caused significant changes in norepinephrine (NE) levels (Figure 5.1E). Neither serotonin (5-HT) nor its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were significantly affected by treatment with either PFAS, leading to no changes in 5-HT metabolism (Figure 5.1F - H).

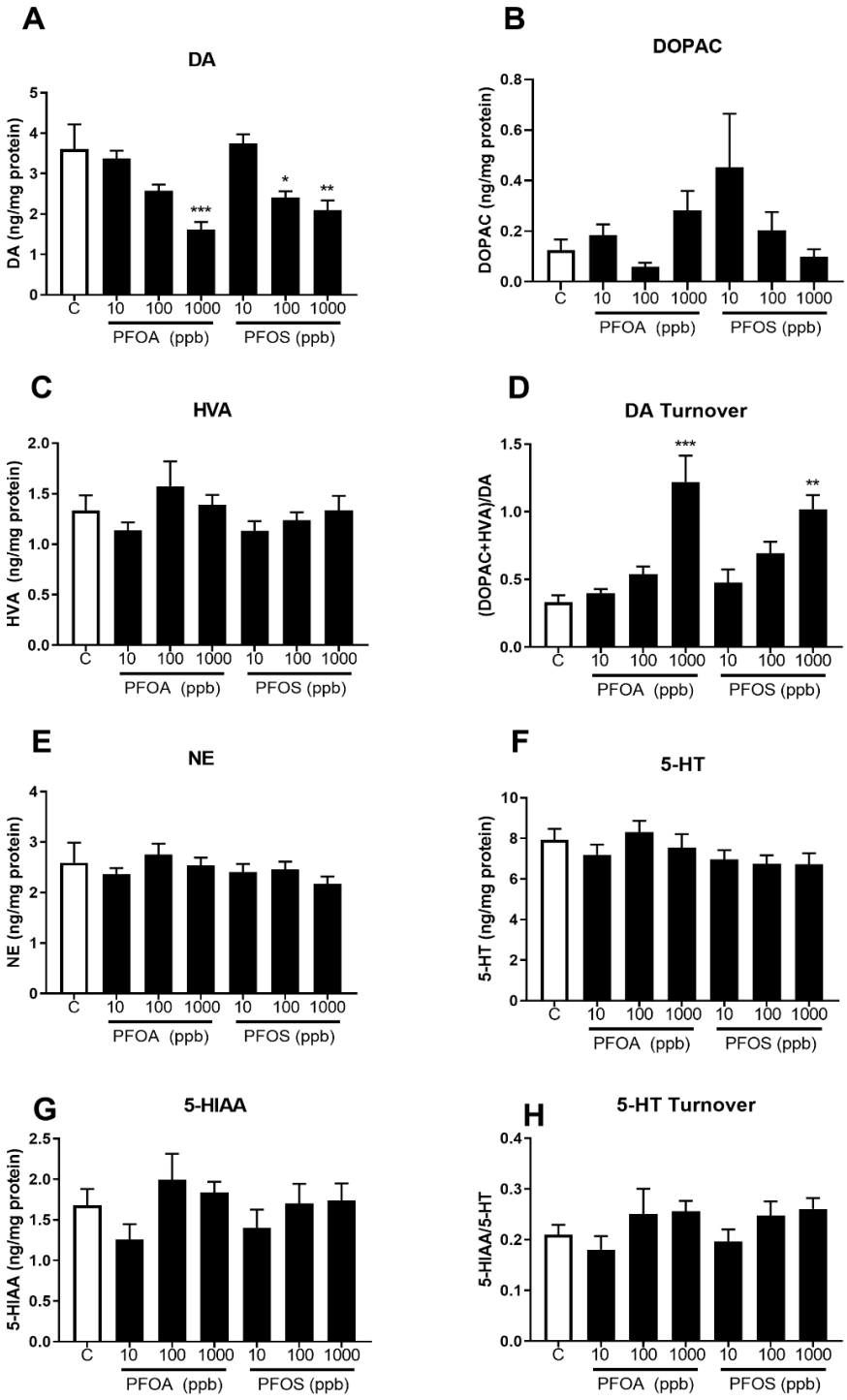

Figure 5.1 PFOS and PFOA significantly decrease dopamine in brain of Northern leopard frogs. A) DA, B) DOPAC, C) HVA, D) DA turnover, E) NE, F) 5-HT, G) 5-HIAA, and H) 5-HT turnover were measured via HPLC in whole brains of frogs treated with PFOS or PFOA.

The amino acid neurotransmitters glutamate and gamma-aminobutyric acid (GABA) were measured and no significant differences were found in treatment groups versus the control for either glutamate or GABA (Figure 5.2A, B). In addition, there was no significant change in ACh levels in any treatment group compared to the control (Figure 5.2C).

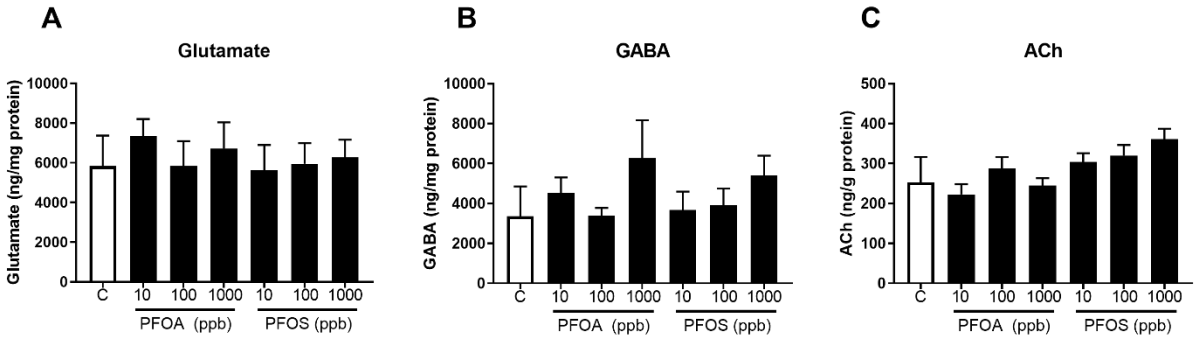

Figure 5.2 Glutamate, GABA, and ACh are not affected by PFOS or PFOA exposure. A) Glutamate, B) GABA, and C) ACh were quantified in whole brains of frogs treated with PFOS or PFOA.

5.4 Discussion

There are major gaps in the literature related to PFAS neurotoxicity. Here, we have shown that developmental exposure within the concentration range documented at contaminated sites produce selective total brain DA depletion in an amphibian sentinel species. Further, in general, we observed that PFAS exposure increased DA turnover, which is typically a compensatory mechanism in response to DArgic neurotoxicity and DA depletion; and an additional metric of damage to DArgic function (Sossi et al., 2002). This short report is expected to prompt research on the neurochemical and neuropathological effects of PFAS on the brain. Further, our findings suggest that the potential role of PFAS in neurological diseases affecting DArgic neurotransmission should be examined.

DA depletion in the frog brain could have several implications, especially given that there are some significant advantages of specific frog species as a model. Leopard frogs are unique in that they have neuromelanin-containing DArgic neurons in the ventral motor neurons which could be related to the DArgic neurons in the nigrostriatal DA system that are affected in Parkinson's

disease (PD). Importantly, these pigmented neurons have been shown to bind PD-relevant toxicants, where high-neuromelanin expressing species exhibit heightened sensitivity with respect to low-neuromelanin expressing species (Karlsson et al., 2009; Lindquist et al., 1988; Sokolowski et al., 1990; Spencer et al., 2004). Taken together, the literature suggests that, with respect to neuromelanin expression and toxicant binding to neuromelanin, certain amphibian species are more representative of human DArgic neurons than low-expressing rodent models. Thus, the potential relevance of DA depletion in PFAS-treated amphibians to PD should be further examined. An additional common DArgic pathway in mammals is the reward system with DArgic neurons extending from the ventral tegmental area to the ventral striatum (Lammel et al., 2014). Frogs have a similar DArgic pathway for reward that contains DArgic neurons in the anteroventral tegmental area, thus the DA depletion that we observed could potentially result in suppression of the reward pathway (O'Connell et al., 2010). DArgic neurons are also important for the hypothalamic function and multiple neuroendocrine axes that control well-conserved behaviors such as metabolic rate stress, digestion, and mood (Hu et al., 2008). Thus, neuroendocrine function in response to PFAS should also be tested.

Another important aspect of these findings potentially relates to developmental origins of adult health and disease (DOHaD). It will be important to examine whether developmental PFAS exposures result in increased susceptibility to neurodegenerative diseases. PD, for example is a disease of aging, where, once the nigrostriatal DA reserve is depleted (i.e., loss of DA neurons reaches a symptomatic threshold), the onset of clinical symptoms appear (Tabbal et al., 2012). Testing whether a developmental PFAS exposure may lower such reserve and increase susceptibility later in life is of importance.

Relationships between PFAS treatments and body burden show log-linear bioaccumulation of PFOS, whereas PFOA did not, which is consistent with the bioaccumulative properties of PFOS, especially in the brain (Maestri et al., 2006). Differences between nominal and measured sediment PFAS levels are due to redistribution of the PFAS between sediment and water, and to a lesser part from a mass balance perspective, to uptake by algal communities and the frogs. Mass balance checks (water, sediment, and frogs) are generally within 15% of the nominal mass added to the system and calculated sediment-water partition coefficients are in the range reported in the literature (Higgins and Luthy, 2006). PFAS levels observed in control animals were low and to be expected, given the ubiquitous global distribution of PFAS. We hypothesize that PFAS detected

in control animals could be derived from the natural water source used to fill tanks, algae, zooplankton and/or leaching from the plastic.

There are some notable limitations in the present study. Neurochemical quantification is a frequent endpoint studied for effects of compounds on the brain in rodents, but has also been measured in aquatic species, both in whole zebrafish larva and brains (Cannon et al., 2009; Dukes et al., 2016; Milanese et al., 2012; Wirbisky et al., 2014; Wirbisky et al., 2015). However, quantification in aquatic species is more difficult and more variable than specific region neurotransmitter quantification in rodents. Clearly, our samples exhibited increased variability relative to micro-dissected rodent studies. Indeed, GABA and ACh analyses exhibited potential nonsignificant trends resulting from PFAS treatment, suggesting further examination. Future studies will require an increased number of animals/group, along with sub-regional analysis.

In summary, our findings suggest developmental PFAS exposure in a sentinel species at environmentally-relevant doses produce selective DA depletion. These findings imply that the role of PFAS in neurological diseases involving DArgic neurotransmission should be evaluated.

5.5 Funding

Ralph W. and Grace M. Showalter Research Trust (to J.R.C.). Strategic Environmental Research and Development Program (ER-2626) (to M.S.S and L.S.L.).

CHAPTER 6. EFFECTS OF ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF PFAS MIXTURES ON FROG NEURODEVELOPMENT

*This chapter has been published in *Neurotoxicology and Teratology* under the title "Single and mixture per- and polyfluoroalkyl substances exposure in developing Northern leopard frogs produces complex neurotransmission alterations". doi: 10.1016/j.ntt.2020.106907

6.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are synthetic compounds that have been used since the 1940s in many different household applications, such as nonstick cookware, stain repellents, water repellents, beauty products, food packaging, and aqueous film forming foam (AFFF) firefighting foam (N. Chen et al., 2014; Moody et al., 2003; Olsen et al., 2007; Onishchenko et al., 2011; T. Stahl et al., 2012). Some longer chain PFAS started being phased out in the United States in 2009, but are still found throughout the world while short chain PFAS are still in use (Moody et al., 2003). Use of AFFF foams on miltary bases can contaminate surface waters with mixtures of PFAS including PFOS, PFOA, PFHxS, PFHxA, and PFPeA, among others (R. H. Anderson, Long, Porter, & Anderson, 2016). PFAS have been shown to accumulate in humans and animals, leading to decreased birth weight-to-height ratios and decreased viability (Olsen et al., 2009). The main organs that accumulate PFAS tend to be liver and blood, but studies in various model systems have found that accumulation and distribution differ widely, with the blood half-life of PFOS in humans being 4.8 years, between 84 and 200 days in cynomolgus monkeys, 24-83 days for rats, and 30-38 days for mice (Johnson, Gibson, & Ober, 1984; Olsen et al., 2007; Pizzurro, Seeley, Kerper, & Beck, 2019; Seacat et al., 2002). However, other PFAS have different accumulation rates. For example, perfluorobutane sulfonate (PFBS) and perfluorooctanoate (PFOA) do not accumulate to the same amount as PFOS, even when exposure levels are the same (F. J. Chen et al., 2018; L. Chen et al., 2018). Furthermore, given the broader diversity of applications associated with PFAS usage, humans and wildlife are exposed to PFAS mixtures rather than individual PFAS (R. H. Anderson et al., 2016). However, few studies have exposed animals to specific mixtures to determine potential changes in toxicity and whether effects are additive, antagonistic, or synergistic.

PFAS have been found in the brain, indicating that they cross the blood brain barrier and are potentially neurotoxic (Eggers Pedersen et al., 2015; Greaves & Letcher, 2013). Previous research in mice and rats have conflicting evidence as to whether PFOS causes decreased motor function after developmental exposure (Johansson et al., 2009; Johansson et al., 2008; Onishchenko et al., 2011). *In vitro* studies have shown that PFOS exposure causes a rat neuroblastoma cell line, PC12, to preferentially differentiate to cholinergic neurons at the expense of dopaminergic neurons (Slotkin et al., 2008). PFOS has also been shown to cause dopaminergic neurotoxicity and decreased motor function in *Ceanorhabditis elegans* (Sammi et al., 2019). In polar bears, various PFAS have been correlated with increased monoamine oxidase activity, muscarinic acetylcholine receptor density, and decreased acetylcholinesterase (AChE) activity (Eggers Pedersen et al., 2015). Female marine medaka exposed to PFBS also had increased acetylcholine (ACh) and choline levels, while males did not (L. Chen et al., 2018). Our laboratory also previously reported that PFOS and PFOA decreased whole brain dopamine (DA) and significantly increased DA turnover in Northern leopard frogs *Rana pipiens* at 100 - 1,000 µg/L (Foguth et al., 2019).

The brain is also key to innervation of the heart, which is regulated by norepinephrine (NE) for sympathetic innervation and ACh for the parasympathetic system. PFAS have been implicated in improper development of the heart, such as increased apoptosis in rats exposed to PFOS *in utero* and decreased expression of cardiac-specific genes in mouse embryonic stem cells during differentiation (W. Cheng, Yu, Feng, & Wang, 2013; Zeng et al., 2015). Furthermore, there are correlations between serum PFAS levels and cardiovascular disease, including congestive heart failure, coronary heart disease, heart attacks, and strokes (M. Huang et al., 2018). PFOS also caused apoptosis of cells while differentiating into cardiomyocytes in rats exposed *in utero* through to 21 days after birth and in cultured mouse embryonic stem cells (W. Cheng et al., 2013; Zeng et al., 2015). Interestingly, the effect of PFAS on the development of the sympathetic and parasympathetic nervous systems have not been tested, which could also be causing heart problems not seen early in development.

In this study, we used Northern leopard frogs as a model for evaluating neurotoxicity resulting from exposure to PFOS or a PFAS mixture. Northern leopard frogs are a suitable model species, especially for neurotoxicity, because they express neuromelanin, a product of DA oxidation that has been implicated in toxicity due to accumulation and later release of neurotoxic

compounds, leading to a large exposure all at once (Karlsson, Berg, Brittebo, & Lindquist, 2009; Kemali & Gioffre, 1985; Lindquist et al., 1988; Zecca et al., 2006). While most rodent models do not produce neuromelanin, Northern leopard frogs do, even as tadpoles (Kemali & Gioffre, 1985; Lindquist et al., 1988). This could indicate enhanced sensitivity to environmental exposures compared to laboratory studies with rodents and other animals lacking neuromelanin. Furthermore, frogs have large broods of eggs that are easily manipulated compared to developmental studies in rodents (Sachs & Buchholz, 2017; Scheenen, Jansen, Roubos, & Martens, 2009). Their eggs are also free living, so it is possible to expose eggs at early life stages directly, with known concentrations, rather than exposing *in utero*. Building on our prior publication on neurotoxic effects (neurotransmitter changes) of PFOA and PFOS individually at relatively high exposure levels in frogs (Foguth et al., 2019), the goals of this study were to assess effects under more environmentally relevant exposures (lower concentrations and for a longer duration) including PFAS mixtures which more commonly occur in nature. We also quantified the effects of PFAS on innervation of the heart, which has not been previously studied.

6.2 Methods

6.2.1 Animals, exposure, survival, and measurement of PFAS levels

All animal studies were approved by the Purdue Animal Care and Use Committee and collection was under IN Scientific Purposes License 19-343 issued to Tyler Hoskins (TDH). On April 1, 2019, six partial Northern leopard frog egg masses were collected from an ephemeral wetland at the Purdue Wildlife Area (40.452275, -87.054853). This wetland hosts a diverse community of native amphibians, lies within a protected area, and has no known history of PFAS contamination. Eggs were grown in outdoor tanks until free swimming stage at Gosner stage (GS) 25 (Gosner, 1960). On April 12, 2019, mesocosms mimicking a simple aquatic food web were prepared by filling twelve 150 L cattle tanks with 100 L of well water and adding 100 g leaf litter (predominantly, oak) from a mixed hardwood forest near the natal site for our animal, along with 5 g Rabbit Chow (Purina) to provide a nutrient base. We selected oak-rich leaf litter because oak tends to decompose more slowly than many other species present at our site and because oak has been shown to positively influence larval survival relative to faster-decaying litter types along with 5 g Rabbit Chow (Purina) to provide a nutrient base. We measured pH, dissolved oxygen, and

conductivity for control mesocosms five times throughout the exposure period (Stoler & Relyea, 2016). Mesocosms were then inoculated with periphyton and phytoplankton (i.e., algal food resources for leopard frog larvae), as well as zooplankton from the wetland where eggs were collected. Twelve days after the first inoculation, mesocosms were spiked with test chemicals to establish chemical treatments (four mesocosms each), which included a no PFAS control, PFOS at a nominal concentration of 10 ppb, or a PFAS mixture totaling 10 ppb (Fig. 1). The PFAS mixture contained 4 ppb PFOS, 3 ppb perfluorohexane sulfonic acid (PFHxS), 1.25 ppb PFOA, 1.25 ppb perfluorohexanoic acid (PFHxA), and 0.5 ppb perfluoro-n-pentanoic acid (PFPeA). The selected mixture components represent the five most commonly detected PFAS in surface waters among 36 aqueous film forming foams (AFFF) impacted sites (R. H. Anderson et al., 2016). Additionally, the relative PFAS proportions were selected to mimic a single AFFF site where data were available (Clark's Marsh, MI; unpublished data, Michigan Department of Environment, Great Lakes, and Energy; Michigan Department of Health and Human Services, 2017) (Services, 2017). The PFOS-only exposure was selected because it is within the range observed in surface waters in the AFFF-impacted Clark's Marsh, is consistently the highest PFAS of the many present, and paralleled the total PFAS concentration in the mixture at Clark's Marsh, MI, where water is contaminated due to being near firefighting training areas (R. H. Anderson et al., 2016). After seven days (May 6, 2019; experimental day 0), 25 leopard frog larvae at GS 25 were added to each mesocosm to initiate the experiment (Gosner, 1960). During the experiment, neither water nor PFAS treatments were replenished.

After 30 days, eight tadpoles were removed from each mesocosm and sacrificed with a solution of buffered 0.4 g/L tricaine methanesulfonate (MS-222). Snout-vent length (SVL), mass, and GS were recorded prior to removal of the brain followed by flash freezing or refrigeration of the brain for neurotransmitter analysis (5 brains per mesocosm) or PFAS brain concentration analysis (3 additional pooled brains per mesocosm), respectively. Mesocosms were checked daily for animals reaching the beginning of metamorphosis, which was defined as the emergence of at least one forelimb (GS 42) (Gosner, 1960). Each day, we captured metamorphs with dip nets, placed animals individually in 16oz deli cups (diameter 4.5") with approximately 1 cm of water from mesocosms where they developed, and held them indoors until completion of metamorphosis with water from the mesocosm and food not replenished (complete tail reabsorption; 1-15 days to GS46) (Gosner, 1960). While animals were in the cups, they were housed individually with a lid

on that contained holes for air and containers were placed at an angle to allow the animals to access either wet or dry areas as needed. At GS 46, frogs were sacrificed with MS-222 and SVL, mass, and date were recorded. The brain and heart were extracted and flash frozen along with the carcass (for neurotransmitter or protein analysis) or refrigerated at 4^{O}C for brain PFAS chemical analysis.

The experiment was terminated after 116 days, when metamorphosis slowed dramatically (0 animals reaching GS 42 for five consecutive days). Tadpoles remaining in mesocosms were collected, sacrificed and measured in the same manner as the tadpoles at the 30-day time point, but all brains and hearts were flash frozen. Survival was calculated as survival to GS 46 and as total survival.

Three brains from individuals collected for each stage were pooled to quantify levels of PFAS. Brains were extracted using a tetrahydrofuran/water solution dosed with mass-labeled PFAS standards as previously described (Foguth et al., 2019; Hoover et al., 2017). Brain extracts along with water samples collected in 50 mL plastic tubes after animals were added and stored at 4^{o}C from each mesocosm were quantified using high performance liquid chromatography tandem mass spectrometry (LC/MS/MS) with isotope dilution as previously reported (Foguth et al., 2019; Hoover et al., 2017).

6.2.2 Neurotransmitter quantification

Neurotransmitters were analyzed from whole brain and heart as previously described (Agim & Cannon, 2018; Jason R. Cannon et al., 2009; Foguth et al., 2019; Wirbisky, Weber, Lee, Cannon, & Freeman, 2014; Wirbisky et al., 2015). Briefly, samples were homogenized in 0.1 M $HClO_4$ and centrifuged at 14,000 RCF for 35 minutes at 4^{O}C. The supernatant was filtered through 0.2 μm filters by centrifugation at 4^{O}C and 1,000 RCF for 15 minutes while the protein pellets were resuspended in 0.1 M NaOH. The majority of neurotransmitters were quantified using high performance liquid chromatography (HPLC) (Dionex Ultimate 3000 model ISO-31000BM pump, WPS-3000TBSL autosampler) with an electrochemical detector (ECD) (Coulochem III electrochemical detector with an ESA Coulochem data station) and a standard curve using the area under the curve. DA, 4-dihydroxylphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and NE were measured using the catecholamine mobile phase (2 mM 1-octanesulfonic acid, 25 μM EDTA, 80 μM sodium phosphate, 200 μM triethylamine, and 10% methanol, pH 2.4), a 0.6 mL/minute flow rate through

a Waters XBridge reverse phase C18 column (15- x 3.0 mm, 3.5 µm particle size), and detector settings set at E1 = -150 mV and E2 = +350 mV with a guard cell at +450 mV to decrease background. To use electrochemical detection for GABA and glutamate analysis, GABA must be derivatized, which was done online using an O-phthaldehyde in β-mercaptoethanol and OPA diluent and a 74/22/4 v/v/v 0.1 M sodium phosphate/methanol/acetonitrile pH 6.75 mobile phase. The flow rate was 0.5 mL/minute and ECD settings were E1 = -150 mV, E2 = +550 mV, and the guard cell was set at +600 mV. Neurotransmitter levels were normalized to protein level quantified using a Bicinchoninic acid assay (Thermo Scientific) in each sample (ng neurotransmitter/mg protein). DA metabolism was calculated using the equation $Dopamine\ metabolism = \frac{DOPAC+HVA}{DA}$ and 5-HT metabolism was calculated as $Serotonin\ metabolism = \frac{5-HIAA}{5-HT}$.

ACh levels and AChE activity were measured in brains prepared in the same way as above using the Invitrogen Molecular Probes Amplex ACh/Acetylcholinesterase Assay kit as used previously (Foguth et al., 2019). Briefly, samples were diluted 1:1 in the reaction buffer for ACh quantification and not diluted for AChE activity before being incubated in a 1:1 ratio with 200 µM amplex red, 1 U/mL horseradish peroxidase, 0.1 U/mL choline oxidase, and 0.5 U/mL acetylcholinesterase or 0.1 mM acetylcholine. Samples and standards were quantified through fluorescence (Ex/Em: 530 nm/590 nm). Acetylcholine levels and acetylcholinesterase activity were normalized to protein levels in the sample (ng ACh/mg protein or U/mg protein).

6.2.3 Statistical analysis

We tested whether treatments affected PFAS brain burdens over time using repeated measures ANOVAs with log-transformed brain burdens as dependent variables and effects of treatment, time, and the treatment x time interaction. Assumptions were checked using diagnostic plots of residuals. For these analyses, we used all values from LC/MS/MS, including samples that were > limit of detection (LOD) but <lower limit of quantification (LLOQ) (Table S2). When main effects of treatment or treatment x time interactions were detected, we used Tukey's post-hoc comparisons to elucidate pairwise differences.

Survival and phenotypic data were analyzed using individual mesocosms as experimental units, taking the mean mass and SVL of frogs from each mesocosm at a certain stage and performing a one-way ANOVA followed by Sidak's post hoc test with significance set at $p<0.05$.

Neurotransmitter levels were analyzed using individual animals as experimental units. Normality of the distribution of data was checked using a D'Agostino and Pearson test. Normally distributed data was analyzed by running a one-way ANOVA followed by Sidak's post hoc test, while non-normally distributed data was analyzed using a Kruskal-Wallis test, with significance set at $p<0.05$. All data are shown as mean ± standard error of the mean (SEM).

AChE activity was analyzed by running simple linear regression analysis to determine whether developmental stage was a factor in activity. Data were then analyzed by treatment group using a Kruskal-Wallis test after determining non-normal distribution through a D'Agostino and Pearson test.

6.3 Results

6.3.1 PFAS in water and brain

Measured water concentrations were slightly above nominals for the majority of chemicals tested (Table 6.1). As expected for such widespread contaminants, we detected background PFAS in some controls and the PFOS-only mesocosms, but concentrations were low relative to exposure concentrations and < LLOQ in most cases (Table 6.1). Among PFAS examined in brain, only PFOS showed clear evidence of bioaccumulation. PFOS and the PFAS mixture treatments increased brain burdens for PFOS in a dose dependent manner ($F_{2,9} = 1048.17$, $p < 0.0001$; Fig. 6.1) and brain concentrations increased from day 30 to completion of metamorphosis ($F_{1, 9} = 19.88$, $p = 0.0016$; Fig. 6.1), but there was no treatment x time interaction ($F_{2,9} = 1.30$, $p = 0.3183$; Fig. 6.1). Post-hoc tests showed that, within each time point, PFOS and mixture treatments always had higher concentrations than controls and that PFOS treatment always had higher burdens than the mixture treatment. PFOS burdens increased from day 30 to GS 46 for both the control and mixture treatments, but not the PFOS treatment (Fig. 6.1). PFPeA was frequently detected but brain burdens were generally < LLOQ (Table 6.1). We observed no effect of treatment ($F_{2, 9} = 0.06$, $p = 0.9399$) or time ($F_{1, 9} = 5.07$, $p = 0.0509$) on PFPeA brain burdens, but we observed a weak treatment x time interaction ($F_{2, 9} = 4.33$, $p = 0.0480$). This interaction was driven by an increase in PFPeA burdens over time for the mixture treatment only; other treatments showed no change

through time, and there were no pairwise treatment differences within time points. Burdens for PFOA, PFHxS, and PFHxA were low and changed over time, but never differed by treatment and no treatment x time interactions were observed.

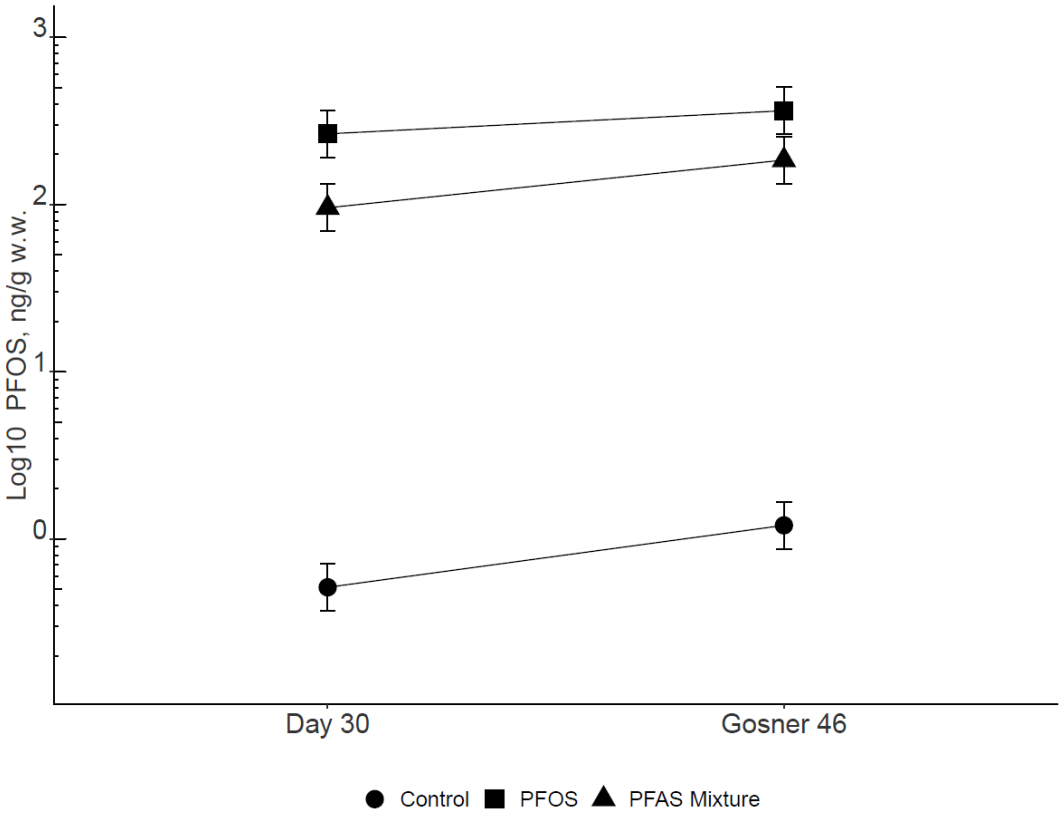

Figure 6.1 Log_{10} brain burdens of PFOS for animals sampled at day 30 of exposure and at completion of metamorphosis (Gosner stage 46), showing the dose dependent increase across chemical treatments and the increase in PFOS burdens from day 30 to completion of metamorphosis. Error bars are 95% C.I.'s.

6.3.2 Effects of PFAS on survival, growth, and development

PFAS exposure did not alter leopard frog survival to GS 42 or 46 compared to controls (data not shown). Averaged across all three treatments, total survival was 78.33% ± 3.191 and 55.56% ± 4.290 reached GS 46. Similar to survival, there were no treatment effects on growth and development response variables at either developmental time point (i.e. 30 days, GS 42 and 46) (data not shown).

6.3.3 Neurotransmitter changes after PFAS exposure

Neurotransmitters quantified in the brain after 30 days of PFAS exposure showed a significant decrease in 5-HT in the PFAS-mixture treatment (control 17.93 ± 2.170 ng/g protein, PFOS 13.09 ± 1.653 ng/g protein, mixture 11.27 ± 0.5686 ng/g protein; $p = 0.0089$ for control vs. mixture, $p = 0.0815$ for control vs. PFOS) (Fig. 6.2G). Both PFAS treatments significantly decreased levels of glutamate compared to the control (13840 ± 1363 ng/g protein, 9810 ± 416.3 ng/g protein, and 9967 ± 285.2 ng/g protein for control, PFOS, and mixture, respectively; $p = 0.0019$ for control vs. PFOS and $p = 0.0029$ for control vs. mixture) (Fig. 6.2J). There were no significant differences in the levels of other neurotransmitters quantified (Fig. 6.2).

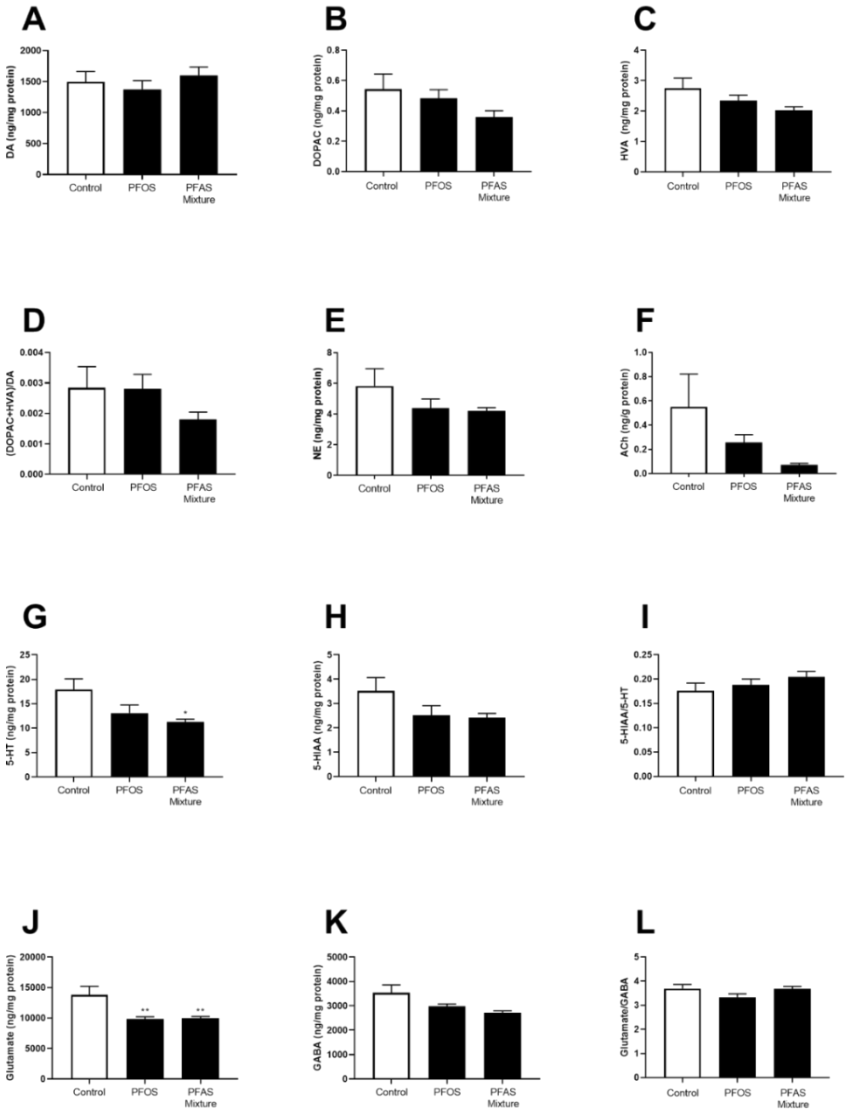

Figure 6.2 Northern leopard frogs exposed to PFOS or the PFAS Mixture for 30 days had decreased 5-HT and glutamate and a trend to decreased ACh. Whole brains from Northern leopard frog tadpoles exposed to PFOS or the PFAS Mixture for 30 days were removed and A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) 5-HT turnover, H) NE, I) 5-HT turnover, J) Glutamate, K) GABA, and L) the ratio of glutamate/GABA were measured.

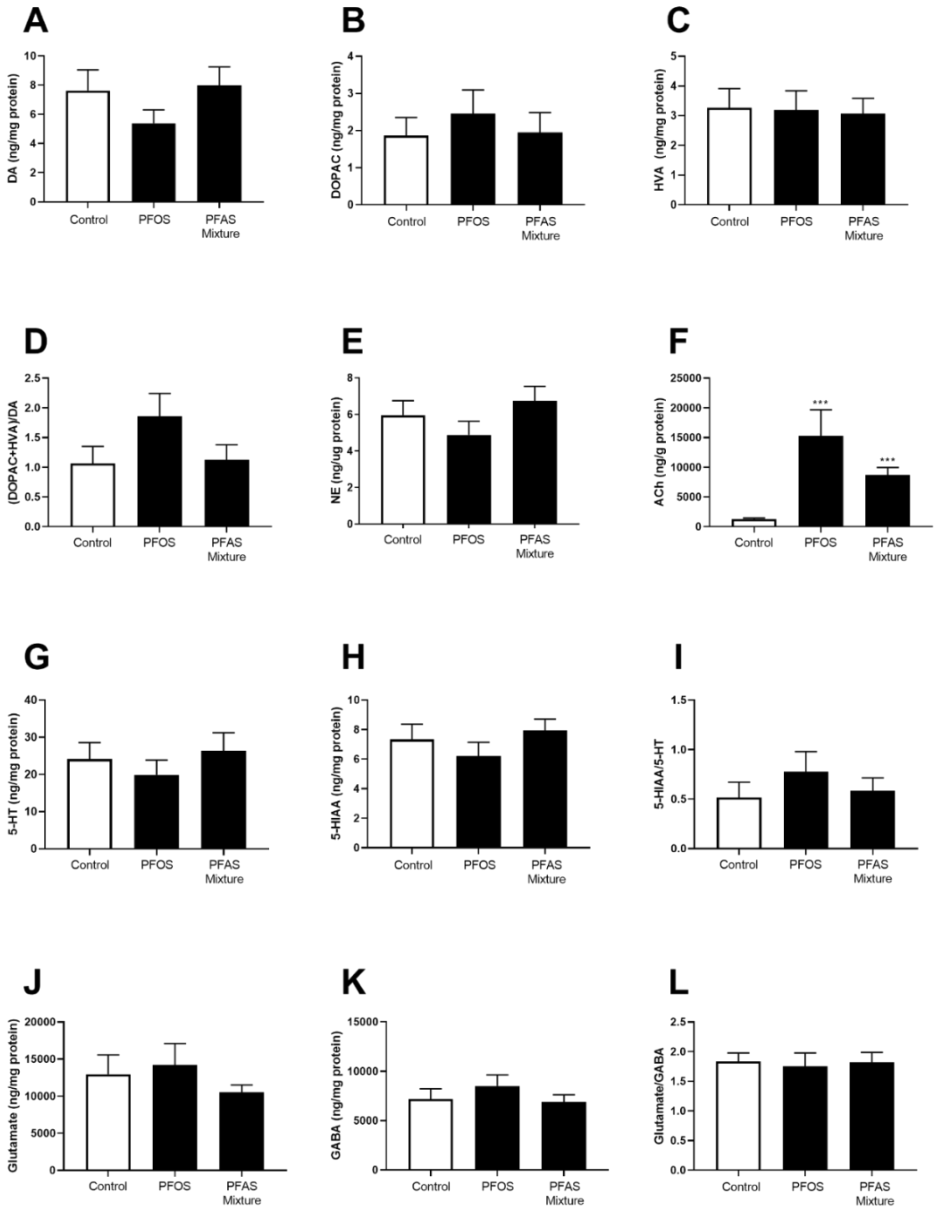

Figure 6.3 Northern leopard frogs exposed to PFOS or the PFAS mixture through development until Gosner stage 46 had increased ACh. Whole brains from Northern leopard frog exposed to PFOS or the PFAS Mixture until Gosner stage 46 were removed and A) DA, B) DOPAC, C) HVA, D) DA turnover, E) 5-HT, F) 5-HIAA, G) 5-HT turnover, H) NE, I) 5-HT turnover, J) Glutamate, K) GABA, and L) the ratio of glutamate/GABA were measured.

Neurotransmitters quantified after PFAS exposure through metamorphosis showed metamorphs at GS 46 exposed to either PFOS or the mixture had significantly increased levels of ACh in the brain compared to controls (1277 ± 171.5 ng/g protein, 15291 ± 4380 ng/g protein, and

8668 ± 1283 ng/g protein for the control, PFOS, and mixture, respectively; $p < 0.0001$ for control vs. PFOS or the mixture) (Fig. 6.3F). Interestingly, the decreases in 5-HT and glutamate found after 30 days of exposure were not found at GS 46 (Fig. 6.3G,J). There were no significant changes in any other neurotransmitters measured (Fig. 6.3).

6.3.4 Acetylcholinesterase activity

Analysis of brains from frogs that were between GS 25 and 42 at the end of the experiment showed no correlation between the developmental stage and AChE activity, so all tadpoles from each treatment were grouped to determine changes in AChE activity after exposure (Fig. 6.4). There were no significant differences in AChE activity after exposure to PFOS or the mixture compared to control (Fig. 6.4).

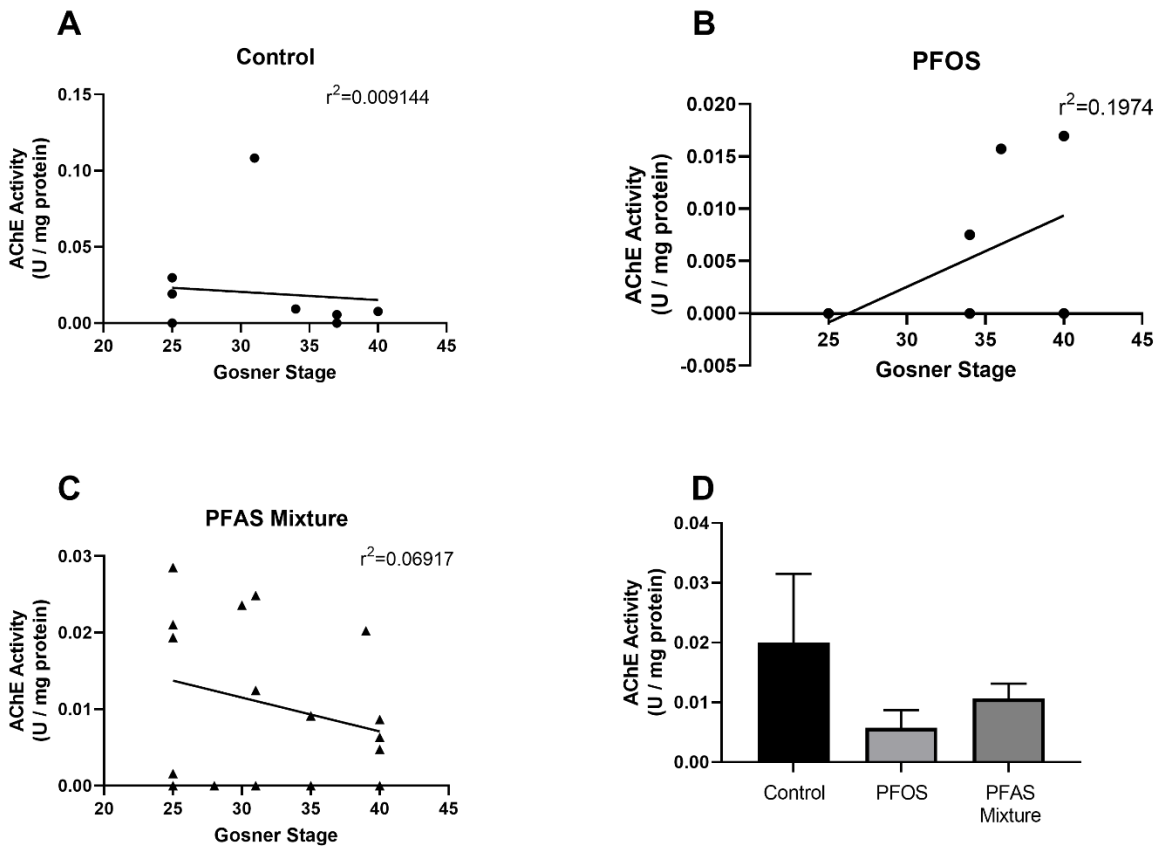

Figure 6.4 There is no correlation between PFAS exposure and activity of AChE. Frogs that had not reached metamorphosis by the end of the exposure were sacrificed and the brains were analyzed for AChE activity**.** A-C) Simple linear regression for ACh activity at various developmental timepoints. D) There was no significant differences in AChE activity in brains of tadpoles exposed to PFOS or the PFAS mixture compared to controls.

6.3.5 Neurotransmitter changes in heart

Hearts were removed from frogs that completed metamorphosis to determine innervation of the heart. There were no significant changes in NE, DOPAC, or HVA with exposure to PFAS (Fig. 6.6). DA, 5-HT, and 5-HIAA were not detected in any samples.

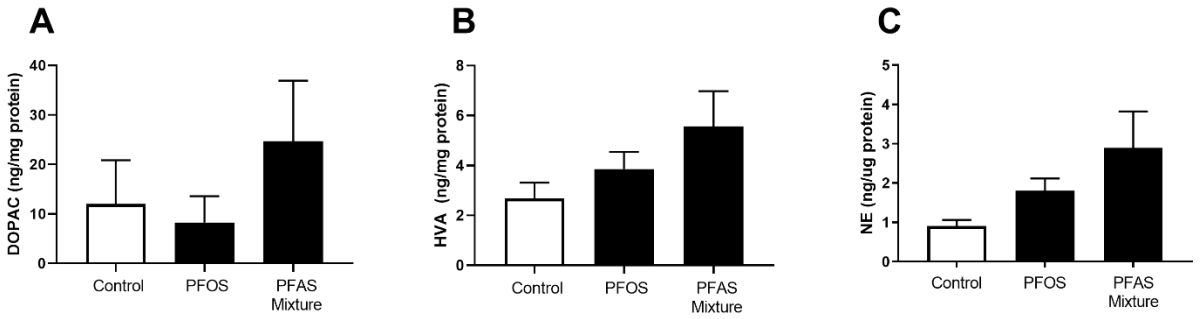

Figure 6.5 Northern leopard frogs exposed to PFOS or the PFAS mixture through development until Gosner stage 46 did not have significant changes in heart neurotransmitter levels. Hearts from Northern leopard frog exposed to PFOS or the PFAS Mixture until Gosner stage 46 were removed and A) DOPAC, B) HVA, and C) NE were measured.

6.4 Discussion

PFAS exposure is a major public health concern. In this study, we exposed Northern leopard frogs, a sentinel species, to environmentally relevant concentrations of PFAS, including a mixture reflective of AFFF impacted sites to assess potential neurotoxicity. We showed brain accumulation and complex neurotransmission changes in the brain. Taken together with previous findings from our group and others, the literature strongly suggest that the nervous system is an important target organ affected by PFAS exposure.

PFOS and other compounds tested here have been shown to bioaccumulate in numerous mammalian taxa, but to our knowledge, this is the first study to demonstrate PFOS accumulation in amphibian brains (Austin et al., 2003; Dassuncao et al., 2019; Eggers Pedersen et al., 2015; Maestri et al., 2006). We measured brain burdens of amphibians exposed to ecologically relevant concentrations of PFAS in water and demonstrate dose-dependent bioaccumulation of PFOS in brain tissues. PFOS burdens observed in PFOS and PFAS mixture treatments across both time points (ranging from 96.2-378.9 ng/g w.w.; Fig. 6.1) were comparable to levels observed in various regions of rat brains following dietary exposure to 1 mg/kg PFOS for 14 days (115-396 ng/g w.w.) (Austin et al., 2003). Polar bears in east Greenland had 128.2 ± 7.14 ng/g w.w. total PFAS while the 10 ppb PFOS exposed frogs had 266.9 ± 21.2 ng/g w. w. and the PFAS mixture group had 96.22 ± 6.23 ng/g w. w. after 30 days of exposure and 378.9 ± 62.38 ng/g w. w. and 202.7 ± 48.38

ng/g w. w. at GS 46, (Eggers Pedersen et al., 2015). While we detected significant, dose-dependent bioaccumulation only for PFOS, our study does not rule out bioaccumulation of PFOA, PFHxS, PFHxA, or PFPeA in amphibian brains. All of these compounds have been detected in environmentally exposed animals, but at much lower concentrations than PFOS, following expected accumulation patterns based on chain length and functional group identity (Dassuncao et al., 2019). Our exposure concentrations for compounds besides PFOS were low in the mixture treatment (Table 6.1), and pooled brain samples were small (range = 5.6-58.4 mg w.w.), which likely reduced our power to discover lower burdens for these compounds.

Our finding that PFOS burdens increased between day 30 of exposure and GS 46 demonstrates that bioaccumulation potential can change through time and/or ontogeny in amphibians. We observed numerical increases in PFOS brain burdens from day 30 to GS 46 in all treatments, but the increase was only detected by Tukey contrasts for the control and PFAS mixture treatments (Fig. 6.1). One potential reason there was not a significant increase in PFOS levels in the brain between 30 days and metamorphosis in the PFOS exposure group could be that the chosen exposure level was sufficiently high to produce significant short-term accumulation, but not a detectable difference over later times. Whether excretion efficiency changes prior to or during metamorphosis is also unknown. Nonetheless, the data suggest that amphibians enter the terrestrial environment with higher PFOS loads than would be predicted based on bioaccumulation studies conducted with early free-swimming larvae (Abercrombie et al., 2019; Hoover et al., 2017).

The months following amphibian metamorphosis are characterized by high mortality and terrestrial life stages have disproportionately large impacts on amphibian population dynamics (Rothermel & Semlitsch, 2006; Vonesh & De la Cruz, 2002). If PFOS carried over from aquatic exposure interacts with natural stressors in the terrestrial environment during this sensitive life stage, this could negatively influence fitness or population growth. Further, because post-metamorphic amphibians serve as prey for a variety of taxa, amphibians may serve as a vector for the transport of PFOS from contaminated wetlands into terrestrial food webs, where both bioaccumulation and biomagnification have been observed (Gronnestad et al., 2019). Lastly, while multiple mechanisms could explain the observed change in PFOS burden over time, increased exposure time alone is unlikely to account for this difference because larval amphibians, including leopard frogs, reach steady state concentrations in < 96 h (Abercrombie et al., 2019). Furthermore, birds have higher levels of PFOS compared to fish, and aquatic respiration compared to aerial

respiration could play a role in the difference (Haukås, Berger, Hop, Gulliksen, & Gabrielsen, 2007). Because GS 46 animals had completed the transition from gilled larvae to air breathing juveniles, it is possible that this change in respiratory mode caused the increase by nullifying gill exchange as an elimination route, a hypothesis that could be tested directly in future work.

We also found that accumulation was dose dependent, with frogs exposed to a mixture of PFAS including 4 ppb PFOS accumulating about 40% of the PFOS accumulated in the frogs exposed to 10 ppb PFOS. This also suggests that co-exposure to other PFAS does not influence PFOS accumulation in the brain. Polar bear brains in East Greenland have been shown to have about 35.2 ng/g w.w. PFOS or 22.92 ng/g wet weight PFOS and approximately 115 ng/g wet weight total PFAS or approximately 28.82 ng/g w.w. total perfluorosulfonates (Eggers Pedersen et al., 2015; Greaves, Letcher, Sonne, & Dietz, 2013). Rats exposed to 20 mg/kg/day PFOS for 28 days had 146 ± 34 µg /g tissue while mice exposed to PFOS during in utero development at 0.3 mg /kg maternal weight/day throughout pregnancy had 3.1 µg/g w.w. PFOS in their brain at birth (Cui et al., 2009; Onishchenko et al., 2011). Comparison of accumulated levels of PFAS can help identify differences in effects between species, because accumulation rate and extraction vary among species. We have previously reported an accumulation of 196 ± 41 ng/g PFOS in bodies of Northern leopard frogs after exposure to 10 ppb PFOS for 30 days during development (Foguth et al., 2019). These levels are not directly comparable due to differences in the exposure route. In the previous study, exposure was through sediment spiked with 10 ppb PFOS whereas in the current study, exposure was through PFOS-spiked water during which all the PFOS added is immediately bioavailable to the frog. It could also be that more PFOS accumulates in the brain of Northern leopard frogs compared to the rest of the body, but that was not determined in this study.

We observed no treatment effects on survival, size, or developmental rates. The absence of treatment effects on survival, size, or development all indicate that PFAS at these concentrations don't significantly affect survival or development in these ways.

After 30 days, there was a significant decrease in 5-HT in the PFAS mixture treatment and glutamate in both the PFOS and the mixture treatment. These effects were not observed in our previous study with exposures ranging from 10-1000 ppb, where there were no changes in 5-HT or glutamate at 10 ppb PFOS in spiked sediment instead of spiked water (Foguth et al., 2019). This indicates that low-level exposure to PFAS causes different effects in neurotransmitter levels compared to the higher exposures we studied previously (Foguth et al., 2019). This is interesting,

because higher levels (100 and 1000 ppb) caused specific dopamine neurotoxicity, while the 10 ppb exposures in the present study did not affect dopamine, but rather 5-HT, glutamate, and ACh, indicating that there is dose-dependent toxicity that significantly changes what neurotransmitters are affected (Foguth et al., 2019). Potential differences could be the bioavailability for PFAS between spiked water vs. spiked sediment, or differences due to the sediment vs. leaf litter. However, after metamorphosis, frogs in both treatment groups had significantly increased ACh compared to controls, with no differences in other neurotransmitter levels. Increased ACh in frogs that had reached GS 46 supports previous research that showed increased differentiation of cells to cholinergic neurons *in vitro* and decreased AChE activity with increased PFAS in polar bear brains, although we did not see an effect on AChE activity in our study (Eggers Pedersen et al., 2015; Slotkin et al., 2008).

To our knowledge, this is the first time neurotransmitter levels were measured in brains of frogs exposed through metamorphosis. Northern leopard frogs are potentially an important model organism for neurotoxicity due to their expression of neuromelanin, which is not present in classic model systems and could better reflect toxicity of certain types of chemicals. Unfortunately, little is known about the basic developmental patterns of neurotransmitter levels in Northern leopard frogs in general. The data presented here indicates that there is a large change in neurotransmitters such as DA, ACh, and GABA. Unfortunately, it is difficult to determine how these changes are affecting general neuronal development of frogs due to the lack of understanding of normal Northern leopard frog neurodevelopment. Furthermore, the differences in neurotransmitter levels between the previous study and this one show that it is important to determine the range that generally occurs in the wild. Levels could potentially be affected by environmental cues, such as weather or predators, and also could be affected by litter used or the presence of different phytoplankton etc in the environment. Knowledge of general development, genome sequence, and basic biology of Northern leopard frogs could help our understanding of how the changes found after PFAS exposure are affecting development. Furthermore, it would be beneficial to perform behavior tests to compare behavioral changes to changes in neurotransmitters after PFAS exposure.

The shift from no significant change in ACh at 30 days to a significant increase in ACh after metamorphosis led us to question whether this was due to an increase in the production of ACh or a decrease in metabolism. Therefore, we performed a preliminary study to measure the activity of AChE in tadpole brains. We found that there was no correlation between developmental

stage and AChE activity in our small sample, and so grouped all animals in each treatment together. There was no significant difference in AChE activity between the treatment groups and the control. Further research needs to be performed to determine the mechanism of ACh increase during later stages of development given the small sample size here for the activity experiment. Furthermore, the production of ACh should also be studied to determine if there is increased production of ACh after PFAS exposure.

In polar bears, there was also a correlation between monoamine oxidase activity and PFAS levels, which could lead to decreased monoamines, such as DA, NE, and 5-HT (Eggers Pedersen et al., 2015). There is potential that an increase in monoamine oxidase activity led to the decrease in 5-HT seen after 30 days of exposure; however, there was also a slight decrease in 5-HIAA, which is produced by metabolism of 5-HT by monoamine oxidase. Furthermore, there was no change in either DA or NE, which are also metabolized by monoamine oxidase. Therefore, further research needs to be performed to determine the mechanism of 5-HT decrease at 30 days of exposure.

Overall, we have shown that PFOS does enter the brain of developing Northern leopard frogs and that exposure levels within ranges observed at AFFF-contaminated site cause neurochemical changes. Our research focused on mesocosm-based exposures of Northern leopard frogs. These exposures more closely reflect an environmental scenario compared to laboratory-based studies; however, they inherently have more variability than controlled laboratory studies. Therefore, the fact that significant changes in neurotransmitter levels were observed during development to PFAS exposures in our mesocosms further supports the significance of the effects observed. Future work using more controlled environments, such as less variable laboratory studies using Northern leopard frogs, are needed to help determine mechanistically what PFAS are affecting leading to these changes in neurotransmitter levels. It is also imperative that research be performed to determine whether these changes are found in other model systems and to determine specific pathways that are being affected by developmental exposure to PFAS.

6.5 Funding

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CHAPTER 7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Rationale

7.1.1 Rationale for studying HAAs

Parkinson's disease is a progressive neurodegenerative disease with more than 90% of cases thought to be sporadic. Therefore, it is imperative to determine risk factors for the disease, including environmental exposures. Although previous studies have shown that certain pesticides, heavy metals, and synthetic drug contaminants can cause PD, few people are exposed to high enough levels for it to cause the disease (J. R. Cannon & Greenamyre, 2010; J. R. Cannon et al., 2009). Therefore, other factors, such as chemicals found in the diet, are of interest for their potential to increase disease risk.

One class of compounds that has sparked interest is the heterocyclic aromatic amines (HAAs), which are found in coffee, tobacco, and meat, as well as other food sources to a lesser extent (Felton et al., 1984; R. J. Turesky, 2007). These compounds are structurally similar to MPTP and MPP+, which are known to cause PD. Furthermore, previous research in our laboratory has shown that PhIP, one of the main HAAs found in charred or overcooked meat, causes DAergic neurotoxicity in a rat primary midbrain culture and decreases DA metabolism after acute exposure in rats (Agim & Cannon, 2018; Cruz-Hernandez et al., 2018).

Harmane and harmine are two other HAAs found in the diet. Harmane is increased in both blood and CSF of patients with PD (Kuhn et al., 1995; Kuhn et al., 1996). While research has shown that derivatives of harmane can cause mitochondrial dysfunction and motor problems, little research has been performed to determine whether harmane also causes this type of toxicity (Albores et al., 1990; Neafsey et al., 1995; Rommelspacher & Subramanian, 1979; Yang et al., 2008).

7.1.2 Rationale for studying PFAS

Per- and polyfluoroalkyl substances (PFAS) are found throughout the planet, especially in contaminated water ways and are present in our drinking water. These compounds have been quantified in brain tissue of polar bears, with PFOS accumulating about 10-100x more than other PFAS (Eggers Pedersen et al., 2015). Behavioral changes, including changes indicating effects on

the motor system, the visual motor system, anxiety, and aggression have been found after developmental exposure in zebrafish (Guo et al., 2018; Jantzen, Annunziato, Bugel, et al., 2016; Jantzen, Annunziato, & Cooper, 2016; Menger et al., 2020; Spulber et al., 2014). Furthermore, changes in enzyme activities, such as MAO and AChE, along with receptor densities for dopamine D2 receptors and GABA-A receptors were found with increasing levels of PFAS in the brains of polar bears (Eggers Pedersen et al., 2015). However, there is still a large gap in the research as to how the majority of neurotransmitters are affected.

7.2 Heterocyclic Aromatic Amine Toxicity

7.2.1 Conclusions from harmane and harmine exposure in vitro

Harmane and harmine both significantly decreased mitochondrial function, with harmane doing so in a dose-dependent manner. Harmane and harmine decreased basal respiration, ATP production, proton leak, and maximal capacity after 24 hours. Both treatments also caused an initial increase in oxidative stress that decreased over time to control levels by 24 hours. Treatment with the antioxidants CoQ10 or NAC did not rescue mitochondrial function, however, indicating that oxidative stress is not the main mechanism of toxicity. They also caused decreased mitochondrial membrane potential after 24 hours, indicated by decreased staining.

Interestingly, isolated mitochondria treated with harmane or harmine for an hour did not have decreased function of mitochondrial complexes I-V. Therefore, we proposed that metabolism was necessary for inhibition of mitochondrial function. However, inhibition of MAO-A and CYP2D through pretreatment with clorgyline or quinidine, respectively, was not able to rescue mitochondrial function.

7.2.2 Conclusions from harmane exposure in rats

Rats exposed to harmane did not have behavioral deficits during the 16 weeks of exposure. Interestingly, the cohort that had all females exposed starting at 9 weeks had significantly increased DA and DOPAC levels in the SN and decreased striatal terminal density which were not present in rats exposed starting at 6 weeks.

7.2.3 Conclusions from PhIP exposure in mice

Mice exposed to PhIP for 8 hours had significantly decreased DA and its metabolites in the striatum. Mice exposed for 4 weeks had a significantly increased ratio of glutamate to GABA, and mice exposed for 16 weeks had significantly increased DA. Interestingly, there was a significantly decrease in striatal terminal density in mice exposed to PhIP for 8 hours, without any changes at later time points.

Furthermore, mice exposed for 16 weeks had decreased motor function, indicated by decreased rears and increased time to descend the pole, starting at 11 weeks of exposure.

7.2.4 Future directions for HAA studies

We found no significant effects of harmane on motor function. It is unclear how the metabolism of harmane differs between humans and rats. It would be beneficial to quantify the metabolism of harmane in rats and measure metabolites in human blood to determine differences in formation of potentially toxic metabolites. This could be performed as a time-course assay to corroborate the estimated oral bioavailability of harmane, 19.2-19.4 % and help determine the oral half-life, which has been calculated to be 29 minutes or 2.26 hours (Guan et al., 2001; S. Li et al., 2016). Furthermore, comparing the half-lives of metabolites to metabolism effects on mitochondrial function could help determine the mechanism of toxicity of harmane and harmine.

Another aspect of harmane exposure to study is essential tremor. Research has shown that patients with essential tremor have increased harmane and norharmane in blood and CSF (Louis, Benito-Leon, et al., 2013; Louis, Factor-Litvak, et al., 2013; Louis et al., 2012). Rats treated with harmane should be studied to determine potential essential tremor, or motor tremor, effects, such as measuring the frequency of tremors, and brain regions affected in essential tremor, such as the olivocerebellum, should be studied to determine potential neurotoxicity (Pan, Ni, Wu, Li, & Kuo, 2018).

PhIP exposure in mice was found to cause decreased motor function after 11 weeks. However, after 16 weeks of exposure, mice treated with PhIP appeared to have more health issues than just motor dysfunction. Therefore, subchronic exposure to a smaller amount of PhIP could help elucidate specific toxicity. Further research should be performed to determine the effects of PhIP on α-syn and oxidative stress in the DA neurons of the SN. We have also shown that there is

an increase in p-tau and amyloid β, indicating potential neurotoxic effects leading to Alzheimer's disease-like pathology (Syeda, Foguth, Llewellyn, & Cannon, 2020). Therefore, testing more behavioral tests, such as those for memory and learning, is imperative.

Another aspect of toxicity that needs to be taken into account for HAAs is the presence of neuromelanin. It is clear that certain chemicals can interact with neuromelanin, in an attempt to decrease toxicity, and then be released all at once, leading to a large exposure (Barbeau, Dallaire, Buu, Poirier, et al., 1985; Barbeau, Dallaire, Buu, Veilleux, et al., 1985; Zecca et al., 2006). These chemicals are structurally very similar to the HAAs we are studying, so it is logical to hypothesize that this can also occur with the HAAs found in the diet. We have shown that different types of melanin, including a DA melanin, can bind to harmane and PhIP, and that the presence of neuromelanin in cells increases their toxicity (Lawana et al., 2020). However, neither mice nor rats express significant amounts of neuromelanin. The mechanism of neuromelanin production is unclear, but one model used is ectopic expression of tyrosinase (Carballo-Carbajal et al., 2019; Hasegawa, 2010). Therefore, using a rat that expresses tyrosinase in DA neurons could closer resemble HAA toxicity than using wild-type rats or mice.

We showed that harmane and harmine decrease mitochondrial function after 24 hours, and propose this is caused by metabolism to a toxic intermediate. While we found that inhibition of MAO-A and CYP2D did not rescue mitochondrial function, there are other enzymes that metabolize harmane and harmine to other compounds. Possible toxic metabolites are harmol, which is produced from harmane through metabolism by CYP2A, which is inhibited with (S)-nicotine (Figure 7.1). Future research should focus on determining the toxic metabolites and quantifying the levels of toxic metabolites after a known exposure. Research should also continue to determine potential therapeutics that decrease the toxic effects of harmane and harmine.

7.2.5 Proposed mechanism of harmane metabolism and potential toxic metabolites

The known first pass metabolites for harmane and harmine are shown in Figure 7.1. Most of this metabolism occurs through enzymatic reactions by cytochrome P450s. Many of the metabolites are mitochondrial respiration inhibitors (Albores et al., 1990). Interestingly, though, the mitochondrial toxicity of harmane has not previously been reported. We have shown that harmane and harmine inhibit mitochondrial function after 24 hours.After 3 hours, harmine started to decrease mitochondrial function, but not significantly, while harmane did not cause any

inhibition. Furthermore, isolated mitochondria treated with harmane or harmine for one hour did not have altered mitochondrial function, either. Therefore, we hypothesize that one of the metabolites is causing the mitochondrial dysfunction.

The proposed mechanism of toxicity is present in Figure 7.1. We showed that inhibition of CYP2D6 and MAO-A through quinidine and clorgyline, respectively, did not significantly rescue the mitochondrial dysfunction caused by harmane and harmine. Future research needs to be performed to determine other enzymes that are important for the metabolism of these compounds and testing the inhibition of specific enzymes to determine if they rescue mitochondrial function.

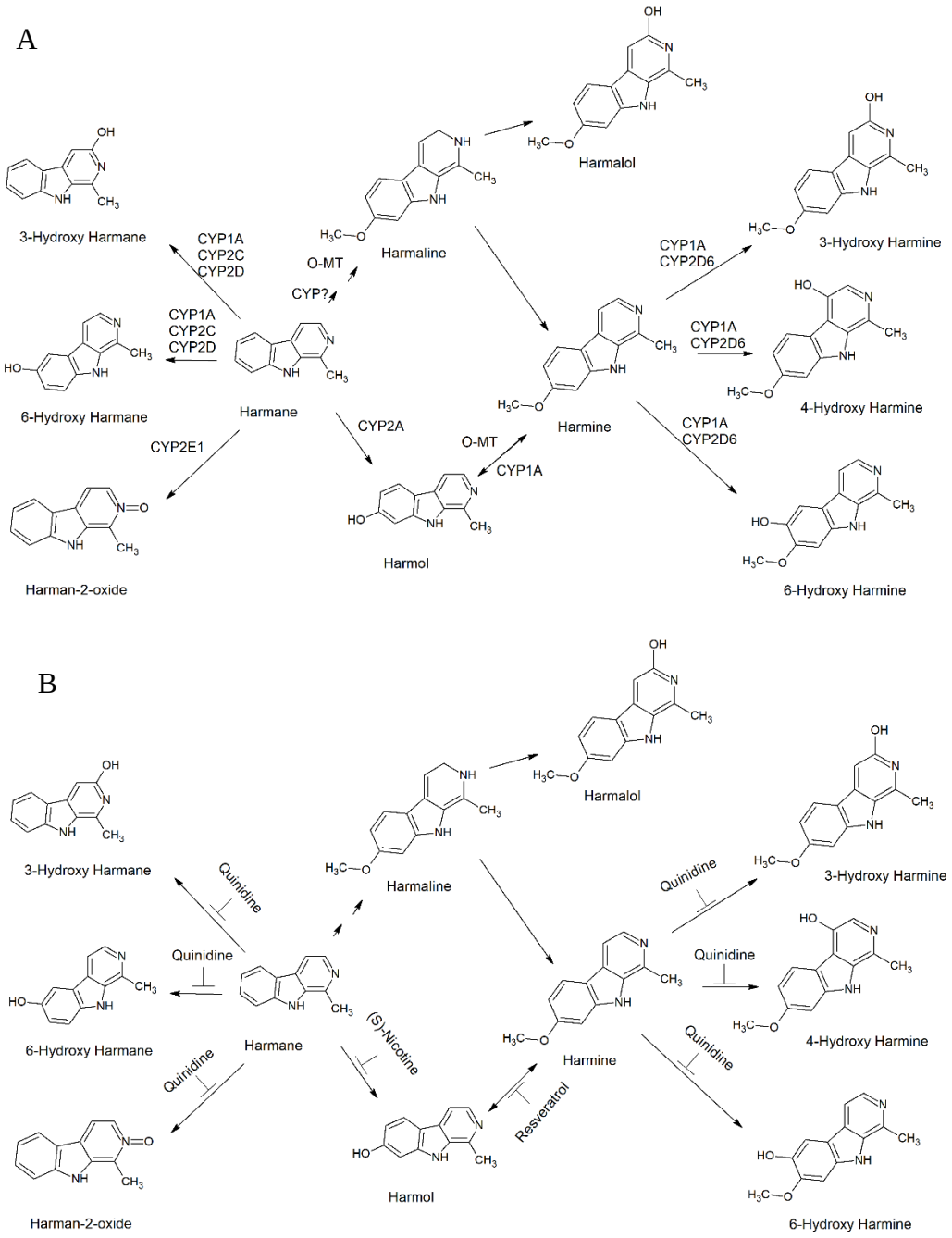

Figure 7.1 Proposed mechanism of harmane toxicity. The proposed mechanism of Phase I metabolism including A) the enzymes that produce the chemicals, or B) the chemicals that inhibit the enzymes.

7.3 PFAS Toxicity

7.3.1 Conclusions for PFAS neurotoxicity

Due to the potential for developmental neurotoxicity of PFAS, we exposed Northern leopard frogs to various PFAS for 30 days or through metamorphosis. We found that 100 and 1000 ppb PFOS as well as 1000 ppb PFOA decreased DA and increased DA turnover without significantly affecting other neurotransmitters. Interestingly, in our study focusing on the lower exposure levels of PFOS or a mixture of PFAS, there were not significant changes in physical features, such as time to metamorphosis, length, or mass. After 30 days of exposure, frogs exposed to the mixture of PFAS had significantly decreased serotonin, while both the mixture and the PFOS only treatment had decreased glutamate and a nonsignificant decrease in acetylcholine. Interestingly, at metamorphosis, there was a significant increase in acetylcholine in both treatment groups and no significant changes in serotonin or glutamate. This indicates that there are dose-dependent changes in neurotransmitter levels in Northern leopard frogs exposed to PFAS, and that the effects differ over time. Furthermore, there was a nonsignificant increase in norepinephrine in the hearts of treated frogs, indicating potential effects on sympathetic innervation of the heart.

7.3.2 Future directions for PFAS studies

Unfortunately, little is known about changes in neurotransmitters in developing Northern leopard frogs during regular development. Therefore, it is important that studies are performed to understand the normal development of these frogs with respect to neurotransmitter levels and amount of neuromelanin present. After this is better understood, it is imperative to determine whether PFAS can bind to neuromelanin, as other chemicals such as HAAs have been shown to do. Furthermore, cell culture studies, such as research in rat primary midbrains or *ex vivo* studies would be useful in doing more high throughput studies to determine how individual PFAS affect neurodevelopment and how mixtures differ.

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VITA

Education

Ph.D. Integrative Neuroscience/Toxicology, Purdue University, West Lafayette, IN 47907

B.S. Biochemistry, Cum Laude, Benedictine College, Atchison, KS 66002, GPA 3.644, May 2015

Certificate of Completion, Purdue Applied Management Principles Program (Purdue's Mini MBA), Purdue University, West Lafayette, IN 47907, May 2019

Industry Experience

Toxicology/Biocompatibility Intern, Cook Biotech (September-December 2019):

Create heat map with completed reports to help gap analysis to determine how to proceed to fulfill ISO 10993 requirements. Perform gap analysis for medical devices regulated by ISO 10993, and determine studies necessary for certification of novel medical device through ISO standards.

Research Experience

Graduate Research, Purdue University (2015-present):

Studying the neurotoxicity of heterocyclic amines found in food and per- and polyfluorinated compounds found in the environment. It is thought that heterocyclic amines and perfluorinated compounds cause mitochondrial dysfunction, leading to dopaminergic neuron loss or neurodevelopmental problems. We are studying this using rats, mice, frogs, and in vitro methods.

Summer Undergraduate Research Program, University of Nebraska-Lincoln (2014):

Studied the mechanism of manganese and alpha-synuclein toxicity on dopaminergic neurons. Manganese toxicity and alpha-synuclein buildup are seen to cause neuronal cell death, as seen in Parkinson's disease.

Summer Undergraduate Research Program, University of Texas Medical Branch (2013):

Studied the Aryl Hydrocarbon Receptor in Mus Musculus and its role in mediating the regulation of gene expression via TCDD and cinnabarinic acid treatment.

Undergraduate Research Assistant, Benedictine College (2012-2014):

Studied the requirement of tes-1 and unc-34 during formation of the C. elegans vulva. Loss of these genes results in aberrations to epithelial cell structure and defects in migration. The tes-

1¬ gene is a homolog of a human gene, testin, that is potentially a tumor suppressor, as it is lost in many metastatic tumors.

Specific Technical Expertise

- Working with ISO standards and GLP toxicity studies
- Managing a laboratory
    - Ensure safe laboratory practices as the Safety Officer of a laboratory, including training laboratory staff and ensuring use of safe practices
    - Inventory and ordering of material for research
    - Coordinating laboratory relocation efficiently
    - Training laboratory members on instrumentation and protocols
- HPLC maintenance, sample preparation, running, and analysis
- Developing and performing animal experiments ethically
    - Rat, mouse, and frog maintenance
    - IP injection, oral gavage, sacrifice, and collection of brain and heart
    - Fine dissection of rat and mouse brains to obtain specific brain regions
    - Rat and mouse behavioral tests for motor dysfunction, including pole test, rearing test, and postural instability test
    - Frog behavioral tests for motor dysfunction, including flip over time and distance hopped
    - C. elegans maintenance including performing genetic crosses and isolation of phenotypic mutants
- Harvesting rat pups and removing the brain for use as primary neurons in cell culture
- Isolation of primary hepatocytes in Mus Musculus
- Isolation of mitochondria and testing of individual mitochondrial complex activities
- Microscopy, extensive use of stereomicroscopes, light microscopes, fluorescent microscopes, and confocal microscopes
- Flow cytometry, use of flow cytometry to look at cell viability, glutathione uptake, and mitochondrial reactive oxygen species
- Immunochemistry, including immunohistochemistry and immunocytochemistry
- Cell culture
    - Maintenance of a cell line, including changing media, infecting, treating, and splitting

- Cell viability assays, including luciferase assay, MTT assay, and cell counts with Trypan blue
- Extracellular Flux Analysis for mitochondrial respiration and glycolysis testing using a Seahorse Extracellular Flux Analyzer

- Media preparation, making buffers
- Protein extraction, Western Blot
- DNA extraction, PCR- based genotyping, RT-PCR -based semi-quantitative analysis, gel electrophoresis
- Mitochondrial isolation

Publications

- Foguth, RM, Hoskins, TD, Clark, GC, Nelson, M, Flynn, RW, de Perre, C, Hoverman, JT, Lee, LS, Sepúlveda, Cannon, JR; "Single and mixture per- and polyfluoroalkyl substances accumulate in developing Northern leopard frog brains and produce complex neurotransmission alterations." *Neurotoxicology and Teratology*. 2020; 81:106907. doi: 10.1016/j.ntt.2020.106907. PMID: 32561179
- Foguth, RM, Sepúlveda, MS, Cannon, JR; "Per- and polyfluoroalkyl substances (PFAS) neurotoxicity in sentinel and nontraditional laboratory model systems: potential utility in predicting adverse outcomes in human health." *Toxics*. 2020; 8(2);E42. doi: 10.1016/j.taap.2019.114623. PMID: 31195004
- Foguth, RM, Cannon, JR; "Emerging contaminants as contributors to parkinsonism: heterocyclic amines." *Parkinsonism and the Environment*. Springer. In press.
- Syeda, T, Foguth, RM, Llewellyn, E, Cannon, JR; "PhIP exposure in rodents produces neuropathology potentially relevant to Alzheimer's disease." *Toxicology*. 2020; 437:152436. doi:10.1016/j.tox.2020.152436. PMID: 32169473
- Patel, SH, Yue, F, Saw, SK, Foguth, RM, Cannon, JR, Shannahan, JH, Kuang, S, Sabbaghi, A, Carroll, CC; "Advanced glycation end-products suppress mitochondrial function and proliferative capacity of achilles tendon-derived fibroblasts." *Scientific Reports*. 2019; 9(1):12614. doi:10.1038/s41598-019-49062-8. PMID: 31471548
- Sammi, SR, Foguth, RM, Nieves, SC, De Perre, C, Wipf, P, McMurray, CT, Lee, LS, Cannon, JR; "Perfluorooctane sulfonate (PFOS) produces dopaminergic neuropathology

in C. elegans." *Toxicological Sciences*. 2019; 172(2):417-434. doi:10.1093/toxsci/kfz191. PMID: 31428778

- Foguth, RM, Flynn, RW, de Perre, C, Iacchetta, M, Lee, LS, Sepúlveda, MS, Cannon, JR; "Developmental exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) selectively decreases brain dopamine levels in Northern leopard frogs." *Toxicology and Applied Pharmacology*. 2019; 377:114623. doi:10.1016/j.taap.2019.114623. PMID: 31195004
- Fernandez, RF, Kim, SQ, Zhao, Y, Foguth, RM, Weera, MM, Counihan, JL, Nomura, DK, Chester, JA, Cannon, JR, Ellis, JM; "Acyl-CoA synthetase 6 enriches the neuroprotective omega-3 fatty acid DHA in the brain." *Proceedings of the National Academy of Sciences*. 2018; 115(49):12525-12530. doi:10.1073/pnas.1807958115. PMID: 30401738
- Hernandez-Franco, P, Anandhan, A, Foguth, RM, Franco, R; "Oxidative Stress and Redox Signalling in the Parkinson's Disease Brain." *Oxidative Stress and Redox Signalling in Parkinson's Disease*. Royal Society of Chemistry. Co-author. 2017.

Abstracts/Posters

- "Effects of Developmental Exposure to Perfluoroalkyl Substances on Brain and Heart Innervation in Northern Leopard Frogs" For the National Society of Toxicology Meeting, Anaheim, CA, 2020.*
- "Developmental Exposure to PFOS and PFOA Produces Selective Dopamine Decreases in Leopard Frogs" Foguth, R; Flynn, R; Sepulveda, M; Cannon, J. Presented at the Parkinson's Disease Gordon Research Conference, Newry, ME, 2019.
- "Neurobehavioral and Neurochemical Effects of Acute to Subchronic PhIP Exposure" Foguth, R; Cannon, J. Presented at the Office of Interdisciplinary Graduate Studies Spring Reception, West Lafayette, IN, 2019.
- "Neurobehavioral and Neurochemical Effects of Acute to Subchronic PhIP Exposure" Foguth, R; Cannon, J. Presented at the National Society of Toxicology Meeting, Baltimore, MA, 2019
- "Heterocyclic Amine-Induced Bioenergetics Alterations" Foguth, R.; Agim, Z. S.; Cannon, J. Presented at the Purdue Office for Interdisciplinary Graduate Programs Spring Reception, West Lafayette, IN, 2018.

- “Heterocyclic Amine-Induced Bioenergetics Alterations” Foguth, R.; Agim, Z. S.; Cannon, J. Presented at the Ohio Valley Society of Toxicology Graduate Summer Meeting, West Lafayette, IN, 2018.
- “Heterocyclic Amine-Induced Alterations in Neuronal Bioenergetics” Foguth, R.; Agim, Z. S.; Cannon, J. Presented at the Indiana Society for Neuroscience Meeting, West Lafayette, IN, 2018.
- “Heterocyclic Amine-Induced Bioenergetics Alterations” Foguth, R.; Agim, Z. S.; Cannon, J. Presented at the National Society of Toxicology Meeting, San Antonio, TX, 2018.
- “Heterocyclic Amine-Induced Bioenergetics Alterations” Foguth, R.; Agim, Z. S.; Cannon, J. Presented at the Ohio Valley Society of Toxicology Meeting, West Lafayette, IN, 2018.
- “Characterization of a Potential Model for Parkinson’s Disease: PhIP Exposure in Mouse” Foguth, R.; Cannon, J. Presented at the Purdue Institute for Integrated Neuroscience Retreat, St. Joseph, MI, 2017.
- "The Use of PCR and Gel Electrophoresis in Looking at Epithelial Cell Morphogenesis in C. elegans" Foguth, R.; Schramp, M. Presented at Benedictine College's Discovery Day 2015, Atchison, KS, 2015.
- "Dopaminergic Cell Death Induced By Manganese and Alpha-Synuclein: Oxidative Stress, p38, and Nuclear Factor-Kappa B Signaling" Foguth, R.; Anandhan, A.; Franco-Cruz, R. Presented at University of Nebraska-Lincoln's Summer Undergraduate Research Program Symposium, Lincoln, NE, 2014.
- "Effects of TES-1 and UNC-34 proteins on the Behavior of Epithelial Cells" Foguth, R.; Schramp, M. Presented at Benedictine College's Discovery Day 2013, Atchison, KS, 2013.
- Formal Presentations
- “Effects of Developmental Exposure to Perfluoroalkyl Substances on Brain and Heart Innervation in Northern Leopard Frogs” Foguth, R.; Hoskins, T.; Clark, G.; Nelson, M.; Flynn, R.; de Perre, C.; Lee, L.; Sepulveda, M.; Cannon, J. Accepted for the National Society of Toxicology Meeting, Anaheim, CA, 2020.*

- “The Link Between Diet and Parkinson’s Disease” Foguth, R.; Cannon, J. Invited presentation presented at the Purdue Lecture Hall Series, 2019.
- "Dopaminergic Cell Death Induced by Manganese and Alpha-Synuclein: Oxidative Stress, p38, and Nuclear Factor-Kappa B Signaling" Foguth, R.; Anandhan, A.; Franco-Cruz, R. Presented at Wakarusa Valley American Chemical Society Student Research Symposium, Atchison, KS, 2014.
- "Observing the Genetic Interactions of TES-1 and UNC-34 in Vulval Development of C. elegans" Foguth, R.; Nickel, N.; Denton, M.; Schramp, M. Presented at Benedictine College's Discovery Day 2014, Atchison, KS, 2014.

Teaching Experience

- Prepared and taught de novo lecture on the toxic effects of tetrahydrocannabinol on the adolescent brain, Spring 2019.
- Health Sciences Seminar Teaching Assistant. Keep attendance, evaluations, and substitutions and assign grades, Fall 2018.
- Undergraduate Research Student Teacher. Taught an undergraduate student laboratory techniques and basic research skills, including immunohistochemistry and cell culture work. Fall 2015-current.
- Organic Chemistry Laboratory Assistant. Assist in teaching organic chemistry laboratory and grade formal laboratory reports, Fall 2014.
- Chemistry Tutor. Tutor students in General Chemistry and Organic Chemistry, Fall 2013 - Spring 2014, Spring 2015.
- Grader for Organic Chemistry Laboratory. Grade both laboratory reports and notebooks of Organic Chemistry students, Fall 2013 - Spring 2015.
- General Chemistry Laboratory Assistant. Helped students with questions about the experiments. Helped students write laboratory reports for one hour each week, Spring 2013.

Honors and Awards

- 3rd Place in Toshio Narahashi Graduate Student Poster Competition Award, Society of Toxicology, Spring 2020
- School of Health and Human Sciences award to attend the Applied Management Principles program at Purdue University, Spring 2019

- Purdue Institute of Integrative Neuroscience Travel Grant, 2018
- Women in Science Travel Grant, 2017
- Compton Graduate Student Research Travel Award, Health and Human Sciences, 2017
- Outstanding Senior Biochemistry/Chemistry Major Award, Benedictine College, ACS Wakarusa Valley Section, Spring 2015
- 1st Place in American Chemical Society Wakarusa Chapter Local Oral Presentation, Benedictine College, Fall 2014
- Department of Toxicology Undergraduate Research Award, University of Texas Medical Branch, Summer 2013
- Sr. Mary Noel Scholarship (Academic Scholarship), Benedictine College, Fall 2012-Spring 2014, $6,000/semester
- Presidential Participation Award, Benedictine College, Fall 2012, Spring 2013, $500/semester
- Dean's List, Benedictine College, Fall 2012, Spring 2013, Spring 2014, Fall 2014
- Dean's List, Waubonsee Community College, Fall 2008-Spring 2012

Extra-Curricular Activities

- President of Purdue Health Sciences Graduate Student Organization, 2019-2020
- Chair of the first annual Purdue Health Sciences Research Retreat, 2019
- Vice President of Purdue Health Sciences Graduate Student Organization, 2018-2019
- Member of the Society of Toxicology, Fall 2016-present
- Member of the American Society for Biochemistry and Molecular Biology, Fall 2013-present
- Member of the American Society of Pharmacology and Experimental Therapeutics, Summer 2013-present
- President of the Benedictine College Biology Club, Fall 2013-Spring 2014
- Member of Phi Theta Kappa Honor Society, Fall 2010-present
- Campus Ministry volunteer, Fall 2013-present

Community Service

- Graduate Judge for Purdue Interdisciplinary Life Sciences Spring Reception, 2017-2019

- Member on panel for first year graduate students of Purdue Interdisciplinary Life Sciences, Fall 2018
- Science in Schools Volunteer helping with science experiments in elementary schools, 2015-2018
- Present research to Animal Sciences class, Fall 2016
- Retreat helper for middle school retreats, Spring 2014
- Spooky Science Fun Bash Volunteer helping with science experiments and baking, 2013, 2014
- Squashing Hunger Volunteer, Fall 2013
- Extraordinary Minister of Holy Communion, Fall 2011-Spring 2015
- Member of youth ministry in St. Gall Catholic Church, Fall 2009-Spring 2012
- Pastoral Council Member, Spring 2011-Spring 2012

* Conference cancelled due to COVID-19 pandemic