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Chronic Effects of Methylphenidate on Neuronal Viability and Plasticity

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Chronic Effects of Methylphenidate on Neuronal Viability and Plasticity

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ABSTRACT

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Methylphenidate (MPH) is the most commonly prescribed drug to treat Attention Deficit Hyperactivity Disorder (ADHD). ADHD is now considered a life-long disorder; therefore, patients take MPH from adolescence into adulthood, highlighting the need for research studying chronic MPH use. MPH increases dopamine and norepinephrine within the synaptic cleft; therefore, chronic use of MPH may lead to changes within important dopaminergic pathways. One pathway, the mesolimbic pathway, includes the hippocampus, an area where adult neurogenesis occurs. We investigated the effects of chronic low and high doses of MPH on neurogenesis and examined levels of a few key proteins linked to cell proliferation in the hippocampus. Low dose MPH appears to increase cell proliferation and cell survival in the hippocampus, and these effects are accompanied by increases in vascular endothelial growth factor (VEGF), the receptor for brain-derived neurotrophic factor (TrkB), and beta-catenin. While high dose MPH may initially increase neuronal proliferation, newly-generated neurons are unable to survive long-term, and decreases in VEGF, TrkB, and beta-catenin are observed with chronic high dose MPH.

Another major dopaminergic pathway is the nigrostriatal pathway, which is involved in motor control and degenerates with Parkinson's disease. Chronic use of MPH appears to sensitize dopaminergic neurons within this pathway to the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP), but the cause of this sensitization is unknown. The autooxidation of excess dopamine forms dopamine-quinones that lead to free radical production, but the antioxidant, glutathione, can protect neurons. However, we showed that chronic MPH increases dopamine-quinone formation and causes a subsequent glutathione depletion within the striatum. Therefore, oxidative stress may sensitize dopamine neurons to MPTP.

We also assessed the vulnerability of dopaminergic neurons in the nigrostriatal pathway to MPTP after chronic MPH in females. Interestingly, proestrus (high estrogen) females were more sensitive to MPTP than anestrus (low estrogen) females. Similar to males, chronic MPH caused a depletion in glutathione that was further decreased following MPTP exposure. However, chronic MPH did not significantly alter dopaminergic neuronal numbers or quinone formation in females. These studies highlight some of the potential effects of chronic MPH use.

DEDICATION

I want to dedicate this work to my family who supported me through this endeavor. To my son, Benjamin Oakes who kept me laughing and my husband, Aaron Oakes whose love and support I could not do without.

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CHAPTER 1. INTRODUCTION

Attention Deficit Hyperactivity Disorder

Attention deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by inattention, hyperactivity, and impulsivity. Over the past few decades, there has been a steady increase in the number of children diagnosed with ADHD. The Centers for Disease Control and Prevention (CDC) reports that as of 2016, 6.1 million children have been diagnosed with ADHD in the United States (Danielson et al. 2018). Additionally, in the past, ADHD was thought of as a childhood disorder; however this perception of ADHD has changed, and ADHD symptoms are known to persist from childhood to adulthood in 80% of individuals diagnosed as children (Faraone 2018; Klein et al. 2019). Thus, many children continue to take medications for ADHD well into adulthood (Bonvicini et al. 2016). Moreover, many individuals are misdiagnosed with ADHD that do not meet the full diagnostic criteria medically, further increasing the number of individuals diagnosed with and treated for ADHD.

ADHD is diagnosed using criteria found in the American Psychiatric Association's Diagnostic and Statistical Manual, fifth edition (DSM-5). The criteria rely heavily on self-reporting or reporting from parents. The DSM-5 lists two categories, inattention and hyperactivity-impulsivity, and multiple symptoms that signify each category. Children up to the age of sixteen must exhibit six or more symptoms that are inappropriate for their developmental level in each category for at least six months. Individuals seventeen years or older must exhibit five or more symptoms in each category. Individuals with inattention may exhibit the following symptoms: fail to give close attention to details resulting in careless mistakes, have difficulty holding their attention on task, fail to follow through with schoolwork, chores, or workplace

tasks, have trouble organizing tasks, reluctant or avoid tasks that require mental effort over an extended period of time, lose things that are needed to complete tasks often, and become easily distracted. Hyperactivity may present as fidgeting, running, and climbing at inappropriate times (often seen as restlessness in adults), unable to do activities quietly, always on the go, and excessive talking. Impulsivity is characterized by blurting out answers before a question has been completed, trouble waiting turns, and often interrupts or intrudes on others (Attention-deficit/hyperactivity disorder (adhd) 2019; Health 2009; Tarver et al. 2014). Moreover, symptoms must have been present before the age of twelve, present in two or more settings, interfere with the quality of life, and not better explained by another mental disorder.

ADHD is further characterized by three different presentations: inattention, hyperactivity/impulsivity, and a combined presentation. An individual is characterized as inattentive when enough symptoms of inattention, but not hyperactivity-impulsivity were present for the past six months. A hyperactivity-impulsivity presentation is characterized by a majority of hyperactivity-impulsivity symptoms within the past six months. A combined presentation is when enough symptoms from all criteria were present for the past six months (Attention-deficit/hyperactivity disorder (adhd) 2019; Posner et al. 2020). At present, four developmental trajectories are known, starting with early onset or preschool ADHD occurring at three to five years of age, middle childhood six to fourteen years, middle childhood with adolescent offset, and adolescent or adult onset from sixteen years and older (Posner et al. 2020). Altogether, the DSM-5 provides a guideline for diagnosing ADHD that is more of a qualitative assessment than a quantitative one.

The cause of ADHD is unknown, but there are many factors that may increase the risk for ADHD. These risk factors include prenatal smoking, prematurity or low birth weight, and diet

(Posner et al. 2020; Tarver et al. 2014). Additionally, males are two times more likely to be diagnosed with ADHD than females (Sridhar et al. 2017). Furthermore, ADHD has been linked to abnormalities in several different neurotransmitter systems. For example, cognitive control and symptom severity has been linked to glutaminergic deficits in frontal cortical and striatal regions. Further, methylation of the serotonin transporter is linked to an increase in the severity of symptoms (Faraone 2018). With so many different pathways with altered functions, individuals with ADHD also have comorbidities such as anxiety, mood, substance use, sleep disturbances, and personality disorders (Faraone 2018). Additionally, single photon emission computed tomography (SPECT) revealed that there is decreased cerebral blood flow in the temporal and cerebellar region and increased blood flow in the subcortical and thalamic regions in the brains of individuals with ADHD (Gustafsson et al. 2000).

Differences in brain morphology may also contribute to ADHD. Individuals with ADHD have decreased global brain volume. In particular, volume reductions have been observed in the nucleus accumbens, amygdala, caudate, hippocampus, and putamen when compared to controls (Faraone 2018). These reductions appear to involve both white and grey matter. Decreased white matter is seen in areas such as the striatum, frontal, temporal, and parietal lobes, and decreased grey matter is noted in areas that form the frontostriatal circuits (Faraone 2018; Nagel et al. 2011; Nakao et al. 2011; Posner et al. 2020; Tarver et al. 2014). There are many brain structures that are altered in individuals with ADHD, but the prefrontal cortex, sensory cortex, motor cortex, anterior cingulate cortex, parietal cortex, striatum, and thalamus are affected more than other brain regions (Frolich et al. 2014). Naturally, research on the pathology of ADHD has expanded to neural circuits involving the areas listed above. For example, individuals with ADHD appear to have reduced activation of the mesolimbic neural circuit when anticipating

rewarding outcomes. Alterations in the mesolimbic circuit are noteworthy, as, it is associated with motivated behaviors, anticipated outcomes, and reinforced learning (Posner et al. 2020).

ADHD has a complex etiology, but evidence indicates that genetics appear to play a role, as there are heritability estimates around 0.7% (Posner et al. 2020; Tarver et al. 2014; Tistarelli et al. 2020). No single gene or genetic risk factor has been identified, suggesting that ADHD may develop from the interactions of several different genes (Tarver et al. 2014; Tistarelli et al. 2020). Much of the research has focused on genes involved in dopaminergic and noradrenergic transmission. Genes of interest include the dopamine transporter, norepinephrine transporter, and dopamine receptors, D4 and D5 (Klein et al. 2019; Tarver et al. 2014). Increased levels of dopamine transporter are seen in ADHD brains (Klein et al. 2019). Inattention presentation has been associated with increased dopamine receptors (D2 and D3) and decreased synaptic dopamine in the striatum and decreased DA synthesis in the nucleus accumbens (Volkow et al. 2012).

The treatment of ADHD includes behavioral modification and pharmacotherapy. Many medical organizations such as the National Institutes of Health recommend the use of medications to manage ADHD symptoms, along with behavioral modification. The medications used to treat ADHD target dopamine and norepinephrine; if the drugs increase dopamine, they are considered psychostimulants. There are some non-stimulant medications that are used to treat ADHD, such as atomoxetine, but psychostimulants are generally favored due to well-established efficacy (Gibson et al. 2006). Psychostimulants were first used in children in the 1930s and are considered first-line treatment for management of ADHD symptoms today.

Norepinephrine and Dopamine

Norepinephrine (NE) is sometimes referred to as noradrenaline; therefore, NE containing neurons are referred to as noradrenergic neurons. Noradrenergic neurons in the locus coeruleus project throughout the brain; however, the projection of noradrenergic neurons to the limbic system is highly relevant to the current work. NE is thought to play a role in sleep and wakefulness, attention, and feeding behavior (Martins et al. 2017). NE is released into the synaptic cleft following an action potential, where it interacts with α- and β-adrenergic receptors on both pre- and postsynaptic sites.

Dopamine (DA) is produced in four major pathways within the CNS, including the mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular pathways. Two of these, the nigrostriatal pathway, and the mesolimbic pathway, are highly relevant to the research in this dissertation. The mesolimbic pathway originates in the ventral tegmental area and projects to the amygdala, hippocampus, and the nucleus accumbens (Juarez Olguin et al. 2016). The mesolimbic pathway is important in reward and motivation (Klein et al. 2019). The nigrostriatal pathway is responsible for motor function, learning, and modulation of pain (Haber 2014). Neurons originating in the substantia nigra project to the caudate and putamen nuclei of the striatum, forming the nigrostriatal pathway. The nigrostriatal pathway plays an important role in voluntary movement control by modulating corticostriatal transmission directly in neurons expressing the DA receptor, D1 resulting in movement activation (Martins et al. 2017). Altogether, DA is thought to play a major role in modulation of behavior and cognition, body movement, motivation, reward and reinforcement, inhibition of prolactin production, nausea, learning, sleeping, and dreaming (Haber 2014; Juarez Olguin et al. 2016; Klein et al. 2019).

DA and NE are both catecholamines that are derived from tyrosine. Tyrosine hydroxylase and the cosubstrate oxygen and cofactor tetrahydrobiopterin catalyze the reaction from the amino acid tyrosine to dihydroxyphenylalanine also known as DOPA. The formation of DOPA is the rate-limiting step in the reaction. DOPA is then catalyzed by DOPA decarboxylase to yield DA. In noradrenergic neurons, the presence of the enzyme dopamine-β hydroxylase catalyzes the conversion of DA to NE (Figure 1.1).

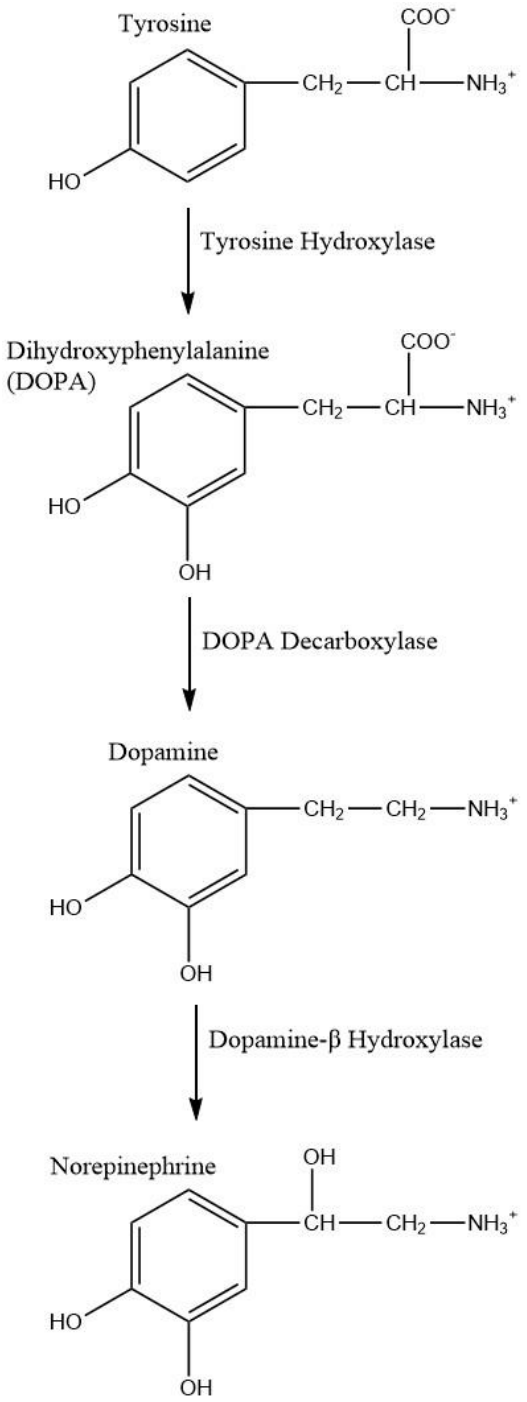

Figure 1.1 Dopamine and Norepinephrine Synthesis. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. DOPA is then converted to dopamine by DOPA decarboxylase. Finally, dopamine is converted to norepinephrine by the catalysis dopamine-β hydroxylase.

Once DA or NE is synthesized within presynaptic neurons, these catecholamines are moved into vesicles by vesicular monoamine transporter 2 (VMAT-2). Vesicles provide a stable environment for catecholamines, because they can maintain a lower pH (Klein et al. 2019) . The catecholamines are then released into the synaptic cleft upon vesicular fusion with the plasma membrane, which occurs following an influx of Ca^{2+}. Dopamine may be released at the axon terminus following an action potential, but dopaminergic neurons may also release DA from their cell bodies and dendrites (Liu and Kaeser 2019). DA signaling is terminated in the synaptic cleft by the reuptake of DA through the dopamine transporter (DAT). DA can then be recycled back into vesicles by VMAT-2 or broken down by the enzymes, monoamine oxidase (MOA) and catechol O-methyltransferase (COMT). Similar to dopamine, NE is cleared from the synaptic cleft by the norepinephrine transporter (NET). NE is then recycled or broken down by the same enzymes that catabolize DA, MOA and COMT. Interestingly, DA can be taken back up into the cell by the NET as well as the DAT (Klein et al. 2019; Volkow et al. 2012). In fact, DA has a greater affinity for the NET than the DAT, implying that DA may be released by noradrenergic neurons as well as dopaminergic neurons (Konova et al. 2013). Interestingly, noradrenergic neurons located in the hippocampus are known to release DA (Klein et al. 2019).

DA released into the synaptic cleft can interact with presynaptic or postsynaptic DA receptors. There is evidence that DA also binds to and activates adrenergic receptors (Klein et al., 2019). There are two groups of DA receptors D1-like and D2-like. D1-like receptors include D1 and D5, whereas D2-like receptors include D2, D3, and D4 (Xing et al. 2016). All DA receptors are metabotropic and lead to the activation of second messengers (Klein et al. 2019). D1 and D5 receptors are mainly found at post-synaptic sites, while D2 and D3 can be found both pre- and post-synaptically. D4 is expressed in the retina (Klein et al. 2019). Moreover, D2-like

receptors have a greater affinity for DA than the D1-like receptors. Thus, it is believed that D1-like receptors are activated by high concentrations of DA (phasic release), and D2-like receptors are activated by low concentrations of DA (tonic release) (Klein et al. 2019).

D1-like receptors are found in the caudate putamen of the striatum, nucleus accumbens, substantia nigra pars reticulata, olfactory bulb, amygdala, and frontal cortex. D-2 like receptors can be found in the striatum, core of the nucleus accumbens, ventral tegmental area, hypothalamus, amygdala, cortical areas, hippocampus, and pituitary. D2 receptors are capable of inhibiting DA synthesis, enhancing DA uptake, and regulating VMAT2 expression (Liu and Kaeser 2019). When presynaptic D2 receptors are activated they inhibit neuron firing (Liu and Kaeser 2019). D2 receptors that inhibit DA synthesis and inhibit neuron firing and thus, the release of DA, are known as autoreceptors and they help to maintain homeostatic levels of DA (Juarez Olguin et al. 2016). Since DA dysfunction is thought to be a major part of ADHD, medications used to treat ADHD often target the DA system and may normalize DA function.

Methylphenidate

In the United States alone, 3.5 million children receive medication to alleviate the symptoms of ADHD (Sridhar et al. 2017). Clinical trials have shown that short-term treatment with medication is effective, but little is known about the long-term effectiveness (Posner et al. 2020). Psychostimulants were first used to treat hyperactivity in the 1930s and continue to be used today. Side-effects of psychostimulants include appetite suppression, dry mouth, and nausea (Golmirzaei et al. 2016). Long-term use of stimulants affects growth trajectories due to appetite suppression; therefore, drug holidays are suggested (Posner et al. 2020). Children often take the medication throughout the weekdays and do not take the medication on weekends.

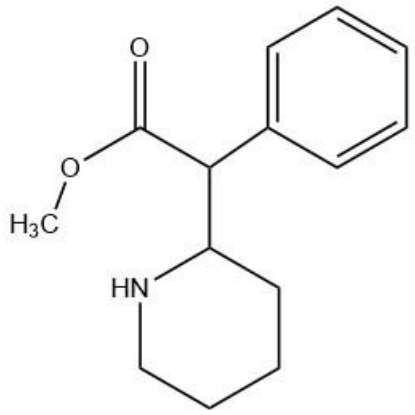

Figure 1.2 The molecular structure of methylphenidate

Methylphenidate (MPH, Figure 1.2), sold as Ritalin®, Concerta™, Metadate©, Methylin®, and Daytrana©, is the most commonly prescribed psychostimulant for the treatment of ADHD. Supporting this, several trials have shown MPH to be more effective than other ADHD medications, such as atomoxetine (Hanwella et al. 2011). MPH is a psychostimulant that inhibits

dopamine (DA) and norepinephrine (NE) reuptake by blocking dopamine and norepinephrine transporters (DAT and NET), thereby increasing the amount of these catecholamines in the synaptic cleft (Figure 1.3). MPH is a racemic mixture composed of d-MPH and l-MPH, both isomers increase extracellular DA and NE; however, the d-isomer is more potent.

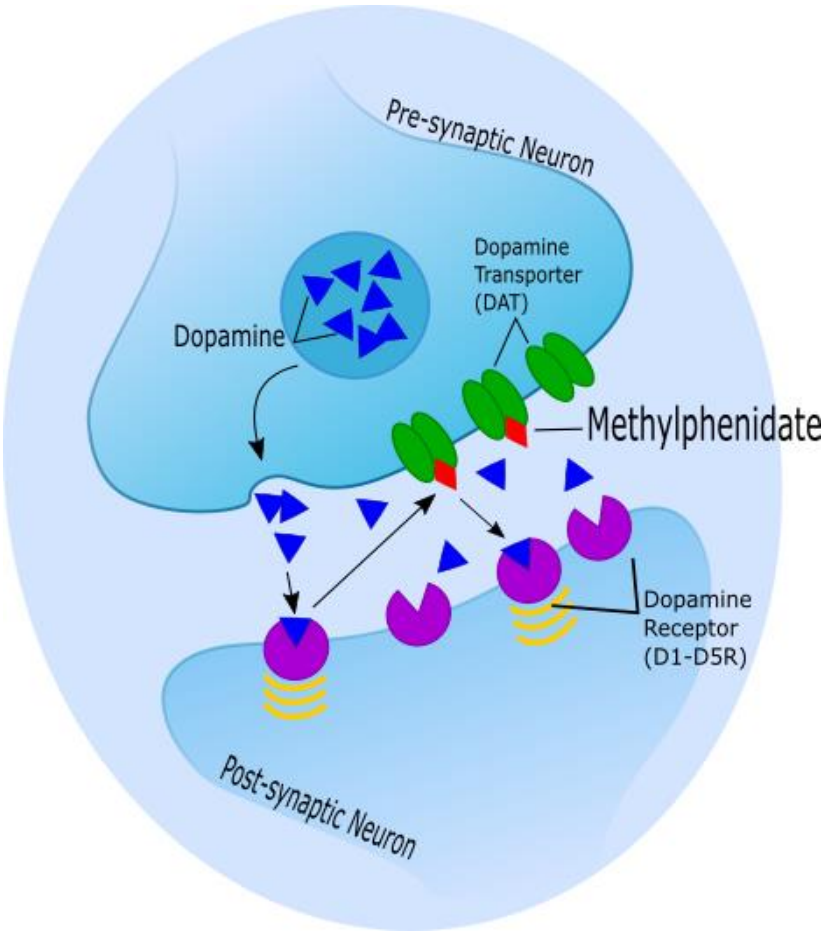

Figure 1.3 Methylphenidate mechanism of action. MPH is a psychostimulant that inhibits dopamine (DA) and norepinephrine (NE) reuptake, increasing the amount of these catecholamines in the synapse. MPH inhibits the reuptake of DA and NE by blocking dopamine or norepinephrine transporters (DAT and NET), respectively. However, MPH has a greater affinity for DAT than NET.

Blockade of DAT by MPH is dose-dependent, and a clinically relevant, oral dose of 0.25 mg/kg or a plasma concentration of 5.7 ng/ml will occupy 50% of total DAT in the brain (Krause et al. 2000). It is commonly thought that MPH has a greater affinity for the DAT than the NET. However, in 2010 Hannestad and colleagues showed that 0.14 mg/kg of MPH is all that is needed to occupy 50% of total NET in the brain (Hannestad et al. 2010). This is a lower dose than is needed to occupy 50% of total DAT in the brain (0.25 mg/kg). This means that an individual taking a MPH dose of 0.5 mg/kg would have about 80% of the total NET in the brain occupied (Hannestad et al. 2010). The average effective MPH dose given to children is 0.7 to 0.9 mg/kg, and for adults, the average dose is 1.1 mg/kg (Hannestad et al. 2010). Moreover, the NET has a greater affinity for DA than the DAT. It appears that DA is cleared by the NET and the DAT in the synaptic cleft, and which one is used depends on which transporter is more abundant and the brain-region of interest. Therefore, MPH binding and blocking both the NET and the DAT may have the overall effect of increasing the amount of extracellular DA, leading to the therapeutic effect of MPH (Hannestad et al. 2010). Additionally, the increase in DA in the synaptic cleft due to MPH is known to upregulate the DAT in the ventral striatum, which could lead to increases in the dose of MPH required to achieve the same therapeutic effect (Wang et al. 2013).

Under normal conditions, synaptic vesicles in dopaminergic neurons may contain concentrations of DA as high as 1.0 M (Monzani et al. 2018). However, MPH is capable of further increasing the amount of DA taken up into vesicles via regulation of VMAT-2 activity (Fleckenstein and Hanson 2003). In fact, a single dose of MPH leads to an increase in the amount of DA released following vesicular fusion. DA in the synaptic cleft may then interact with D2 receptors. Activation of D2 receptors helps to redistribute vesicles from the membrane

to the cytoplasm, where they begin reuptake of cytosolic DA via VMAT-2. Additionally, there is a redistribution of VMAT-2 from membrane-associated vesicles to cytoplasmic vesicles further increasing the reuptake of DA (Sandoval et al. 2002; Volz et al. 2008). Moreover, DA reuptake into membrane-associated vesicles is also increased because VMAT2 activity is upregulated in vesicles that remain associated to the membrane. Overall, more DA is taken up into vesicles leading to an increase in DA release (Sandoval et al. 2002; Volz et al. 2008).

Extracellular DA levels can be homeostatically controlled via presynaptic autoreceptors activation. Specifically, the activation of presynaptic D2 and D3 receptors decreases impulse-triggered vesicular DA release, leading to reduced signaling at postsynaptic D1 and D2 receptors (Frolich et al. 2014). Autoreceptor activity leads to decreased background firing rates and increased signal to noise ratio, which MPH has been shown to do in striatal cells (Volkow et al. 2001). In fact, both a D1 and D2 antagonist are both needed to block the effects of MPH (Riddle et al. 2005).

Different responses to MPH may be due to an individual's baseline dopaminergic tone, DAT availability, and sex. A good clinical outcome is associated with a high baseline DA release and high baseline of DAT (Krause et al. 2000; Volkow et al. 2002). One's sex can also affect the response to MPH. Women have lower plasma concentrations of MPH when compared to men (Markowitz et al. 2003). This has also been shown in the case of rodents, where females have higher brain concentrations of MPH than males (Bentley et al. 2015). There are sex differences in DAT density and extracellular DA clearance, and an upregulation of male striatal D1 receptor when compared to females (Cummins et al. 2014; Frolich et al. 2014). These differences in DAT and D1 receptor may possibly be related to the sex differences that are seen with MPH use.

MPH can cause changes in brain plasticity in addition to changes in dopaminergic tone. As mentioned earlier individuals with ADHD have altered brain structure and cerebral blood flow. Interestingly, MPH increases cerebral blood flow in the frontal lobes, caudate nuclei, and thalamic areas (Frolich et al. 2014; Gustafsson et al. 2000). Additionally, MPH strengthens the connectivity of corticolimbic connections (Konova et al. 2013). Use of MPH has been associated with increased neuroinflammation and oxidative stress in the brain (Faraone 2018). Additionally, several studies indicate that MPH use may be a risk factor for addiction (Ashok et al. 2017; Castells et al. 2016; dela Pena et al. 2014).

Due to the stimulant properties of MPH, high school and college students often misuse MPH (Bonvicini et al. 2016). MPH may be abused as a "cognitive enhancer", an appetite suppressant, or for the euphoria it can produce following intravenous (IV) use (Advokat and Scheithauer 2013; Posner et al. 2020). In 2017, an estimated 12% of college students ages 18-25 years old reported using ADHD medication as a cognitive enhancer or study drug (Abelman 2017). Additionally, one study found that individuals were injecting MPH to get "high" (Frauger et al. 2016). IV injections of MPH have a similar pharmacodynamic effect as cocaine, as it allows for rapid MPH uptake into the brain and a surge in extracellular DA levels within the nucleus accumbens (Baladi et al. 2014; Frolich et al. 2014; Volkow et al. 2002). In fact, MPH is more potent at the DAT than cocaine (Calipari et al. 2015) However, oral doses of MPH produce a slower rate of distribution to the brain than cocaine, which helps prevent the misuse of MPH through an oral route (Konova et al. 2013; Volkow et al. 2002). Therefore, the proper administration of MPH, both the route of administration and dose, are critical in preventing abuse and addiction. However, given the rise in MPH use and misuse, studies to determine the

long-term effects of MPH on the brain are necessary. Important dopaminergic pathways may be affected by MPH, including the mesolimbic pathway, which innervates the hippocampus.

Neurogenesis

As mentioned earlier, brain structure is altered in individuals with ADHD. One of the areas that is impacted is the hippocampus, where volume reductions are seen hippocampus in individuals with ADHD (Faraone 2018). The hippocampus is one of the few places in the brain where neurogenesis occurs throughout an individual's life. Neurogenesis is the "birth of new neurons" and it occurs during development and into adulthood in these neurogenic niches (Kuhn et al. 2018). Adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ), below the lateral ventricles. New neurons produced by the SVZ migrate to the olfactory bulb, where they act as interneurons. My research focused on neurogenesis in the hippocampus, where newly generated neurons are incorporated into the DG of the hippocampus as granular cells (Figure 1.2).

The hippocampus is responsible for memory, learning, and emotion, and is comprised of different areas that perform different functions. Of note, neuronal cell bodies within the hippocampus lie within the Cornu Ammonis fields (CA1, CA2, and CA3) and the DG. Neurons within the DG project to CA2, an area known to play a role in social memory and contextual discrimination (Fares et al. 2019). In adulthood, newly formed DG neurons also excite CA3 neurons, an area of the brain that is important for memory recovery (Fares et al. 2019). The rate at which neurogenesis occurs in the SGZ of the DG may be affected by many factors; these

include aging, stress, genetics, medications, and exercise. Additionally, neurogenesis can be influenced through modulation of neurotransmitter systems, including DA and NE.

The mesolimbic pathway provides dopaminergic input to the hippocampus and thus may influence neurogenesis in the SGZ. Immunohistochemical analysis has shown dopaminergic afferent fibers extending into the hippocampus, and that the D2 receptor is expressed in the DG (Veena et al. 2011). Additionally, lesions of the dopaminergic afferent fibers to the hippocampus or blockade of the D2 receptor decreases neurogenesis and impairs memory, while; activating D2 receptors increases neurogenesis and stabilizes memory (Aimone et al. 2014; McNamara et al. 2014; Veena et al. 2011).

Noradrenergic neurons originate from the locus coeruleus and project to many different parts of the brain. Importantly, they also project to the limbic system, including the hippocampus. Like DA, NE also appears to play an important role in neurogenesis, as lesions to noradrenergic fibers projecting to the hippocampus decrease neurogenesis rate (Aimone et al. 2014). In addition, several classes of drugs that increase NE have been shown to increase neurogenesis rates, including the serotonin-norepinephrine reuptake inhibitors (SNRIs). SNRIs block the NET and serotonin transporter (SERT). These medications are used to treat mood disorders such as depression and anxiety, and the increase in hippocampal neurogenesis that SNRIs produce is believed to contribute to their efficacy (Lee et al. 2012). Given the fact that both NE and DA appear to play a role in neurogenesis, it is possible that the NET and DAT inhibitor, MPH, may influence neurogenesis.

Interestingly though, not all monoamine transport inhibitors have been shown to increase neurogenesis. Results of experiments examining cocaine's effect have been mixed, cocaine is a psychostimulant that blocks the DAT, NET, and SERT. Studies with cocaine have shown that it

can either increase or decrease the rate of neurogenesis (Castilla-Ortega et al. 2016; Garcia-Fuster et al. 2010; Lloyd et al. 2010; Sudai et al. 2011). For example, chronic subcutaneous injections of cocaine (2 x 10 mg/kg/day) increased the rate of neurogenesis in the hippocampus of mice (Lloyd et al. 2010). In contrast, impaired neurogenesis and decreased cell proliferation was seen in rats that self-administered cocaine (1.5 mg/kg intravenous infusions) for 14 days (Sudai et al. 2011). These discrepancies may be due to varied doses and animal models that are used for these experiments. MPH has a similar mechanism of action to cocaine and may also affect hippocampal neurogenesis. Therefore, we explore the effect that MPH has on neurogenesis in this work.

A few studies have looked at the effects of MPH on neurogenesis, but these have yielded contradicting results. An increase in neurogenesis was observed in adolescent rats treated orally with MPH at low (5 mg/kg) or high (10 mg/kg) doses for 28 days (van der Marel et al. 2015). Yet in another study, low oral doses of MPH (2.5 mg/kg or 5 mg/kg) for 21 days had no effect on neurogenesis in adolescent mice, but a high dose (10 mg/kg) appeared to increase neurogenesis when compared to controls (Lee et al. 2012). In agreement, another study found no effect on neurogenesis when gerbils received 5 mg/kg MPH orally for 30 days (Schaefers et al. 2009). Still, other studies have shown that intraperitoneal injections of a low (2 mg/kg) dose of MPH for 15 days given to adolescent rats resulted in no effect on the rate of neurogenesis, but did affect the survival of newly generated neurons (Lagace et al. 2006). These conflicting results may be due to differences in the length of treatment, doses of MPH, drug delivery, and the animal model; moreover, these studies highlight the need for further investigation. In this dissertation a dose of 1 mg/kg MPH given intraperitoneal (i.p.) twice daily was utilized as this dose and method of delivery has been shown to be equivalent to a therapeutic oral dose given for the

clinical treatment of ADHD (Gerasimov et al. 2000; Koda et al. 2010). The dose of 10 mg/kg MPH i.p. was also used as this dose is equivalent to a therapeutic dose given for the treatment of narcolepsy and representative of the recreational misuse of MPH (Valvassori et al. 2007).

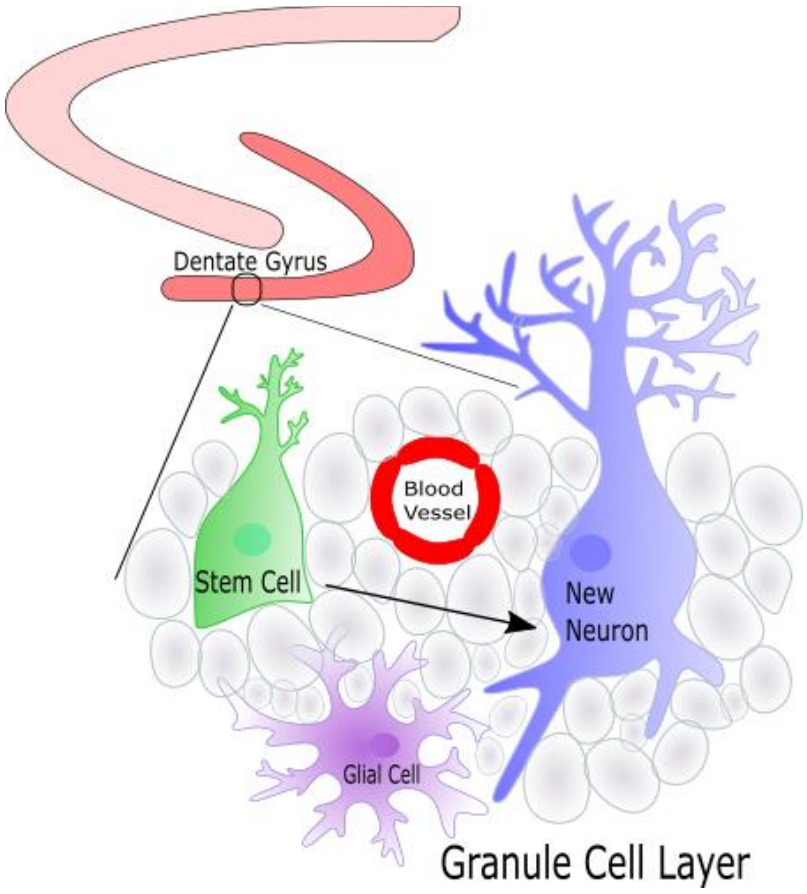

Figure 1.4 Neurogenesis in the dentate gyrus. Adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus.

Proteins Related to Neurogenesis in the Hippocampus

The exact mechanism of how DA and NE affect neurogenesis is unknown. However, it has been proposed that these neurotransmitters lead to differences in expression of several proteins that may modulate neuronal development, growth, and survival. Several studies have indicated that monoamines alter the expression of beta-catenin and vascular endothelial growth factor (VEGF). In fact, other NET inhibitors, such as desipramine have been found to increase expression of beta-catenin and VEGF (Rolando and Taylor 2014; Warner-Schmidt and Duman 2007; Zhao et al. 2008). Beta-catenin is a downstream effector of the Wingless-Int (Wnt) pathway. Wnt proteins are glycoproteins that are known to affect cell proliferation and differentiation, and beta-catenin is a key player involved in this signaling cascade (Hayat 2006). Beta-catenin can be found associated with the cell membrane or in the cytoplasm near the nucleus (Hayat 2006). Under normal conditions, beta-catenin levels are kept low, as it is constantly phosphorylated and degraded, preventing the protein from traveling to the nucleus and activating gene transcription. However, with the activation of the signaling cascade from Wnt, beta-catenin is stabilized and can travel to the nucleus (Baines and Renaud 2017). Beta-catenin then interacts with transcription factors that regulate genes associated with cell survival and proliferation. In fact, increased beta-catenin levels can cause the transformation of normal cells into malignant cells, and overexpression of beta-catenin has been reported in many cancers (Averett et al. 2014).

Beta-catenin is important for normal hippocampal development (Rolando and Taylor 2014) by promoting proliferation of neuronal progenitor cells (Warner-Schmidt and Duman 2006; Yu et al. 2014). There is a decrease in neurogenesis when beta-catenin expression is inhibited (Hui et al. 2014; Mostany et al. 2008; Rolando and Taylor 2014). Moreover, when the

Wnt pathway is activated by antidepressants that block the reuptake of NE, quiescent neuronal progenitor cells begin to proliferate (Rolando and Taylor 2014). As further evidence of beta-catenin's complex role in neurogenesis, beta-catenin is able to promote the synthesis of two other proteins involved in neurogenesis, VEGF and brain-derived neurotrophic factor (BDNF) (Hui et al. 2014; Tayyab et al. 2018).

VEGF has been shown to act as a neurotrophic factor in addition to promoting angiogenesis. VEGF contributes to the neurogenic niche needed to promote the survival of newly formed neurons. Neuronal progenitor cells, neurons, glial cells, and endothelial cells in the hippocampus all express VEGF and its receptors; however, VEGF appears to affect neural precursors separately from the effects it has on endothelial cells (During and Cao 2006). A direct infusion of VEGF into the hippocampus results in increased neurogenesis (Warner-Schmidt and Duman 2007; Zhao et al. 2008). Moreover, antidepressants that increase neurogenesis are known to increase VEGF in the hippocampus, and blocking VEGF signaling inhibits the neurogenic effect of antidepressants (Cao et al. 2004; Zhao et al. 2008). Exercise and an enriched environment are known to increase neurogenesis within the hippocampus and VEGF appears to play an important role in mediating this. VEGF is needed for exercise-induced neurogenesis (Bettio et al. 2019) and VEGF knockdown inhibits neurogenesis in the hippocampus that is produced when animals are subjected to an enriched environment (During and Cao 2006). Additionally, overexpression of VEGF in rats was able to enhance hippocampal spatial memory formation (Tillo et al. 2012).

BDNF is a neurotrophic factor that is responsible for neuronal growth and survival. Upregulation of BDNF increases cell proliferation in neuronal cultures, and beta-catenin helps to promote the synthesis of BDNF through the Wnt pathway (Tayyab et al. 2018). High

concentrations of BDNF are found in the hippocampus (Erickson et al. 2012), and infusion of BDNF into the hippocampus of rats increases neurogenesis within the DG (Scharfman et al. 2005). Interestingly, neurogenesis can be induced in rodents even when BDNF is administered peripherally (Erickson et al. 2012). The increased hippocampal neurogenesis produced by SSRIs and SNRIs is associated with increases in BDNF in the hippocampus of rodent models of depression and human patients treated by antidepressants (Jiang et al. 2014; Sairanen et al. 2005; Tayyab et al. 2018).

Furthermore, BDNF's receptor, tropomyosin receptor kinase B (TrkB) is expressed on neural progenitor cells in the DG and is required for hippocampal neurogenesis, as deletion of TrkB from neuronal progenitor cells hinders neurogenesis (Li et al. 2008). Activation of TrkB by BDNF is known to promote cell growth and survival in serotonergic neurons (Erickson et al. 2012). Additionally, chronic treatment with antidepressants, which increases neurogenesis, also leads to an upregulation TrkB mRNA in the hippocampus (Sairanen et al. 2005). Some studies have shown that MPH may alter expression of BDNF and/or tropomyosin receptor kinase B (TrkB) in the hippocampus and prefrontal cortex (Fumagalli et al. 2010); however, they have yielded conflicting results. Some studies have found an increase in BDNF expression while others have found a decrease in BDNF expression after MPH exposure (Fumagalli et al. 2010; Lagace et al. 2006; Lee et al. 2012). As above, these differences may be due to the dose of MPH used or the area of the brain studied. For example, one lab has shown an increase in BDNF in the subgranular zone of the hippocampus with a high dose of MPH (10 mg/kg) (Lee et al. 2012), while another has shown a decrease in BDNF in the hippocampus after exposure to a low dose of MPH (2 mg/kg) (Lagace et al. 2006). Moreover, another lab found an increase in BDNF in the striatum of animals exposed to a low dose of MPH (1 mg/kg) (Fumagalli et al. 2010).

Additionally, age may also play an important role. One study showed that BDNF mRNA decreased when juvenile rats were exposed to MPH but increased when adult rats were exposed to MPH (Banerjee et al. 2009). This is interesting because natural aging has been associated with a decrease in neurogenesis. As age increases BDNF levels remain unchanged, while TrkB expression decreases (Aimone et al. 2014). Of note, MPH has been shown to either increase or decrease TrkB, again depending on the area of brain studied. One study found that exposure to MPH resulted in a decrease in BDNF and TrkB in the prefrontal cortex and an increase in BDNF with no change in TrkB in the striatum (Fumagalli et al. 2010).

Another neurotrophic factor that is important for cell growth and survival is glial cell line-derived neurotrophic factor (GDNF). GDNF and its receptor GDNF family receptor alpha 1 (GFRa1) are both expressed on adult-born granule cells in the hippocampus (Bonafina et al. 2019). Moreover, GDNF is decreased in the hippocampus of stressed or depressed rodents, and antidepressants increase the mRNA of GDNF in rat astrocytes (Kajitani et al. 2012; Popova et al. 2017). Furthermore, exercise is known to increase neurogenesis, and voluntary running triggers an increase in GDNF expression (Bonafina et al. 2019). Additionally, in vivo infusion of GDNF into the hippocampus of rats results in increased neurogenesis (Chen et al. 2005). Additionally, GDNF has been shown to support the survival of DA neurons (Allen et al. 2013). While a few studies have looked at the effects MPH on BDNF, no studies have examined the effects of MPH on GDNF.

Altogether, these results highlight the need to further investigate the effect of MPH on neurogenesis. In chapter 2 of this dissertation, we look at how chronic MPH treatment affects the proliferation and survival of granule cells within the DG of the hippocampus. Additionally, we

examine the mechanism for this interaction by measuring the expression of these proteins related to neurogenesis, beta-catenin, VEGF, BDNF, TrkB, or GDNF, are altered.

Dopamine-induced Oxidative Stress

Another important dopaminergic pathway is the nigrostriatal pathway, where neurons project from the substantia nigra to the caudate and putamen nuclei of the striatum. Approximately 80% of the DA in the brain is contained within the nigrostriatal pathway (Golan D 2011; Stahl 2008). This pathway is primarily responsible for purposeful movement and damage to this pathway can result in tremors, spasms, tardive dyskinesia, and Parkinson's disease. Unfortunately, it appears that DA may be capable of causing such damage.

Excess DA not stored in vesicles can contribute to oxidative stress. Oxidative stress occurs when reactive oxygen species (ROS) overwhelm the body's natural antioxidants. These ROS include the, superoxide anion ($0_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$). Certain ROS known as free-radicals are unstable due to an unpaired electron. Depending on the free-radical, it will readily donate or accept electrons to become stable. However, by donating or accepting an electron, the free-radical sets off a chain reaction, further increasing the number of free-radicals that are then able to interact with yet more, molecules (Betteridge 2000). Oxidative stress becomes a problem, because free-radicals interact with macromolecules causing lipid peroxidation, protein damage, cross-linking and fragmentation of proteins, and even DNA double strand breaks (Betteridge 2000; Salim 2017). In addition, oxidative stress in the brain increases the blood-brain barrier permeability, changing brain morphology, increasing neuroinflammation, and can cause neurodegeneration (Salim 2017). As mentioned earlier, ROS

also includes H_2O_2 as well as free-radicals. H_2O_2 can easily pass through cell membranes, and when it comes into contact with transition metal ions, H_2O_2 produces hydroxyl radicals (Betteridge 2000). Furthermore, the brain is enriched with transition metals such as iron (Fe) and copper (Cu). In fact, chelated Fe and Cu levels can reach into the mM range; this is due to the brains ability to use transition metals to increase the rate of a biochemical reaction (Cobley et al. 2018). Fe is also capable of assisting in many other neuronal functions including synthesis of the myelin sheath (Todorich et al. 2009). Due to the importance of Fe, unsurprisingly, neurons contain a reserve of chelated Fe that can be used when needed.

Indeed, the brain is particularly susceptible to oxidative stress. Another source of ROS are the mitochondria. Mitochondria are cell organelles responsible for converting glucose to a useful form of energy, adenosine triphosphate (ATP) using the electron transport chain and O_2 in a process known as oxidative phosphorylation. The brain consumes 20% of the total basal oxygen due to the ATP needed for neurons to maintain ionic gradients and release neurotransmitters as needed in addition to other cellular functions (Cobley et al. 2018). Mitochondria have other functions in addition to the production of ATP. One function is the breakdown of DA by enzymes located in the mitochondria (Figure 1.5). These enzymes are known as monoamine oxidase A (MOA-A) and monoamine oxidase B (MOA-B). However, DA is preferentially broken down by MOA-B, which is located on the outer space of the mitochondrial inner membrane where few antioxidants exist (Cobley et al. 2018). Unfortunately, when DA is broken down, H_2O_2 is produced as a byproduct (Stokes et al. 2000). The capacity of MOA-A and MOA-B to generate H_2O_2 depends on the concentration of oxygen. When they are saturated with oxygen, they produce a considerable amount of H_2O_2 (Cobley et al. 2018). Then,

transition metal ions (especially Fe) are capable of converting H_2O_2 to $^{\cdot}OH$, further increasing oxidative stress. In certain conditions, DA itself can also lead to oxidative stress.

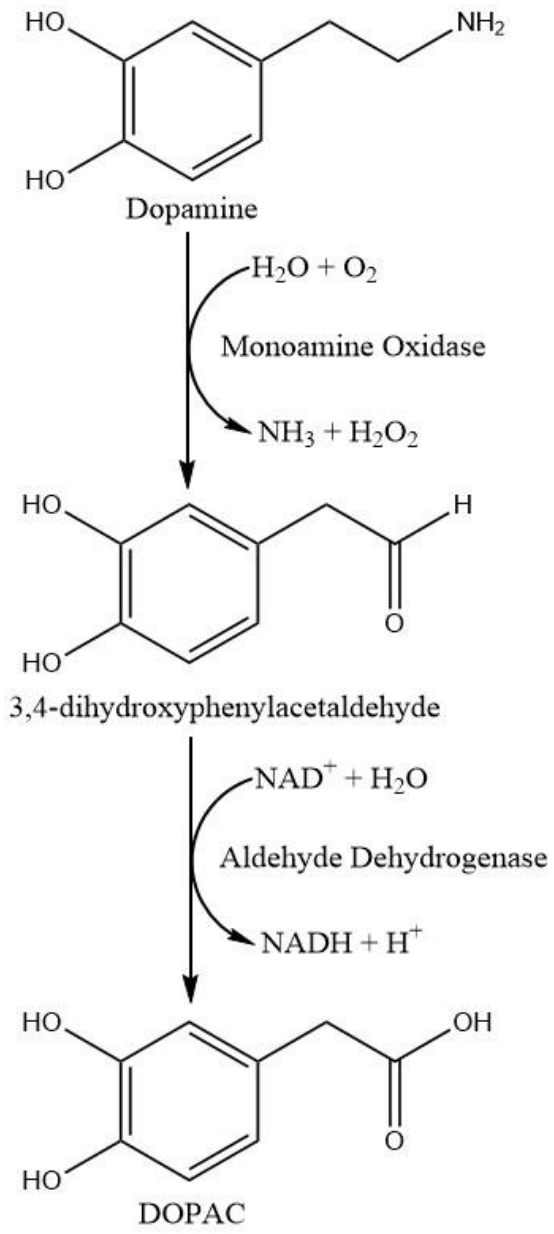

Figure 1.5 The breakdown of Dopamine by monoamine oxidase. Dopamine can be metabolized by the enzyme monoamine oxidase (MAO) within the mitochondria. MAO produces H_2O_2 when it catalyzes dopamine to 3,4-dihydroxyphenylacetaldehyde. H_2O_2 may interact with Fe or Cu producing a hydroxyl radical.

DA is capable of autoxidizing at a physiological pH by dissociation of a hydroxyl group creating a DA o-quinone which then forms an aminochrome and eventually forms H_2O_2 and hydroxyl radicals (Figure 1.6). Under normal conditions, excess DA is sequestered in vesicles with a lower pH to prevent DA autoxidation and contact with catalytic factors (Klein et al. 2019). However, DA is capable of leaking out of synaptic vesicles and collecting in the cytosol (Zhang et al. 2019). This means that free DA may be autoxidized without a catalysis and produce ROS. However, Fe and Cu, as well as enzymes with peroxidase activity are capable of catalyzing DA into DA o-quinone, speeding up the reaction and increasing the levels of DA o-quinones. The capability of Fe and Cu to catalyze DA into harmful ROS and the stores of these transition metals in neurons highlight the susceptibility that dopaminergic pathways have to oxidative stress. For example, the nigrostriatal DA neuronal pathway accumulates ferrous ions that promote DA oxidation (Klein et al. 2019). Quinones and other ROS generated from the formation of quinones interrupt normal cell function by inducing oxidative stress throughout the cell and indiscriminately damaging proteins, lipids, DNA, and other important macromolecules which could potentially trigger apoptosis (Park et al. 2007).

DA o-quinones disrupt normal cell function by targeting cysteine residues on proteins. If cysteine is not available, these quinones may target histidine and lysine residues as well (Monzani et al. 2018). DA o-quinones reacting and forming adducts with cysteine residues is particularly alarming, because cysteine residues are often found in the active site of enzymes. This could be detrimental to the cell if enough enzymes are unable to participate in essential reactions. Some proteins that DA o-quinones are known to form adducts with are crucial to dopaminergic neurotransmission, including the DAT and tyrosine hydroxylase, the enzyme responsible for DA synthesis. Of note, adducts can also be formed with parkin, a protein known

to be altered in familial forms of Parkinson's disease. Finally, DA o-quinones will also form adducts and alter the functioning of proteins that have pivotal roles in maintaining ROS levels such as, glutathione peroxidase, superoxide dismutase, and mitochondrial complexes in the electron transport chain (Zhang et al. 2019; Zucca et al. 2017).

Oxidative phosphorylation occurs in mitochondria and is the process of using oxygen to synthesize ATP. This is accomplished by the electron transport chain. The electron transport chain consists of 4 protein complexes known as complex I, complex II, complex III, and complex IV. Cells use NADH to carry electrons to complex I where they enter the electron transport chain. Electrons usually flow through complexes I, III, and IV to release energy in a step-wise fashion, leading to the production of ATP. Complex I is also known as NADH:ubiquinone oxidoreductase is responsible for catalyzing the first step in the electron transport chain by oxidizing NADH and transferring that energy to ubiquinone forming ubiquinol. Ubiquinol carries two electrons to complex III. Complex III is also known as ubiquinone-cytochrome c oxidase. Complex III uses the energy gained from the electrons to oxidize semiquinone and reduce cytochrome c, pumping protons from the mitochondrial matrix to the inner membrane space. If there is a decrease in electron transfer, oxygen can accept electrons from complex III, resulting in superoxide formation (Dias et al. 2013). In fact, complex I and III are both known to produce superoxide anions under normal physiological conditions (Murphy 2009). Mitochondria have the enzyme superoxide dismutase and others to combat the harmful ROS it produces. Unfortunately, these enzymes are the target of DA o-quinones.

When adducts are formed between DA o-quinones and enzymes, the enzymes are unable to bind and remove ROS. Therefore, the ROS produced by the mitochondria are free to cause mitochondrial damage, eventually leading to apoptosis. Furthermore, quinones are known to

interrupt the normal functioning of mitochondria, further increasing the formation of radicals (Segura-Aguilar et al. 2014). In response to DA quinones, isolated mitochondria exhibit significant decreases in the activity of all 4 complexes (Jana et al. 2011). In fact, DA o-quinone has been shown to form adducts with proteins in complexes I, III, and IV, greatly reducing their function and producing free radicals (Zucca et al. 2017). Others have shown mitochondrial membrane depolarization and even toxic effects via interactions with the electron carrier, NADH (Bisaglia et al. 2010; Jana et al. 2011). Moreover, derivatives of DA quinones are also capable of damaging mitochondria and contributing to oxidative stress.

DA o-quinone cycles to aminochrome and leukoaminochrome. Both conjugates are unstable; however, aminochrome is more stable than leukoaminochrome. Aminochrome is known to form adducts with and inhibit complex I in vitro in SHSY5Y cells that have been differentiated into dopaminergic cells (Aguirre et al. 2012). Additionally, mitochondrial dysfunction is seen when aminochrome is directly injected into the striatum (Zhang et al. 2019). In addition, aminochrome accepts electrons from NADH to form leukoaminochrome-o-semiquinone radical (Figure 1.6) which is a free-radical that is highly reactive with oxygen. Leukoaminochrome-o-semiquinone will autoxidize back to aminochrome, while reducing oxygen to create more ROS. Moreover, aminochrome continues to cycle to leukoaminochrome-o-semiquinone and back causing the depletion of NADH (Zhang et al. 2019; Zucca et al. 2017). NADH is an important electron carrier needed in the electron transport chain and without NADH, ATP production is greatly reduced. Taken together, it is clear that aminochrome and leukoaminochrome can be detrimental to mitochondrial function, and without ATP production from mitochondria neurons may become apoptotic. Perhaps more significant is that aminochrome is able to promote the formation of α-synuclein.

The protein, α-synuclein has been implicated in neurodegenerative diseases including Alzheimer's and Parkinson's disease (Segura-Aguilar et al. 2014). Normally, α-synuclein can be found at the synapse within active and reserved synaptic vesicles in dopaminergic neurons, where it plays a role in DA release. The importance of α-synuclein in regulation of DA release is evidence that knock-out mice have decreased DA release within the striatum (Villar-Piqué et al. 2016). However, overexpression of α-synuclein can induce neurodegeneration in dopaminergic neurons (Asanuma et al. 2003). When α-synuclein is overexpressed or mutated, it can begin to aggregate, elongate, and assemble into an enriched β-sheet structure (Villar-Piqué et al. 2016). These insoluble aggregates can form in the cell body or processes of neurons and are known as Lewy bodies or Lewy neurites, respectively (Kalia and Lang 2015). Aggregated α-synuclein can form toxic protofibrils known to destroy synaptic vesicular membranes, inhibit movement of the cell cytoskeleton and accumulate in mitochondria where they inhibit complex I (Asanuma et al. 2003; Villar-Piqué et al. 2016). The damage to vesicular membranes and mitochondria can lead to an increase in ROS. To guard against this, fibrils can be formed from α-synuclein protofibrils to help prevent this damage.

Fibrils are larger and more stable than protofibrils and prevent the smaller protofibrils from diffusing into surrounding cells where they can cause additional damage to surrounding cells (Siddiqi et al. 2019). However, aminochrome as well as DA quinones will bind to α-synuclein and protofibrils, stabilizing them and preventing the formation of fibrils (Asanuma et al. 2003; Miyazaki and Asanuma 2008; Monzani et al. 2018; Zucca et al. 2017). In addition, increased ROS promotte the aggregation of α-synuclein in dopaminergic neurons, contributing further to this vicious cycle (Salim 2017). This may be one mechanism by which dopaminergic neurons are lost in Parkinson's disease. Increased DA induces α-synuclein aggregation and the

degeneration of neurons in the nigrostriatal pathway (Monzani et al. 2018). In fact, DA has long been known to have a role in the degeneration of DA terminals (LaVoie and Hastings 1999). To help prevent degeneration, dopaminergic neurons have evolved mechanisms to prevent oxidative stress.

Neuromelanin

One such mechanism is the formation of neuromelanin. Free DA and metal ions can be incorporated into neuromelanin to prevent oxidation. Neuromelanin is a polymer that makes up the dark pigment found within the substantia nigra, where it is formed as a neuroprotective action against DA oxidation. Once DA o-quinone forms aminochrome, it can be rearranged to 5,6 dihydroxyindole, then oxidized to 5,6 indolequinone which is polymerized to form neuromelanin (Zhang et al. 2019). Neuromelanin can also be formed when DA o-quinone interacts with cysteine forming cysteinyl-DA (Zucca et al. 2017). Neuromelanin also functions as an antioxidant by scavenging ROS and binding and inactivating transition metals (Zhang et al. 2019). This chelation of metal ions prevents them from interacting with and oxidizing free DA. Once neuromelanin is formed, it is sequestered in vesicles where it is stored. Additionally, the number of vesicles and concentration of neuromelanin within them increase as we age and are generally considered neuroprotective.

However, when dopaminergic neurons degenerate, as with Parkinson's disease, neuromelanin is released from the neuron into the extracellular space. Neuromelanin can then act as a chemotaxin for microglia, leading to an immune response (Zucca et al. 2014; Zucca et al. 2017). Microglia phagosize neuromelanin and release pro-inflammatory factors such as tumor

necrosis factor α (TNFα), interleukin 6, nitric oxide, superoxide, and hydrogen peroxide (Cobley et al. 2018; Zucca et al. 2014). Indeed, inflammation in the substantia nigra is part of the pathology of Parkinson's disease. Given these points, it is clear to see how neuromelanin can be neuroprotective under normal physiological conditions but can also become neurotoxic.

Glutathione

Under normal physiological conditions, small amounts of DA o-quinone are made, and the cell prevents any damage before it is done. In addition to incorporating DA o-quinone derivatives into neuromelanin, the brain utilizes the antioxidant glutathione (GSH) to protect neurons from the toxicity of quinones (Motaghinejad et al. 2016). In fact, GSH has been shown to prevent DA-induced cell death in some rodent models (Park et al. 2007; Stokes et al. 2000; Zhou and Lim 2009). GSH forms conjugation reactions with quinones or aminochromes eventually leading to the formation of 5-S-cysteinyl DA or 4-S-glutathionyl-5,6-dihydroxyindoline, respectively. 5-S-cysteinyl DA and 4-S-glutathionyl-5,6-dihydroxyindoline may then be incorporated into neuromelanin (Zhou and Lim 2009) and some 5-S-cysteinyl DA is released into cerebral spinal fluid. After the conjugation reactions, GSH becomes glutathione disulfide (GSSG). Following the catalysis by GSH reductase and the input of energy in the form of NADH, GSSG may be converted back into its reduced form GSH, so it can participate in another conjugation reaction. Unfortunately, aminochrome is also competing for NADH to form leukoaminochrome o-semiquinone radical and hydroxyl radicals (Figure 1.3) (Dagnino-Subiabre et al. 2000; Munoz et al. 2012). The consequences of this are two-fold, excess quinone production will deplete GSH as well as deplete the cofactor (NADH) needed to convert GSH back to its reduced form. Thus, the depletion of GSH leads to the production of more free

quinones, and therefore more radicals, leading to oxidative stress and eventually neurotoxicity via apoptosis (Stokes et al. 2000). This becomes a vicious cycle when oxidative stress leads to degradation of neuromelanin and the release of additional DA and metal ions, further increasing the potential for oxidative stress (Meiser et al. 2013). Moreover, GSH levels are lower in neurons when compared to other cells (Cobley et al. 2018), and glutathione activity is reduced as we age (Juarez Olguin et al. 2016). Taken together it becomes apparent that the brain is particularly susceptible to DA-induced oxidative stress. Therefore drugs, such as psychostimulants, that increase synaptic DA levels may have detrimental effects on the dopaminergic pathways within the brain.

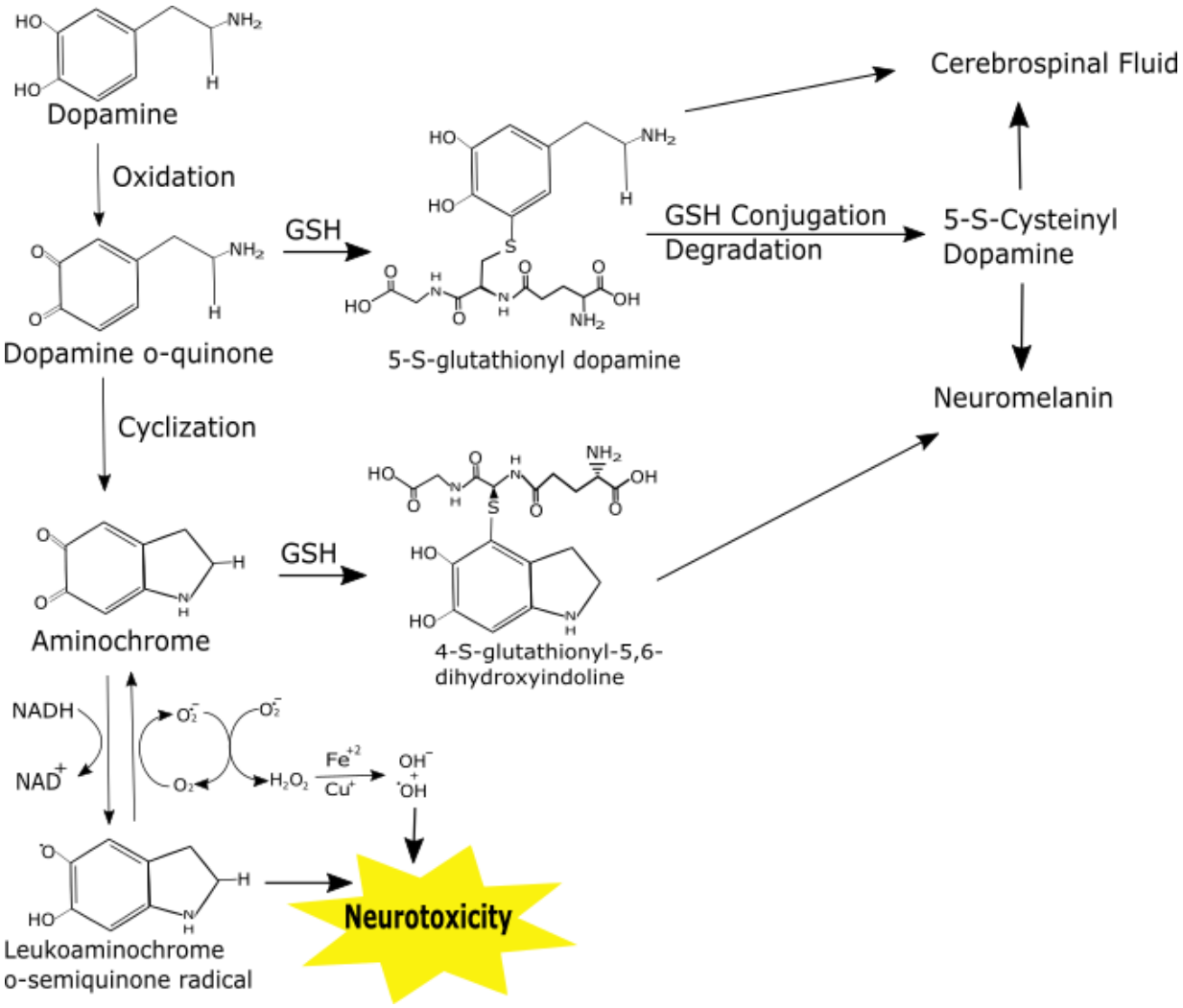

Figure 1.6 Dopamine and Oxidative Stress. DA can be oxidized to DA o-quinone at a physiological pH. DA o-quinone then forms an aminochrome and with the aid of oxygen and NADH generates radicals. Glutathione (GSH) conjugates to the dopamine o-quinone, which then degrades to form 5-S-cysteinyl dopamine. 5-S-cysteinyl dopamine may be then incorporated into neuromelanin or dumped into the cerebral spinal fluid. GSH can also conjugate to aminochrome and become incorporated into neuromelanin. Neurotoxicity results when radicals are formed and is further exacerbated when GSH is depleted.

Oxidative Stress and Psychostimulants

Given the fact that psychostimulants increase levels of free DA in the synaptic cleft, some studies have examined whether psychostimulants could contribute to oxidative stress. Cocaine is a psychostimulant that has a similar mechanism of action to MPH, inhibiting the reuptake of DA and NE, but also serotonin. Cocaine has been shown to increase H_2O_2 production and lipid peroxidation in the striatum (Korpi et al. 2015; Lipaus et al. 2019; Pomierny-Chamioło et al. 2013; Sharma et al. 2009; Vitcheva et al. 2015). Furthermore, treatment with antioxidants lessened the oxidative stress (Korpi et al. 2015). Unfortunately, the antioxidant GSH appears to be depleted in the striatum of rodent brains exposed to cocaine (Hirsch et al. 2018; Pomierny-Chamioło et al. 2013; Vitcheva et al. 2015). Additionally, exposure to cocaine can increase the expression of proinflammatory cytokines such as TNFα and IL-1β in the nigrostriatal pathway (Sajja et al. 2016). Interestingly though, many studies have indicated that cocaine alone does not cause blatant neurotoxicity. For example, high doses of cocaine resulted in no significant degeneration of axons in the striatum (Korpi et al. 2015). Moreover, rats treated with cocaine exhibited no dopaminergic cell loss in the nigrostriatal pathway (Little et al. 2009).

The amphetamine psychostimulants have also been examined for their potential to induce oxidative stress and neurotoxicity. These include methamphetamine, a popular drug of abuse, and amphetamine, another commonly prescribed drug for ADHD. Amphetamines are substrates for the monoamine transporters, competing with endogenous neurotransmitters for transporter-mediated uptake into the presynaptic neurons (Fleckenstein et al. 2007; Sitte et al. 1998). Once the amphetamines are transported into presynaptic neurons, they promote the release of DA, NE, and/or serotonin back into the synaptic cleft via reversal of monoamine transporter flux (Jones et al. 1999; Rothman and Baumann 2003) In addition, amphetamines are capable of inhibiting

MAO causing increases in the overall concentration of the monoamine transmitters (Korpi et al. 2015). Neurotoxicity can occur following amphetamine exposure and appears to result from a combination of hyperthermia, excitotoxicity, mitochondrial dysfunction, and oxidative stress (Seger 2010). Oxidative stress following amphetamine exposure likely occurs because of the large increases in both intracellular and extracellular DA. As mentioned earlier, excess DA is susceptible to autoxidation or catalytic oxidation to form toxic quinones. Multiple animal studies have revealed the significant DA neurotoxicity that can be induced by methamphetamine and the contributing role of DA quinones in this toxicity (Baldwin et al. 1993; Guillot et al. 2008; Kita et al. 2003; LaVoie and Hastings 1999). Moreover, DA cell loss and GSH depletion in the nigrostriatal pathway was also observed in postmortem brain tissue of chronic methamphetamine users (Tong et al. 2018). Increases in intracellular DA appear to be more detrimental for the cell than increases in extracellular DA. In support of this, blockade of DAT or inhibition of DA synthesis after methamphetamine exposure reduces the toxicity associated with methamphetamine (Kita et al. 2009; Korpi et al. 2015). The loss of dopaminergic neurons is evident in experiments with chronic amphetamine use as well. Amphetamine exposure can induce a significant loss in dopaminergic neurons that is accompanied by a decrease in DA, DAT, and tyrosine hydroxylase activity in the striatum (Fleckenstein et al. 2009; Kita et al. 2009; Little et al. 2009). Significantly, these effects are attenuated by pretreatment with antioxidants (Kita et al. 2003).

In addition to dopaminergic oxidative stress, amphetamines also induce a proinflammatory response that results in the activation of microglial cells. Microglia then release TNFα and other molecules such as superoxide in an effort to damage pathogens (Guillot et al. 2008). Oxidative stress is further exacerbated by a decrease in the activity of mitochondrial

complexes I, II, and IV in the striatum due to amphetamine use (Korpi et al. 2015). The decreased activity of these complexes will result in decreased levels of ATP and more chances of superoxide to be leaked out of the electron transport chain. In fact, dopaminergic cell damage after amphetamine treatment can be prevented with superoxide dismutase an enzyme that targets superoxide (Kita et al. 2009). Altogether, it appears that amphetamines can cause oxidative stress to dopaminergic neurons resulting in dopamine cell loss and eventually in an overall decreased level of DA and its related proteins.

Another psychostimulant, MPH, increases synaptic DA levels as high as amphetamines (Little et al. 2009). Although repeated high doses of MPH were unable to produce the long-term dopaminergic damage that is seen after repeated high doses of methamphetamine (Kita et al. 2009), MPH may be capable of increasing oxidative stress in the brain.

Oxidative Stress and Methylphenidate

Chronic MPH has been shown to induce oxidative stress and neuroinflammation in the hippocampus of the rat brain by increasing lipid peroxidation and inflammatory cytokines and decreasing GSH (Martins et al. 2006; Motaghinejad et al. 2016). Additionally, chronic, high dose MPH was shown to deplete GSH and GSH reductase activity in the mitochondria in the hippocampus of adult rats (Motaghinejad et al. 2016). Furthermore, chronic exposure to low (1 mg/kg) and high (10 mg/kg) doses of MPH led to an increase in inflammatory cytokines TNFα and IL-1β and decreased the levels of BDNF and GDNF in the substantia nigra (SN), and that this response sensitized DA neurons to further oxidative stress (Sadasivan et al. 2012).

Moreover, age appears to play a role in the extent of oxidative stress. Some labs have

shown that young rats but not adults treated with chronic low (1 mg/kg or 2 mg/kg) doses of MPH showed an increased index of DNA damage and lipid peroxidation in the striatum, hippocampus, prefrontal cortex, and cerebellum (Andreazza et al. 2007; Comim et al. 2014; Martins et al. 2006; Schmitz et al. 2012b). Additionally, acute MPH exposure increased superoxide production in whole rat brain in young rats but not adult rats (Comim et al. 2014; Gomes et al. 2009; Martins et al. 2006; Sadasivan et al. 2012). In contrast, some studies have found that MPH did not induce oxidative stress and in some cases may protect against it. Notably, low concentrations of MPH with methamphetamine appear to protect against the loss of DA neurons due to blocking the DAT and preventing an influx of DA in to the cytosol (El Ayadi and Zigmond 2011; Korpi et al. 2015; Little et al. 2009; Ludolph et al. 2006; Perfeito et al. 2013). Other studies were able to show that chronic exposure to MPH did not increase reactive oxygen species in the blood of rats but did increase the activity of superoxide dismutase and deplete total antioxidant capacity as determined by total radical-trapping antioxidant potential (TRAP) (Schmitz et al. 2012a). When the hippocampus, striatum, prefrontal cortex and, cerebral cortex of adult rats was analyzed, both acute and chronic exposure to MPH resulted in decreased activity of all four complexes of the electron transport chain (Fagundes et al. 2010b). However, in young rats, acute MPH appears to decrease the activity of mitochondrial complexes I and IV in the cerebellum and prefrontal cortex but does not affect the striatum (Fagundes et al. 2010a). In another study, chronic MPH exposure in young rats was found to increase the activity of complexes III and IV and decrease the activity of complexes I and II (Comim et al. 2014). Altogether, the effects of oxidative stress induced by MPH seem to depend on the dose used, length of treatment, area of the brain studied, and age. Interestingly, dopamine-induced oxidative stress and neurotoxicity has been linked to Parkinson's disease, a neurodegenerative disorder,

and individuals with a history of psychostimulant use have a higher rate of Parkinson's disease when compared to control populations (Curtin et al. 2015; Kita et al. 2009; Moratalla et al. 2017; Perfeito et al. 2013).

Parkinson's Disease

Parkinson's disease was first described in 1817 by Dr. James Parkinson, who characterized it as a shaking palsy (Emamzadeh 2017). Today, it is the second most common neurodegenerative disorder, affecting 1% of the population over the age of 65 and 3.5% of individuals 85 to 89 years old (Haddad et al. 2017; Zhang et al. 2019). Parkinson's disease pathology is characterized by dopaminergic cell loss in the substantia nigra pars compacta and loss of dopaminergic input into the caudate and putamen nuclei of the striatum. The disease leads to motor symptoms such as bradykinesia, resting tremor, rigidity, and postural instability. Additionally, non-motor symptoms including impaired olfactory function, insomnia, incontinence, depression, anxiety, and cognitive impairment (Klein et al. 2019; Zhang et al. 2019; Zucca et al. 2017). Motor symptoms are not seen until there is at least an 80% loss of dopaminergic neurons in the substantia nigra, and they usually begin on one side of the body, eventually affecting both sides (Emamzadeh 2017). However, non-motor symptoms can occur at any stage of the disease and usually appear before motor symptoms (Emamzadeh 2017).

A distinguishing characteristic of Parkinson's disease is the appearance of Lewy bodies made up of aggregated α-synuclein (Asanuma et al. 2003; Dias et al. 2013; Villar-Piqué et al. 2016). In fact, as the disease progresses, α-synuclein levels are increased in the cerebral spinal fluid and plasma of individuals with Parkinson's disease as compared to controls (Emamzadeh

2017). The appearance and increase in α-synuclein can be used to determine the stage of the disease. Parkinson's disease pathology of Lewy bodies has been proposed to occur in six stages, known as Braak staging of Lewy pathology (Kalia and Lang 2015; Zucca et al. 2017). Stage one begins with the appearance of α-synuclein aggregations in the peripheral nervous system, olfactory bulbs, and medulla oblongata. The Lewy bodies will then proceed to affect the locus coeruleus, caudal raphe nuclei, and magnocellular reticular formation in stage two. Stage three begins five years before the onset of motor symptoms when the Lewy bodies begins to infiltrate the substantia nigra, amygdala central subnucleus, pedunculopontine tegmental nucleus, and Meynert's nucleus. In stage four, individuals have lesions that extend to the temporal mesocortex. After approximately ten years of motor symptoms, stage five is initiated when the disease begins to affect tertiary sensory association areas and prefrontal cortex. Finally, in stage six, secondary and primary motor and sensory areas are affected (Kalia and Lang 2015; Zucca et al. 2017).

Most individuals develop idiosyncratic Parkinson's disease; however, some individuals develop familial Parkinsonism which is due to mutations in genes related to autophagy, lysosomal function, oxidative stress response, and maintenance of mitochondrial integrity (Klein et al. 2019). One such mutation is in the α-synuclein gene (SNCA gene) (Dias et al. 2013). As mentioned earlier, α-synuclein normally associates with vesicular and membrane structures and aids in the recycling and storage of neurotransmitters in synaptic vesicles (Dias et al. 2013; Fleckenstein and Hanson 2003). Normally, α-synuclein is cleared by proteasomes, but mutant α-synuclein cannot be degraded by proteasomes and forms harmful aggregates (Dias et al. 2013; Emamzadeh 2017; Monzani et al. 2019). When α-synuclein is not degraded, it can interfere with cellular function, rendering dopaminergic neurons more susceptible to damage. As mentioned

earlier, conjugation of DA to α-synuclein inhibits the transition of protofibrils to fibrils, causing an accumulation of toxic protofibrils (Dias et al. 2013; Monzani et al. 2019). Furthermore, oxidative stress may also induce α-synuclein aggregation (Salim 2017). This may perpetuate the problem as α-synuclein aggregation can negatively affect the structure and function of mitochondria (Dias et al. 2013; Emamzadeh 2017). Specifically, α-synuclein can bind to the inner mitochondrial membrane and associate with complex I, decreasing its activity and allowing for more opportunities for superoxide to form (Dias et al. 2013). Relevant to this work, a variant of the gene coding for α-synuclein is seen in individuals that have been diagnosed with both, ADHD and Parkinson's disease (Gerlach et al. 2019). Furthermore, the risk of developing Parkinson's disease before the age of fifty was increased in individuals with a history of using psychostimulants to treat ADHD (Curtin et al. 2018; Walitza et al. 2007).

Additionally, age is a risk factor to developing Parkinson's disease (Haddad et al. 2017; Kordower et al. 2013; Zhang et al. 2019). In fact, natural aging leads to a 5 to 10% loss of dopaminergic neurons every decade (Juarez Olguin et al. 2016; Salim 2017). However, within a decade, individuals with Parkinson's disease lose up to ten times more neurons than age-matched controls (Lappin et al. 2018). Moreover, levels of the antioxidant, GSH decrease due to natural aging, and the level is further decreased in age-matched individuals with Parkinson's disease (Bisaglia et al. 2010; Dias et al. 2013; Smeyne and Smeyne 2013). Fe levels have also been observed to increase in the nigrostriatal pathway with natural aging, and Fe levels are further increased in age-matched controls of individuals with Parkinson's disease (Monzani et al. 2018; Obata 2002; Sun et al. 2018). Depleted GSH and increased Fe levels provide the perfect environment for the oxidation of DA. In fact, DA quinone-induced oxidative stress is considered

one of the key factors leading to and exacerbating Parkinson's disease (Bisaglia et al. 2010; Cobley et al. 2018).

Interestingly, epidemiological studies relevant to this work also indicate that chronic use of amphetamines appear to increase the risk of developing Parkinson's disease (Curtin et al. 2015; Lappin and Sara 2019; Moratalla et al. 2017; Perfeito et al. 2013). In fact, one epidemiological study found that methamphetamine users were 3.1 times more likely to develop Parkinson's disease (Curtin et al. 2015), and the median age of Parkinson's disease onset was 6 years younger among chronic methamphetamine users (Lappin et al. 2018). Moreover, females appear to have a greater risk of developing Parkinson's disease after chronic exposure to amphetamines than males (Curtin et al. 2015). This is particularly interesting, because the disease is usually more prevalent in men than women (Kalia and Lang 2015). In general, methamphetamine users can develop chronic deficits in fine motor dexterity and timed gait tasks, and these effects can be seen in individuals who have been abstinent up to 12 months (Lappin et al. 2018). Motor deficits with psychostimulant use are not unique to methamphetamine users, chronic use of cocaine can also result in motor deficits. Some individuals that are known to have abused MPH (26%) or cocaine (38%) develop punding (Fasano et al. 2008). Punding is when individuals repeat a certain task compulsively with no purpose, a behavior that is often seen with Parkinson's disease. Additionally, chronic cocaine abusers are known to develop a resting tremor that is associated with the degree and length of use (Brust 2010; Deik et al. 2012). Dystonia or chorea has been observed with cocaine abuse; sometimes these symptoms do not appear until after abstinence, and symptoms have been known to last up to twenty months after abstinence (Brust 2010). Indeed, chronic psychostimulant use is associated with changes in the substantia nigra, and this is a risk factor for movement disorders such as Parkinson's disease (Mursaleen

and Stamford 2016). Moreover, neurodegenerative changes in the nigrostriatal pathway can occur with either methamphetamine or cocaine abuse. For example, DA levels in the striatum of chronic methamphetamine users has been shown to be reduced by 50% (Brust 2010; Lappin et al. 2018; Wilson et al. 1996), and loss of dopaminergic and serotonergic neurons are seen with chronic use of methamphetamine, even after several years of abstinence (Mursaleen and Stamford 2016). Neurodegenerative changes have also been seen in dopaminergic neurons of chronic cocaine abusers, such as overexpression of α-synuclein (Deik et al. 2012; Illés et al. 2019). Additionally, cocaine is capable of binding to α-synuclein possibly leading to aggregation (Illés et al. 2019). Decreased levels of DA and VMAT-2 are also seen with chronic abuse of cocaine (Deik et al. 2012). Furthermore, post-mortem brain tissue of chronic cocaine users showed decreased DA neuronal markers in the nigrostriatal pathway (Mursaleen and Stamford 2016). Lloyd and colleagues also observed this phenomena in mice (Lloyd et al. 2006). However, this was not seen in other rodent studies (Lappin et al. 2018; Little et al. 2009). Altogether, these studies suggest that chronic use of psychostimulants may have detrimental effects on the nigrostriatal pathway, possibly leading to Parkinson's disease.

Parkinsonian Toxin MPTP

In California, in the late 1970s and early 1980s a few unusual medical cases came to the attention of neurologist, Dr. Langston. The patients in these cases were in a catatonic state unable to move their muscles. They were even unable to speak. They displayed the classic symptoms of Parkinson's disease. However, the ages of the individuals ranged from 20 to 40 years old and their symptoms developed into the advanced stages of Parkinson's disease in a matter of days. Parkinson's disease is known to occur almost exclusively in the elderly, and the advanced stages

take many years, if not decades, to develop. Dr. Langston gave his patients L-DOPA, and they responded very well to the treatment and began to move again. Through careful detective work, Dr. Langston soon discovered that the individuals had taken a new "synthetic heroin" and took the next steps to acquire the product and analyze the compounds in the drug. The synthetic drug contained meperidines. Among the meperidines found, the product contained 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Ballard et al. 1985). Dr. Langston soon discovered that MPTP was capable of causing Parkinson's disease-like symptoms in animal models too, revolutionizing Parkinson's disease research (Langston 2017; Palfreman 2014). Previously, there had been no animal model for Parkinson's disease. Interestingly, MPTP leads to changes in the brain that are remarkably similar to the changes in the brain of individuals with Parkinson's disease.

Following systemic administration, MPTP crosses the blood-brain barrier. Then, glial cells take up MPTP, where it is converted to MPP^+ by monoamine oxidase B (MAO-B). MPP^+ is then taken up by DA neurons through the DAT, resulting in a number of effects that lead to neurotoxicity (Blandini and Armentero 2012). In fact, mice that have a double knockout of DAT are resistant to the toxic effects of MPTP (Little et al. 2009). Once inside the DA neurons, MPP^+ inhibits complex I in the mitochondria and causes DA release, eventually leading to cell death. The neurotoxicity is likely a result of oxidative stress produced by complex I inhibition and DA release (Figure 1.7). Indeed, antioxidants can protect against MPP^+ toxicity, and MPP^+ has been shown to cause lipid peroxidation and production of hydroxyl radicals (Lee et al. 2003; Obata 2002; Seyfried et al. 2000). However, in an effort to prevent toxicity, cells sequester MPP^+ into vesicles. VMAT-2 helps move MPP^+ into the vesicle, and VMAT-2 inhibitors and VMAT-2 heterozygote knockout mice have enhanced MPTP toxicity (Fleckenstein and Hanson 2003).

However, MPP^+ displaces DA from vesicles into the cytosol; therefore, it is possible that MPP^+ may be capable of inducing oxidative stress by creating the right conditions for the autoxidation of DA (Lee et al. 2003). In fact, MPP^+ is known to instigate a large release of DA in the striatum (Obata 2002). Moreover, MPP^+ has a half-life of more than a week; therefore, MPP^+ is capable to causing a sustained release of DA (Obata 2002). As mentioned earlier, DA can easily form free-radicals that lead to the formation of DA-o-quinones, further exacerbating oxidative stress. GSH tries to protect against the oxidative stress produced by MPP^+ and the DA release that it produces but can become depleted (Gaki and Papavassiliou 2014; Seyfried et al. 2000).

MPTP has become a standard model for Parkinson's disease for several reasons. Firstly, MPTP leads to a significant loss of DA neurons in the nigrostriatal pathway, a hallmark pathology of Parkinson's disease. Furthermore, as discussed above, MPTP results in oxidative stress, and studies show that oxidative stress also contributes to the development of Parkinson's disease (Hald and Lotharius 2005). In fact, post-mortem brains of Parkinson's disease patients have depleted GSH (Gaki and Papavassiliou 2014; Stokes et al. 2000). In addition, MPTP shares a similar structure to the pesticide paraquat and a similar mechanism of action to the pesticide rotenone, and pesticide exposure may contribute to idiopathic Parkinson's disease (Drechsel and Patel 2008; Gaki and Papavassiliou 2014). In fact, epidemiological studies indicate that Parkinson's disease is more prevalent in rural areas, where it is more likely that these pesticides or similar compounds are used (Cerri et al. 2019). Furthermore, as mentioned earlier, α-synuclein is implicated in Parkinson's disease and aggregates of α-synuclein are seen after rodents are exposed to the pesticides rotenone, dichlorvos, or paraquat (Dias et al. 2013). Interestingly, single doses of MPTP do not result in Parkinson's disease-like symptoms; however, PET studies showed that asymptomatic individuals did have decreased dopaminergic neurons (Brust 2010).

This suggests that multiple doses of the neurotoxin overtime, may eventually lead to a neurodegenerative disorder. Moreover, males appear to be more susceptible to the neurotoxin, MPTP.

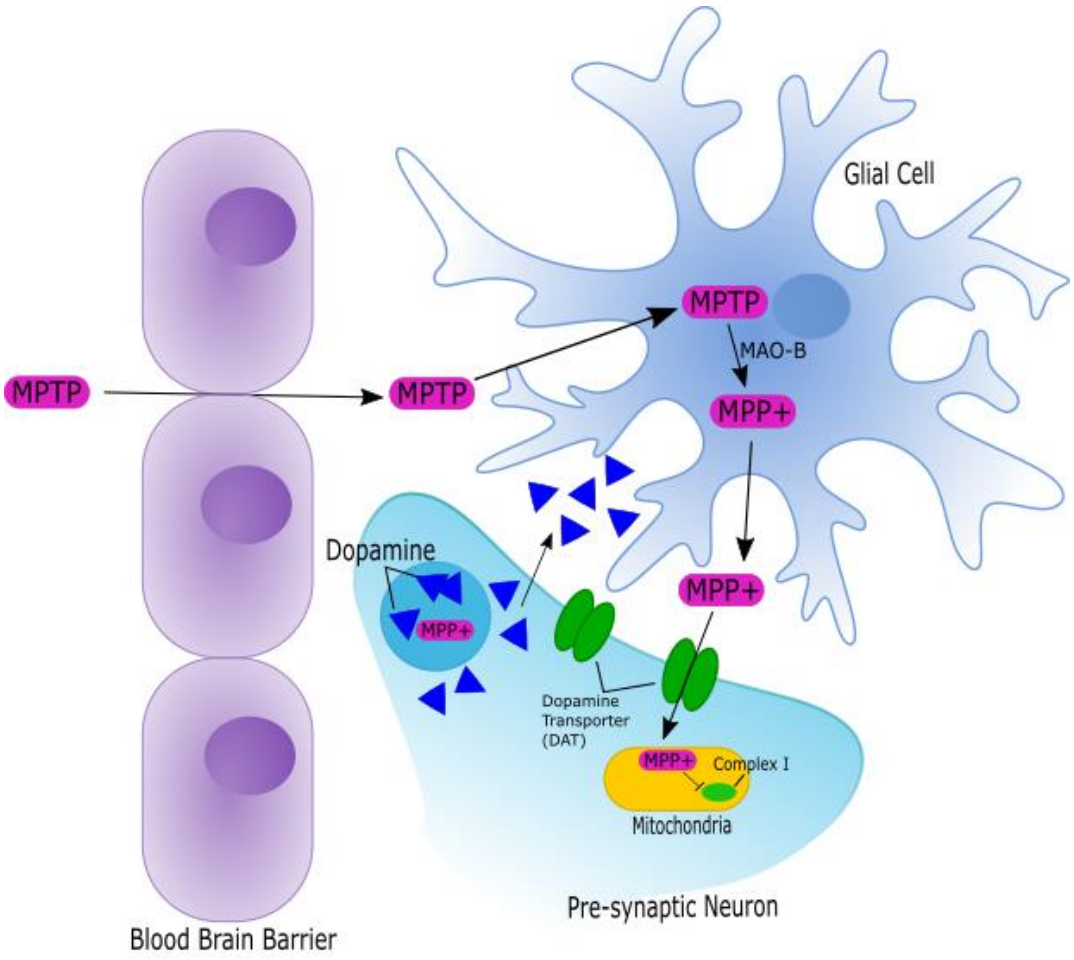

Figure 1.7 MPTP mechanism of action. MPTP targets and destroys dopaminergic neurons, resulting in Parkinson's-like symptoms. MPTP crosses the blood-brain barrier, where it is oxidized by MAO-B to MPP^+ in glial cells. MPP^+ is transported out of glial cells, where it is then taken up by neurons through the dopamine transporter (DAT). MPP^+ then inhibits complex I of mitochondria, leading to apoptosis.

Parkinson's Disease, MPTP, and Gender

Parkinson's disease has a higher prevalence in males than females (Kalia and Lang 2015). Furthermore, Parkinson's disease affects males and females differently. For instance, females tend to present with different motor symptoms, and motor symptoms appear later within the disease progression when compared to males (Cerri et al. 2019; Picillo et al. 2017). Reduced rigidity and tremor are often seen in females whereas males are more susceptible to developing freezing gait (Cerri et al. 2019; Picillo et al. 2017). Nonmotor symptoms such as depression, anxiety, fatigue, and loss of taste or smell are more common and more severe in females while males are more likely to develop impulse control disorders such as pathological gambling and hypersexuality (Cerri et al. 2019). It is hypothesized that differing levels of estrogen may be one reason Parkinson's disease affects males and females differently, because the incidence of Parkinson's disease is similar when males are compared to post-menopausal females (Cerri et al. 2019). Of note, both estrogen receptor α and estrogen receptor β are found in the substantia nigra, and estrogen receptor β can be found on substantia nigra pars compacta projections to the ventral striatum (Al Sweidi et al. 2012). Estrogen is believed to be neuroprotective in Parkinson's disease, and females with higher estrogen exposure throughout their lifetime have a decreased risk of developing the disease (Cerri et al. 2019; Jurado-Coronel et al. 2018; Picillo et al. 2017). Additionally, estrogen replacement therapy reduces the risk of developing Parkinson's disease and increases DAT density in the striatum of post-menopausal females (Al Sweidi et al. 2012; Jurado-Coronel et al. 2018). In contrast, administration of testosterone 24 hours before methamphetamine increased DA depletion in the striatum of male mice and had no effect on female mice (Jurado-Coronel et al. 2018).

The most abundant estrogen in humans is 17 β-estradiol, which has been shown to improve Parkinson's disease symptoms for both males and females (Al Sweidi et al. 2012; Cerri et al. 2019). The activity of 17 β-estradiol may be due to its modulation of DA and/or the DAT. 17 β-estradiol is capable of increasing the synthesis, release, reuptake, and turnover of DA (Al Sweidi et al. 2012; Cerri et al. 2019; Morissette et al. 2008). Additionally, 17 β-estradiol is known to modulate the levels of DAT in the striatum (Morissette et al. 2008). To illustrate, DAT expression in the striatum of female mice was significantly increased during diestrus (low estrogen) when compared to proestrus (high estrogen) (Jurado-Coronel et al. 2018). Furthermore, there is a greater depletion of DA in the striatum when methamphetamine is administered during the proestrus phase as compared to diestrus (Jurado-Coronel et al. 2018). One way that estrogen may be neuroprotective is by modulating DAT levels (Ragonese et al. 2006). Of note, young to middle-aged females have higher levels of DAT binding when compared to males, which may be one possible reason pre-menopausal females are at a lower risk of developing Parkinson's disease (Jurado-Coronel et al. 2018).

Additionally, 17 β-estradiol has been shown to reduce oxidative stress and apoptosis in Parkinson's disease models (Cerri et al. 2019; Ragonese et al. 2006). The mitochondria of female rats generally show less damage and oxidative stress than male rats in animal models of aging and neurodegenerative disease (Cerri et al. 2019). This may be because neurons in females have higher electron transport chain activity, which helps reduce the loss of superoxide radicals (Cerri et al. 2019). Furthermore, estrogen is capable of decreasing DA-induced oxidative stress as it plays a role in Fe metabolism, and males are more susceptible to Fe accumulation (Cerri et al. 2019). Furthermore, estrogens are known to prevent α-synuclein aggregation and, therefore, prevent Lewy body formation (Picillo et al. 2017).

Males appear to have an increased susceptibility to neurotoxicity induced by MPTP (Jurado-Coronel et al. 2018; Morissette et al. 2008). Studies show that estrogen may be responsible for conveying neuroprotection against MPTP. For example, MPTP caused a significant decrease in dopaminergic neurons in the substantia nigra of ovariectomized female mice, and this effect was attenuated with estrogen replacement (Jurado-Coronel et al. 2018). Moreover, 17 β-estradiol was not neuroprotective against MPTP in estrogen receptor knock out mice (Al Sweidi et al. 2012). However, estrogen may only be neuroprotective in females as estrogen treatment in gonadectomized female mice decreased DA release in the striatum caused by MPP^+ but this effect was not seen in gonadectomized male mice (Disshon and Dluzen 2000). In contrast, pretreatment with a low, physiological dose of estrogen before administering MPTP yields neuroprotection to male and female mice, while a high dose of estrogen does not (Al Sweidi et al. 2012; Morissette et al. 2008). Clearly, more research is needed to further elucidate how mechanisms within the nigrostriatal pathway in females may differ from males.

Research Objectives

In recent years, ADHD has become an important health issue as more and more individuals are diagnosed. Psychostimulants are first-line treatment, and MPH is the most commonly prescribed drug to treat ADHD. MPH increases the levels of DA and NE in the synapse by blocking DAT and NET, respectively. ADHD was initially thought to be a childhood disorder and MPH was given for short periods of time; however, it is now thought to extend into adulthood (Bonvicini et al. 2016). As a consequence, individuals are taking MPH for longer periods of time, highlighting the need to study the chronic use of MPH. Additionally, since MPH increases DA, it can be a drug of abuse. In this study, we analyzed the long-term consequences

of exposure to low (therapeutic) and high (abusive) doses of MPH. MPH targets dopaminergic neurons; therefore, chronic use of MPH may lead to changes within important dopaminergic pathways, including the mesolimbic and nigrostriatal pathways. The mesolimbic pathway is well-known for its role in reward; however, there are projections along this pathway that lead to the hippocampus. The hippocampus plays an important role in memory and recall; and memory and recall can be altered with MPH. In addition, adult neurogenesis is known to occur within the dentate gyrus of the hippocampus. Mesolimbic dopaminergic neurons innervate the hippocampus, and thus, it is possible that alterations in dopaminergic signaling may affect neurogenesis. The nigrostriatal pathway is important for motor function. Indeed, a loss of the DA neurons in this pathway leads to the movement disorder, Parkinson's disease. The nigrostriatal pathway is particularly vulnerable to oxidative stress due to its high concentration of dopaminergic neurons. In fact, oxidative stress related to DA has been linked to the pathology of Parkinson's disease. When DA is at a physiological pH, it readily autoxidizes to form quinones and free-radicals. These free-radicals can disrupt normal cellular function, possibly leading to cell death. The mechanism of action of MPH leads to an increase of DA in the synaptic cleft; since this increased DA may be oxidized to quinones and produce free-radicals, long-term MPH exposure could increase oxidative stress. Therefore, we aimed to see if chronic MPH use increased indicators of oxidative stress within the nigrostriatal pathway.

In Chapter 2, we investigate whether chronic low or high doses of MPH affect the proliferation and survival of granule cells within the DG of the hippocampus. To investigate the proliferation and survival of newly generated cells after chronic MPH treatment, we utilized 5'-ethinyl-2'-deoxyuridine (EdU), a thymidine analog which incorporates into DNA when the cell is dividing. Number of EdU+ cells to total neurons, which were labeled using

immunohistochemistry for neuronal nuclei (NeuN), were calculated and compared between groups. Interestingly, chronic low and high doses of MPH were found to increase hippocampal neurogenesis rate. However, if MPH injections were not continued, then the newly generated cells did not survive. If treatment was continued, the newly generated neurons survived only in the mice receiving low dose methylphenidate.

To investigate the mechanism for this effect, we examined levels of proteins linked to cell proliferation in the hippocampus, including BDNF, GDNF, VEGF, TrkB, and beta-catenin after chronic MPH treatment. BDNF and GDNF were investigated in the dentate gyrus of the hippocampus using ELISA-based assays. BDNF and GDNF levels were not significantly different between groups. The protein levels of VEGF, TrkB, and beta-catenin were determined using a Simple Western assay. Hippocampal VEGF, TrkB, and beta-catenin were significantly increased in mice receiving low dose MPH for 28 days compared to controls. Interestingly, high dose MPH significantly decreased beta-catenin after 28 days and decreased VEGF, beta-catenin, and TrkB after 56 days compared to controls. Thus, low dose MPH appears to increase cell proliferation and cell survival in the hippocampus, and these effects may be mediated by increases in VEGF, TrkB, and beta-catenin. While high dose MPH may initially increase neuronal proliferation, newly-generated neurons are unable to survive long-term, possibly due to decreases in VEGF, TrkB and beta-catenin.

In Chapter 3, we examine the mechanism by which long-term MPH exposure sensitizes nigrostriatal DA neurons to the Parkinsonian toxin, MPTP. We hypothesized that chronic MPH exposure leads to an increase in DA-o-quinone production and a depletion in the antioxidant GSH. To investigate this, male mice received chronic low or high dose MPH or saline and were then challenged with MPTP or saline. The striatum was extracted, and DA-o-quinone levels was

examined using near-infrared fluorescence dot blots, while a glutathione assay was used to measure glutathione content. DA o-quinone formation increased with increasing doses of MPH. Additionally, MPH dose-dependently resulted in a depletion of glutathione, which was further depleted following treatment with the Parkinsonian toxin, MPTP. Thus, the increased sensitivity of dopamine neurons to MPTP toxicity following chronic MPH exposure may be due, at least in part, to quinone production and subsequent depletion of glutathione.

In Chapter 4, we investigated the interaction between long-term MPH exposure and MPTP in a female model. We hypothesized that chronic exposure to MPH will render dopaminergic neurons within the nigrostriatal pathway more sensitive to MPTP, and that estrogen may play a protective role. To investigate this, female mice received chronic low or high dose MPH or saline and were then challenged with MPTP or saline. Female mice were grouped and analyzed as either proestrus (high estrogen) or anestrus (low estrogen) at the time of MPTP (or saline) exposure. Animals were sacrificed 7 days after MPTP or saline exposure, and midbrain tissue was sectioned, immunostained for tyrosine hydroxylase, and the number of dopaminergic neurons in the substantia nigra were counted using stereology. In addition, as above, the striatum was extracted, and oxidative stress related to increased dopamine levels was examined using a glutathione assay to measure glutathione content and near-infrared fluorescence to measure free and protein-bound ortho-quinones. Interestingly, dopaminergic neurons in the nigrostriatal pathway are sensitized to MPTP in female mice that are in proestrus at the time of MPTP exposure. However, anestrus females exhibited no neurotoxicity to MPTP after long-term exposure to MPH. This same trend was observed in proestrus females that received long-term MPH, but MPTP effect was not significant. Chronic MPH exposure contributed to GSH depletion, but surprisingly, it did not increase dopamine quinone levels or

dopaminergic cell loss. There were no significant differences in anestrus animals, with the exception of a depletion in glutathione seen when animals received long-term high dose (10 mg/kg) MPH followed by MPTP. Thus, estrogen may sensitize neurons to MPTP in this model, and long-term MPH may contribute to glutathione depletion within the striatum. This study provides insight into how long-term psychostimulant use may affect males and females differently.

Altogether, these studies have shown that chronic treatment with MPH is changes neuronal viability and plasticity in multiple dopaminergic pathways. Chronic MPH at low and high doses affects mesolimbic DA input to the hippocampus, altering neurogenesis within the dentate gyrus. Additionally, chronic MPH treatment sensitizes dopaminergic neurons in the nigrostriatal pathway to further oxidative stress, possibly leading to neurodegeneration. Moreover, chronic MPH treatment appears to affect males and females differently, possibly due to estrogen. These data are particularly relevant as there are an estimated 35 million stimulant users worldwide (Lappin et al. 2018). Furthermore, many of these stimulant users are children diagnosed with ADHD (Sridhar et al. 2017). Importantly, these data indicate that long-term MPH may have profound effects on adolescent brain development. Therefore, knowledge gained about the chronic use of MPH can be used to make more informed decisions on the length of treatment for individuals with ADHD.

CHAPTER 2. NEUROGENESIS WITHIN THE HIPPOCAMPUS AFTER CHRONIC METHYLPHENIDATE EXPOSURE

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Abstract

Methylphenidate is a psychostimulant used to treat Attention Deficit Hyperactivity Disorder. Neurogenesis occurs throughout adulthood within the dentate gyrus of the hippocampus and can be altered by psychoactive medications; however, the impact of methylphenidate on neurogenesis is not fully understood. We investigated the effects of chronic low (1 mg/kg) and high (10 mg/kg) intraperitoneal doses of methylphenidate on neurogenesis in mouse hippocampus following 28 days and 56 days of treatment. Interestingly, methylphenidate, at both doses, increased neurogenesis. However, if methylphenidate treatment was not continued, the newly generated cells did not survive after 28 days. If treatment was continued, the newly generated neurons survived only in the mice receiving low dose methylphenidate. To investigate the mechanism for this effect, we examined levels of proteins linked to cell proliferation in the hippocampus, including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), tropomyosin receptor kinase B (TrkB), and beta-catenin. BDNF or GDNF levels were not significantly different between groups. However, hippocampal VEGF, TrkB, and beta-catenin were significantly increased in mice receiving low dose methylphenidate for 28 days compared to controls. Interestingly, high dose methylphenidate significantly decreased beta-catenin after 28 days and decreased VEGF, beta-catenin, and TrkB after 56 days compared to controls. Thus, low dose methylphenidate appears to increase cell proliferation and cell survival in the hippocampus, and these effects may be mediated by increases in VEGF, TrkB, and beta-catenin. While high dose methylphenidate may initially increase neuronal proliferation, newly-generated neurons are unable to survive long-term, possibly due to decreases in VEGF, TrkB and beta-catenin.

Keywords: Neurogenesis, Methylphenidate, Hippocampus, Dentate Gyrus

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a neurobehavioral developmental disorder that affects 11% of children ages 4-17 years old in the U.S. alone (Hamed et al., 2015). The most commonly prescribed drug to treat ADHD is methylphenidate (MPH; Ritalin®). MPH is a psychostimulant that inhibits dopamine (DA) and norepinephrine (NE) reuptake, increasing the amount of these monoamines in the synaptic cleft. MPH inhibits the reuptake of these monoamines by blocking dopamine or norepinephrine transporters (DAT and NET) but has a greater affinity for DAT than NET. Recently, there has been an increase in the number of high school and college students abusing MPH and an increase in children continuing to take MPH for ADHD symptoms into adulthood (Bonvicini et al., 2016). Despite the increasing use and abuse of MPH, few studies have been conducted that examine the neurologic effects of chronic treatment with MPH.

Neurogenesis or the "birth of new neurons" occurs throughout life within the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus, where the newly generated cells become incorporated into the local DG as granular cells. The rate of hippocampal neurogenesis as well as the survival of these cells is known to be altered by exposure to psychoactive compounds, such as psychostimulants and antidepressants (Lee et al., 2012). Antidepressants such as selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs) and drugs that block only the NET increase neurogenesis, increasing both proliferation and survival of hippocampal neurons. In contrast, chronic exposure to cocaine, a psychostimulant that inhibits the DAT, NET, and the serotonin transporter (SERT), has been shown to either increase or decrease neurogenesis rate, depending on the animal model (Castilla-Ortega et al., 2016; García-Fuster et al., 2010; Lloyd et al., 2010; Sudai et al., 2011). In one study, chronic cocaine exposure

(2 x 10 mg/kg/day, subcutaneous injections) for 28 days increased hippocampal neurogenesis rate in mice (Lloyd et al., 2010). In another study, rats trained to self-administer cocaine (1.5 mg/kg intravenous infusions) for 14 days displayed impaired neurogenesis, with decreases in cell proliferation, yet no effect on cell survival (Sudai et al., 2011). Thus, given the number of serotonin, norepinephrine, and dopamine-targeting drugs that alter cell proliferation within the hippocampus, these monoamine neurotransmitters appear to play a modulatory role in hippocampal neurogenesis. This idea is further supported by studies demonstrating that lesions of noradrenergic fibers projecting to the dentate gyrus decrease hippocampal neurogenesis (Aimone et al., 2014). Other studies support a role for DA in hippocampal neurogenesis; specifically, immunohistochemical analyses of the hippocampus have shown areas of dense dopaminergic afferent fibers within the DG where the DA receptor, D_2 is expressed (Srikumar et al., 2011). Activation of D_2 receptors increases adult neurogenesis and stabilizes memory, while blockade of the D_2 receptors or lesions of dopaminergic afferent fibers leads to a decrease in neurogenesis and impairs memory (Aimone et al., 2014; McNamara et al., 2014; Srikumar et al., 2011). Given the substantial evidence for the role of NE and DA in both hippocampal development and plasticity, it is likely that chronic exposure to MPH, a NET and DAT inhibitor, could influence neurogenesis in the dentate gyrus.

The monoamines DA and NE may modulate hippocampal neurogenesis by a number of mechanisms, which may include regulation of proteins known to affect cell proliferation, including beta-catenin and vascular endothelial growth factor (VEGF). Other NET and DAT inhibitors have been shown to affect neurogenesis within the hippocampus via upregulating expression of these proteins (Rolando and Taylor, 2014; Warner-Schmidt and Duman, 2007; Zhao et al., 2008). Beta-catenin is a downstream effector of the Wnt pathway, which mediates

the proliferation of neuronal progenitor cells (Warner-Schmidt and Duman, 2006) and is important for the normal development of the hippocampus (Rolando and Taylor, 2014; Yu et al., 2014). When beta-catenin expression is inhibited, there is a decrease in hippocampal neurogenesis (Hui et al., 2015; Mostany et al., 2008; Rolando and Taylor, 2014). Moreover, antidepressants that block the reuptake of NE stimulate proliferation of quiescent neuronal progenitor cells in the adult hippocampus through activation of the Wnt pathway (Rolando and Taylor, 2014). Additionally, beta-catenin signaling is known to stimulate the synthesis of VEGF (Hui et al., 2015). VEGF and its receptors are expressed in and on neuronal progenitor cells, neurons, glial cells, and endothelial cells in the hippocampus, where VEGF appears to promote neurogenesis. Antidepressants, which again are known to increase neurogenesis, induce increases in VEGF, and the blockade of VEGF signaling inhibits the neurogenic effects of antidepressants (Cao et al., 2004; Zhao et al., 2008). Finally, direct infusion of VEGF into the hippocampus increases neurogenesis (Warner-Schmidt and Duman, 2007; Zhao et al., 2008).

Another growth factor that is known to promote neurogenesis in the hippocampus is brain-derived neurotrophic factor (BDNF), and some studies have indicated that MPH can alter expression of BDNF and/or its receptor, TrkB (Fumagalli et al., 2010). SSRIs and SNRIs known to increase hippocampal neurogenesis also cause an increase in BDNF in the hippocampus of rodent models of depression and in post-mortem human brain tissue from individuals who had been treated with antidepressants (Jiang et al., 2014; Sairanen et al., 2005; Tayyab et al., 2018). BDNF has a high affinity for TrkB, and this receptor is expressed in neural progenitor cells of the dentate gyrus (Li et al., 2008). TrkB is required for hippocampal neurogenesis, and deletion of TrkB from neuronal progenitor cells impairs neurogenesis (Li et al., 2008). Chronic antidepressant treatment upregulates mRNA of BDNF and TrkB in the hippocampus (Sairanen et

al., 2005), and infusion of BDNF into the hippocampus of rats increases neurogenesis within the dentate gyrus (Scharfman et al., 2005). Of note, natural aging leads to a decrease in adult neurogenesis, and this decline is associated with decreases in TrkB expression, while BDNF levels are unchanged (Aimone et al., 2014). Interestingly, there is evidence for a modulatory role of BDNF by Wnt signaling, as beta-catenin signaling promotes synthesis of BDNF, and upregulation of BDNF has been found to increase the growth of neurons in culture through activation of the Wnt pathway (Tayyab et al., 2018). Another neurotrophic factor thought to be involved in hippocampal neurogenesis is glial cell line-derived neurotrophic factor (GDNF). Antidepressants increase GDNF mRNA expression in rat astrocytes *in vitro* (Kajitani et al., 2012), and infusion of GDNF into the hippocampus of rats *in vivo* increases neurogenesis within the dentate gyrus (CHEN et al., 2005). Thus, MPH and other drugs that influence monoamine neurochemistry may influence hippocampal neurogenesis through altering the expression of these proteins.

Despite the extensive use of MPH and the supporting evidence that this NET/DAT inhibitor may influence neurogenesis, few studies have examined the impact of this drug on hippocampal plasticity (Lagace et al., 2006; Lee et al., 2012; Schaefers et al., 2009; van der Marel et al., 2015). Furthermore, the studies that have been conducted are conflicting. In some models, treatment with MPH appears to increase neurogenesis (Lee et al., 2012; van der Marel et al., 2015), while others reveal that the number of proliferating neurons in the hippocampus is not altered with MPH (Lagace et al., 2006; Schaefers et al., 2009). Additionally, in one study, a decrease in survival of newly generated neurons was seen in adult rats that were exposed to MPH as juveniles (Lagace et al., 2006). These conflicting results and the overwhelming evidence that MPH can alter neurogenesis highlights a need for further study. Here, we examine how treatment

with chronic MPH affects the proliferation and survival of granule cells within the DG of the hippocampus.

Materials and Methods

Mice and Drug Treatment

All experiments and procedures with animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the University Committee on Animal Care (UCAC) at East Tennessee State University. Juvenile male Swiss-Webster mice were acclimated to the animal facility for a week prior to treatment. Mice were allowed food and water *ad libitum* and were kept on a 12-hour light/dark cycle. Beginning at post-natal day 28 (PND 28), the mice received intraperitoneal (i.p.) injections twice daily with saline, 1 mg/kg, or 10 mg/kg MPH (Sigma Aldrich, dissolved in 0.9% sterile saline) for 28 or 56 days, ending at PND 56 or PND 84, respectively. Five animals were included in each treatment group for both 28 and 56 days of treatment. This period would correspond with the time of early adolescence (PND 28) through late adolescence (PND 56) or adulthood (PND 84) (Laviola et al., 2003). As doses of less than 5 mg/kg MPH i.p. in rodents may model the use of MPH in the clinical treatment of ADHD (Gerasimov et al., 2000; Koda et al., 2010), the 1 mg/kg MPH dose was considered therapeutic. In contrast, the high dose of 10 mg/kg MPH i.p. may represent recreational misuse or the use of MPH in the treatment of narcolepsy (Valvassori et al., 2007). The injections were administered using a school week (5 days/week) dosing schedule to prevent MPH from impeding normal weight gain and/or growth (Martins et al., 2004; Roche et al., 1979).

EdU injections and Immunohistochemistry

After 28 days of treatment with saline, 1 mg/kg, or 10 mg/kg MPH, all mice received a single i.p. dose of 50 mg/kg EdU. EdU is a thymidine analog that is incorporated into DNA during cell division (Chehrehasa et al., 2009). In order to examine neurogenesis rate, some mice were sacrificed 1 day after EdU injection, whereas survival of newly generated cells was examined by sacrificing other mice 28 days after EdU injection. Of note, in order to determine how MPH affects the survival, during the 28 days post-EdU, 1 group received no injections of saline or MPH, while the other group continued injections of saline or MPH. Mice were sacrificed via transcardial perfusion with saline, followed by 4% paraformaldehyde, and brain tissue was subsequently collected. Brains were removed and placed in cryoprotectant (30% sucrose in phosphate buffer) and then sectioned on the cryostat at 20 µm and collected onto slides. Tissue sections were then stained for EdU using Click-iT EdU Alexa Fluor 594 imaging kit (Molecular Probes) and double-labeled for the neuronal marker, NeuN using immunohistochemistry (1:400 mouse anti-NeuN (Millipore) and 1:750 AlexaFluor 488 Goat anti-Mouse (Molecular Probes). Approximately 20 slides were made from each animal with 6 sections per slide. Every other slide was taken and double-labeled for EdU and NeuN.

Sections were analyzed using an epifluorescence microscope (Olympus BX41) equipped with an Olympus Q-color 3 digital camera and Q-Cap Pro 7 software (Fig. 2.1). The number of EdU positive cells were counted in the subgranular zone of the dentate gyrus, and the number of NeuN positive cells were counted throughout the dentate gyrus. The ratio of Edu positive cells/ NeuN positive cells were calculated for each section and total ratios were compiled for each brain/mouse. Counts were made in both hemispheres at 200 X, averaged, and compared between the groups.

Analysis of proteins related to cell proliferation in the hippocampus

The expression of proteins known to influence cell proliferation in the hippocampus were examined following chronic exposure to saline, 1 mg/kg, or 10 mg/kg MPH; these included brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), tropomyosin receptor kinase B (TrkB), and beta-catenin. For these analyses, mice were sacrificed by decapitation, and the hippocampus was removed, flash-frozen in liquid nitrogen, and stored at -70°C until analysis. The hippocampal tissue was homogenized in lysis buffer (300 µl/2 mg of tissue) and normalized for protein content following a Bradford protein assay. The quantification of BDNF and GDNF in the hippocampus utilized an enzyme-linked immunosorbent assay (ELISA, Boster Biological Technology, Pleasanton, CA). The protein levels of VEGF, TrkB, and beta-catenin were determined using a Protein Simple, Simple Western assay (anti-rabbit HRP 12-230 included with kit or anti-goat HRP, 1:50, R&D Systems). The antibodies rabbit anti-VEGF (0.5 µg/mL, Millipore), goat anti-TrkB (10 µg/mL, R&D Systems), and goat anti-beta-catenin (50 µg/mL, R&D Systems) were utilized, and the samples were normalized to GapdH expression (rabbit anti-GapdH, 1:1000, Novus). The protocol included with the kit was followed with no deviations. The Compass software (Protein Simple, Simple Western) was run on its default settings.

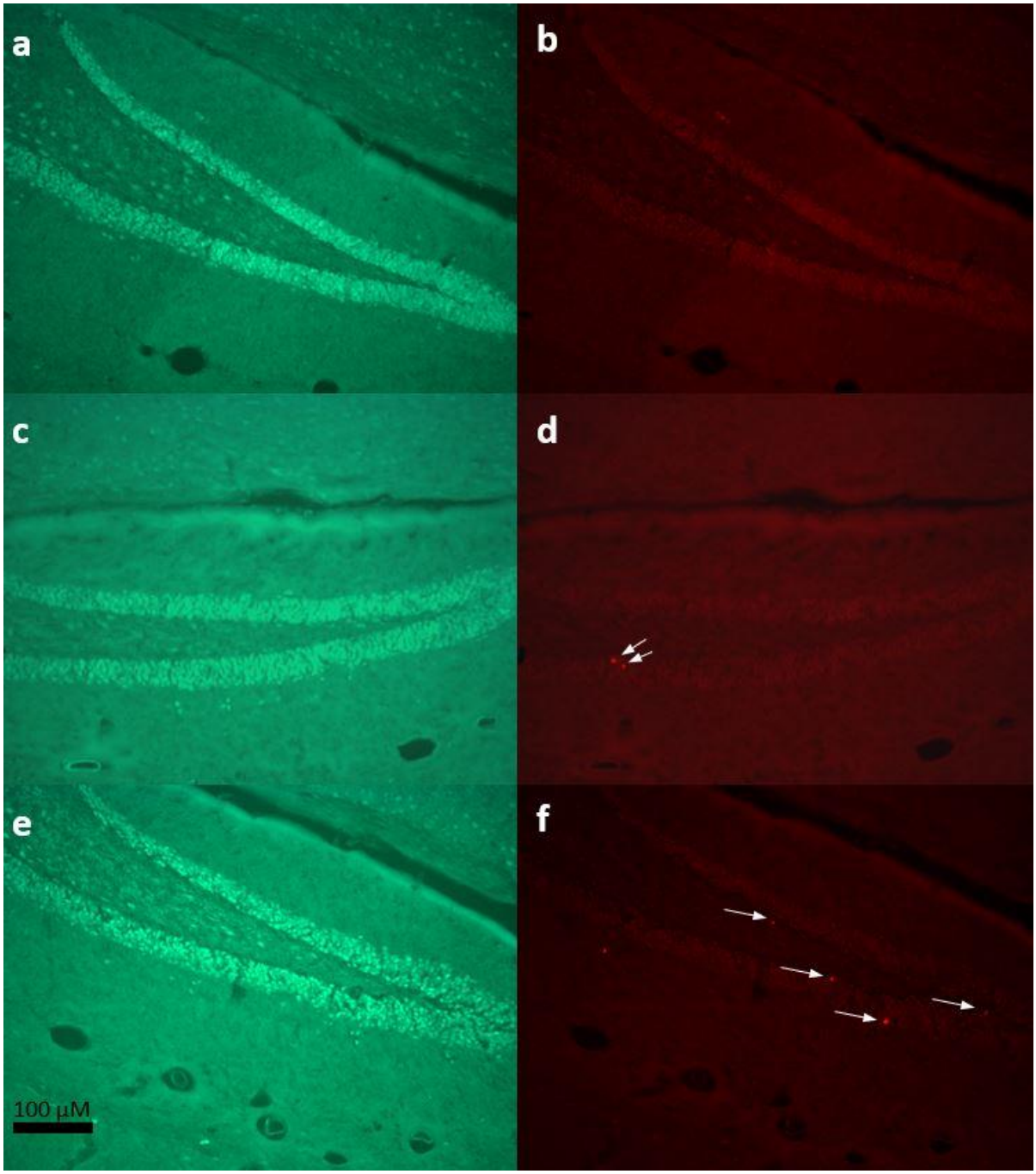

Figure 2.1 Representative fluorescent photomicrographs of the hippocampal dentate gyrus from mice receiving saline (a & b), 1 mg/kg MPH (c & d), or 10 mg/kg MPH (e & f). The brain sections were stained to identify the neuronal marker NeuN (green) seen in panels c, d, & e and the newly generated EdU positive cells (red) seen in b, d, & f

Statistical Analysis

Descriptive statistics were completed on all data and are reported as means +/- SEM. All Statistical analysis were performed using multiple variable analysis of variance (ANOVA), followed by Tukey's multiple comparisons test using GraphPad Prism software version 5. P values of < 0.05 were considered significant.

Results

Chronic Exposure to MPH Increases Neurogenesis

In order to examine the effect of chronic MPH on neurogenesis rate, mice receiving saline, 1 mg/kg, or 10 mg/kg MPH were injected with EdU, and brains were collected the following day. The ratio of EdU+ cells to NeuN+ cells were calculated and compared between groups. Interestingly, both the low and high doses of MPH significantly increased neurogenesis rates compared to saline control (Fig. 2.2). In fact, ratios were approximately 5-fold greater following chronic MPH exposure, indicating that chronic exposure of MPH markedly increases neurogenesis in the DG.

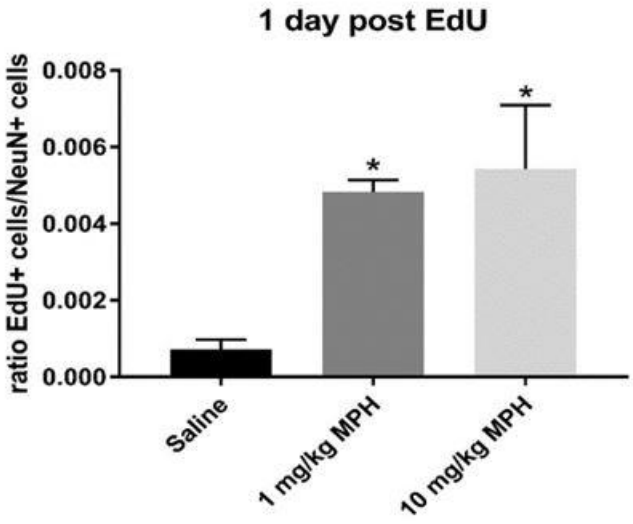

Figure 2.2 Chronic MPH exposure increases in neurogenesis within the dentate gyrus (DG). The ratios of EdU+ cells/ NeuN+ cells in the DG of the hippocampus were compared in mice receiving saline, 1 mg/kg, or 10 mg/kg MPH, injected with EdU, and brains collected 1 day later. Data are expressed as means ± SEM (n=5) and were subjected to a one-way ANOVA followed by Tukey's post-hoc analyses, *p=0.0306 vs Saline

Survival of Newly Generated Neurons is Drug and Dose-Dependent

To determine if the new cells created in response to MPH exposure survive and are incorporated into the DG, mice receiving saline, 1 mg/kg, or 10 mg/kg MPH were injected with EdU, and brains were collected 28 days later. In one experiment, mice did not continue to receive treatment during the 28 days post-EdU and were kept in standard housing (Fig. 2.3a). In the second experiment, mice continued to receive twice-daily injections 5 days/week per their treatment group (saline, 1 mg/kg, or 10 mg/kg MPH). Again, the ratios of EdU+ to NeuN+ cells were calculated and compared. There were no significant differences between any of the groups

in mice where treatment was discontinued, indicating that the new neurons "born" as a result of MPH exposure do not survive and integrate into the DG (Fig. 2.3a). Furthermore, when treatment was continued during the 28 days following EdU exposure, a significant difference in EdU+/NeuN+ ratios was only seen with the low (1 mg/kg) dose of MPH (Fig. 2.3b). Mice receiving chronic exposure to high dose (10 mg/kg) MPH had EdU+/NeuN+ ratios that were equivalent to control animals. However, again, there still appears to be a loss of cells as ratios in 1 mg/kg MPH mice were only about 2-fold higher than saline-controls (as compared to 5-fold higher 1-day post-EdU). Thus, although both low and high doses of MPH may increase neurogenesis rate, neurons are maintained and incorporated into the DG only in mice who continue to receive low dose MPH and not all cells survive.

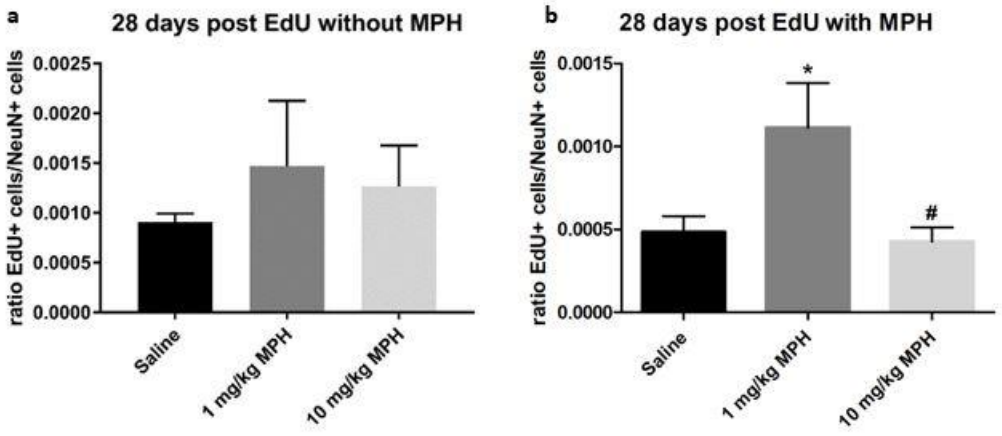

Figure 2.3 Survival of newly generated hippocampal neurons following chronic MPH exposure. The ratios of EdU+ cells/NeuN+ cells in the dentate gyrus of the hippocampus were compared in mice receiving saline, 1 mg/kg, or 10 mg/kg MPH, injected with EdU, and brains collected 28 days later. a) Newly generated hippocampal neurons do not survive when MPH is not continued. b) The newly generated neurons survive when only therapeutic doses of MPH are continued. All

data are expressed as means ± SEM (n=5) and were subjected to one-way ANOVA followed by Tukey's post-hoc analyses, *p=0.0413 vs Saline, #p=0.0333 vs 1 mg/kg MPH

Chronic Exposure to MPH and Growth Factors

In order to elucidate the mechanism by which MPH influences neurogenesis rate and survival of neurons in the hippocampus, we examined the protein levels of various neurotrophic factors in the hippocampus following chronic MPH exposure. Given the wealth of evidence for the role of BDNF, GDNF, and VEGF, we performed ELISA-based assays to examine hippocampal BDNF and GDNF levels and simple westerns to examine levels of VEGF. Surprisingly, there were no significant differences in BDNF and GDNF between any of the drug groups (Fig. 2.4a and 2.4b). However, BDNF does appear to increase with age in all treatment groups. After 28 days of exposure to 1 mg/kg MPH, VEGF was increased compared to the saline group. Conversely, there were no significant differences in hippocampal VEGF in mice exposed to 10 mg/kg MPH despite the profound effect of this dose on neurogenesis rate. Additionally, in mice treated to the match the neuronal survival experiments (28 days treatment, followed by an additional 28 days of treatment = 56 days treatment), VEGF was significantly decreased in mice receiving the high (10 mg/kg) dose of MPH compared to both saline and low dose (Fig. 2.4c).

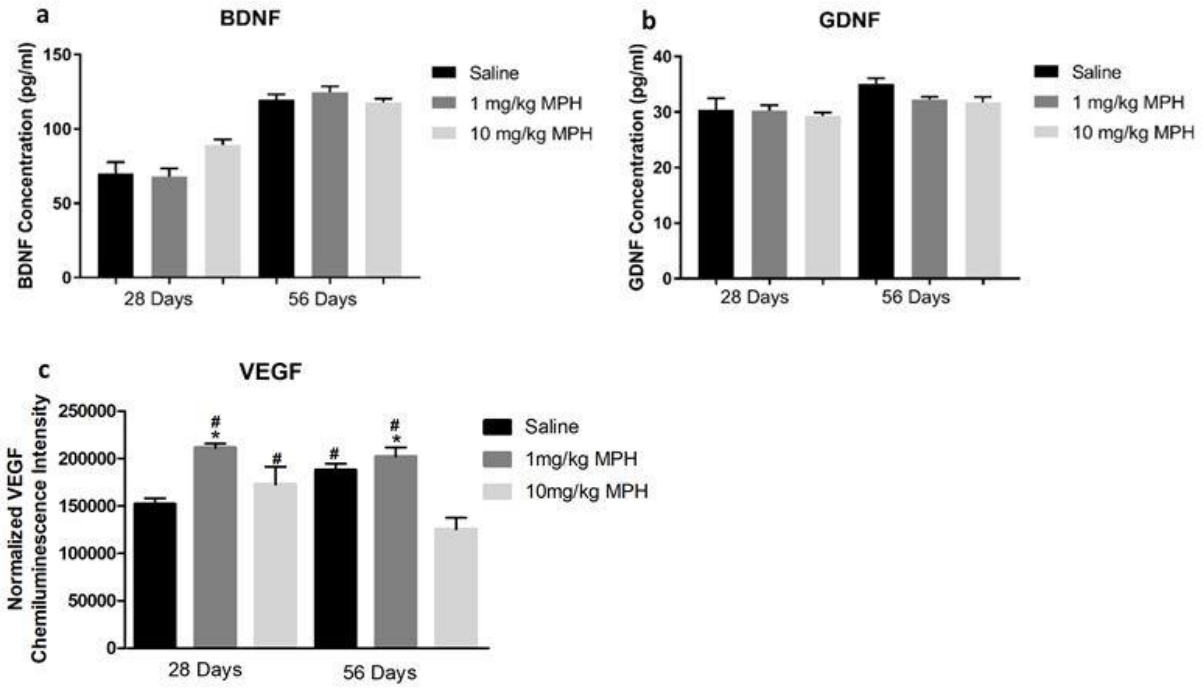

Figure 2.4 Hippocampal growth factors following chronic MPH. Protein levels of BDNF (a), GDNF (b), and VEGF (c). Data are expressed as means ± SEM (n=5). One-way ANOVA followed by Tukey's post-hoc analysis, *p<0.05 vs 28 Days Saline (p=0.0076 28 Days Saline vs 28 Days 1 mg/kg MPH, p=0.0311 28 Days Saline vs 56 Days 10 mg/kg MPH), #p<0.05 vs 56 Days 10 mg/kg MPH (p=0.0001 56 Days 10 mg/kg MPH vs 28 Days 1 mg/kg MPH, p=0.0476 56 Days 10 mg/kg MPH vs 28 Days 10 mg/kg MPH, p=0.0046 56 Days 10 mg/kg MPH vs 56 Days Saline, p=0.0005 56 Days 10 mg/kg MPH vs 56 Days 1 mg/kg MPH)

Chronic Exposure to MPH and Proteins: TrkB and Beta-catenin

To further investigate the mechanism by which MPH influences neurogenesis rate and neuronal survival, we examined the levels of a number of other proteins known to modulate hippocampal neurogenesis, including the BDNF receptor, TrkB. While no significant differences

were seen in BDNF (Fig. 2.4a), the expression of its receptor appears to be influenced by MPH. Following 28 days of 1 mg/kg MPH, TrkB is significantly increased in the hippocampus compared to saline-control animals. However, a decrease in hippocampal TrkB was observed in mice receiving 10 mg/kg MPH for both 28 days and 56 days (Fig. 2.5).

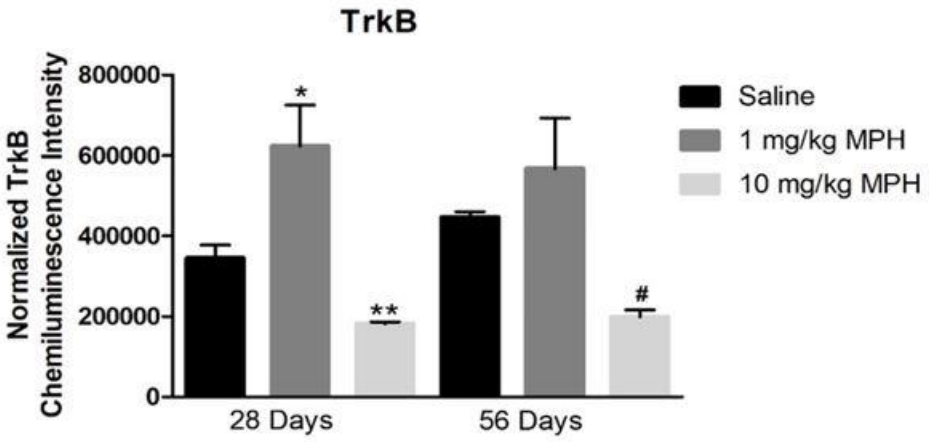

Figure 2.5 Protein levels of the BDNF receptor, TrkB normalized to GapdH. Data are expressed as means ± SEM (n=5). One-way ANOVA followed by Tukey's post-hoc analysis, *p=0.0148 vs 28 Days Saline, **p<0.0001 vs 28 Days 1 mg/kg MPH, #p=0.0003 vs 56 Days 1 mg/kg MPH

Given the considerable evidence for the role of the Wnt pathway in hippocampal neurogenesis, we examined an important downstream effector of this pathway, beta-catenin. Notably, after 28 days of 1 mg/kg MPH, beta-catenin was significantly increased compared to levels in saline-control animals; however, there are no significant differences between saline and 1 mg/kg MPH after 56 days of treatment. Similar to the pattern seen with TrkB, animals that

received 10 mg/kg MPH for either 28 or 56 days had significantly less hippocampal beta-catenin than animals that received either saline or 1 mg/kg MPH (Fig. 2.6).

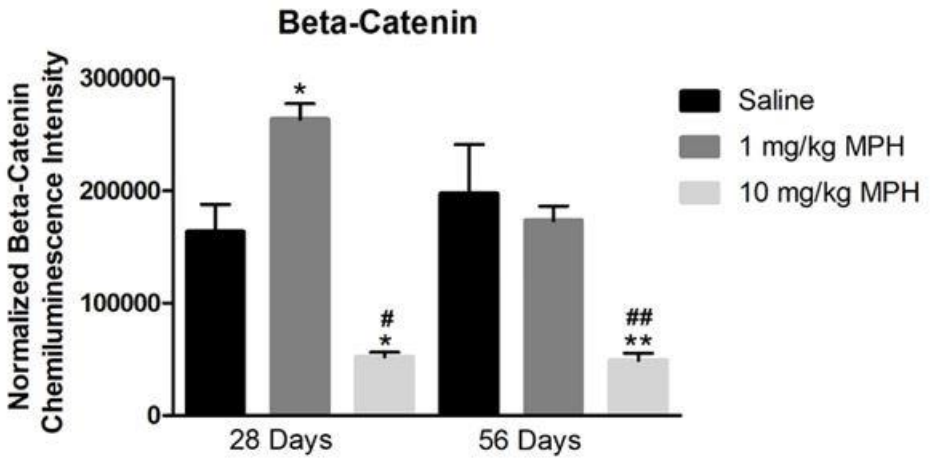

Figure 2.6 Protein levels of beta-catenin normalized to GapdH. Data are expressed as means ± SEM (n=5). One-way ANOVA followed by Tukey's post-hoc analysis, *p<0.05 vs 28 Days Saline (p=0.0389 28 Days Saline vs 1 mg/kg MPH, p=0.0184 28 Days Saline vs 10 mg/kg MPH), #p<0.0001 vs 28 Days 1 mg/kg MPH, **p=0.0010 vs 56 Days Saline, ##p=0.0068 vs 56 Days 1 mg/kg MPH

Discussion

The present study examined how exposure to chronic MPH at low and high doses can induce changes in neurogenesis within the DG from childhood to adulthood, *in vivo.* We demonstrated that both low and high doses of MPH increase neurogenesis, as indicated by increased EdU+/NeuN+ neuron ratios 1-day post EdU exposure. However, the survival of the

newly divided neurons 28 days later is drug- and dose-dependent. Chronic exposure to low doses of MPH appears to support the maintenance and integration of the new neurons into the hippocampus, as EdU+/NeuN+ ratios in animals exposed to low dose MPH were significantly higher than controls. However, chronic exposure to high doses of MPH does not have the same effect, as EdU+/NeuN+ ratios 28 days after EdU exposure were not significantly different than controls. Additionally, newly generated neurons do not survive if MPH is not continued.

Previous studies of the effect of MPH on hippocampal neurogenesis produced mixed results. Studies have shown that low, oral doses (2.5 or 5 mg/kg) of MPH given to adolescent mice for 21 days have no impact on the rate of neurogenesis, but high doses (10 mg/kg) increase hippocampal neurogenesis compared to controls (Lee et al., 2012). Others have shown an increase in neurogenesis when adolescent rats were treated orally with MPH at low (5 mg/kg) or high (10 mg/kg) doses for 28 days (van der Marel et al., 2015). In contrast, one study noted a decrease in survival of newly generated neurons into adulthood in rats exposed to MPH as adolescents (Lagace et al., 2006). Other studies showed that MPH had no effect on neurogenesis in rats when administered i.p. for 15 days (2 mg/kg MPH) or gerbils when administered orally for 30 days (5 mg/kg MPH) (Lagace et al., 2006; Schaefers et al., 2009). These seemingly conflicting observations are likely due to differences in the length of treatment, doses of MPH, drug delivery, and animal model.

The growth factors BDNF, GDNF and VEGF have been shown to effect neurogenesis within the DG (Cao et al., 2004; Fumagalli et al., 2010; Kirby et al., 2015; Lee et al., 2012; Warner-Schmidt and Duman, 2007, 2006). We investigated these proteins in our model and have shown that the mechanism by which MPH increases neurogenesis does not appear to involve BDNF or GDNF. In this study, MPH, at neither low nor high doses, induced a change in BDNF

or GDNF. Although no previous studies investigated the effect of MPH on GDNF expression, studies examining the effect of MPH on BDNF have been mixed. While some groups observed increases in BDNF expression in the hippocampus, others have reported a decrease in BDNF expression with MPH exposure (Fumagalli et al., 2010; Lagace et al., 2006; Lee et al., 2012). Previous studies have shown increases in BDNF in the subgranular zone with 10 mg/kg (Lee et al., 2012), an increase in BDNF in the striatum with 1 mg/kg (Fumagalli et al., 2010), and a decrease in BDNF in the hippocampus using a 2 mg/kg dose of MPH (Lagace et al., 2006). Another study shows a decrease in BDNF mRNA in the hippocampus of juvenile rats, and an increase in adult rats treated with MPH (Banerjee et al., 2009). Additionally, MPH has been shown to decrease BDNF and TrkB in the prefrontal cortex and increase BDNF in the striatum (Fumagalli et al., 2010). These differences may be due to the dose of MPH, animal model, and the area of the brain studied.

In this study, VEGF, TrkB, and beta-catenin were significantly increased by low dose MPH after 28 days compared to saline-treated animals, correlating to the increase in the rate of neurogenesis seen in the DG after 28 days of 1 mg/kg MPH. Interestingly, although exposure to high doses (10 mg/kg) of MPH for 28 days induced an increase in neurogenesis rate, none of these factors were increased in response to 28 days of exposure to 10 mg/kg MPH. In fact, there was a significant decrease in beta-catenin after 28 days and a significant decrease in VEGF, beta-catenin, and TrkB was observed after 56 days of treatment. Of note, the decreased expression of these proteins may explain why neurons generated in response to 10 mg/kg MPH were unable to survive, with or without continued treatment, 28 days later. Although BDNF was unaltered by methylphenidate (only increased by age), TrkB was decreased by high dose MPH. Other studies have seen no change in BDNF and a decrease in TrkB with natural aging (Aimone et al., 2014).

Chronically, low doses of MPH appear to increase cell proliferation and cell survival in the hippocampus, and these effects may be mediated by increases in the expression of VEGF, TrkB, and beta-catenin. While chronic high doses of MPH may initially increase neuronal proliferation, newly-generated neurons are unable to survive long-term, possibly due to decreases in VEGF, TrkB and beta-catenin. Neurogenesis is capable of improving cognitive function (Villeda et al., 2011), and the ability of MPH to impact hippocampal neurogenesis may play an important role in the mechanism by which MPH increases cognitive functions.

Compliance with Ethical Standards

Ethical Approval: All experiments and procedures with animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the University Committee on Animal Care (UCAC) at East Tennessee State University.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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CHAPTER 3. CHRONIC METHYLPHENIDATE INDUCES INCREASED QUINONE PRODUCTION AND SUBSEQUENT DEPLETION OF THE ANTIOXIDANT GLUTATHIONE IN THE STRIATUM

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Oakes H.V., Ketchem S., Hall A., Ensley T., Medlock K., Pond B.B. 2019. Chronic methylphenidate induces increased quinone production and subsequent depletion of the antioxidant glutathione. *Pharmacological Reports.* doi:10.1016/j.pharep.2019.08.003

Abstract

Background: Methylphenidate (Ritalin®) is a psychostimulant used chronically to treat attention deficit hyperactivity disorder. Methylphenidate acts by preventing the reuptake of dopamine and norepinephrine, resulting in an increase in these neurotransmitters in the synaptic cleft. Excess dopamine can be autoxidized to a quinone that may lead to oxidative stress. The antioxidant, glutathione helps to protect the cell against quinones via conjugation reactions; however, depletion of glutathione may result from excess quinone formation. Chronic exposure to methylphenidate appears to sensitize dopaminergic neurons to the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We hypothesized that oxidative stress caused by the autooxidation of the excess dopamine renders dopaminergic neurons within the nigrostriatal pathway to be more sensitive to MPTP.

Methods: To test this hypothesis, male mice received chronic low or high doses of MPH and were exposed to saline or MPTP following a 1-week washout. Quinone formation in the striatum was examined via dot blot, and striatal GSH was quantified using a glutathione assay.

Results: Indeed, quinone formation increased with increasing doses of methylphenidate. Additionally, methylphenidate dose-dependently resulted in a depletion of glutathione, which was further depleted following MPTP treatment.

Conclusions: Thus, the increased sensitivity of dopamine neurons to MPTP toxicity following chronic methylphenidate exposure may be due to quinone production and subsequent depletion of glutathione.

Keywords: Methylphenidate, Oxidative stress, Glutathione, Dopamine quinone

Introduction

According to the Centers for Disease Control and Prevention, 6.4 million children have been diagnosed with attention deficit hyperactivity disorder (ADHD) in the U.S. alone. The psychostimulant, methylphenidate (MPH) is the most commonly prescribed drug for the treatment of ADHD. MPH acts by blocking dopamine (DA) and norepinephrine (NE) transporters, preventing the reuptake of these catecholamines following release. Thus, MPH causes a profound, acute increase in DA levels within the striatum (STR) [1]. Previous research has shown that chronic exposure to MPH causes dopaminergic neurons within the nigrostriatal pathway to be more sensitive to the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [2]. These findings are supported by epidemiological data that indicates use of psychostimulants, such as amphetamine and methamphetamine, increases the risk of developing Parkinson's disease [3-5]. Of note, a significant loss of dopaminergic neurons within the nigrostriatal pathway is a hallmark of this disease. Given that oxidative stress is known to be involved in the degenerative process of Parkinson's disease [6] and that excess dopamine may cause oxidative stress [7], we hypothesize that chronic MPH exposure leads to oxidative stress when the autoxidation of excess DA renders dopaminergic neurons within the STR to be more sensitive to MPTP.

At a physiological pH, the hydroxyl group in DA is able to dissociate, creating DA o-quinone. Thus, DA can be autoxidized without catalysis, producing DA o-quinone which cycles to aminochrome and eventually forms hydrogen peroxide (H_2O_2) (Figure 3.1). The H_2O_2 may be converted to superoxide and hydroxyl radicals in the presence of the metal ions, leading to oxidative stress (Figure 3.1). In addition to this, excess DA that is not sequestered in vesicles can form the DA o-quinone in the presence of metal ions and enzymes with peroxidase activity.

Interestingly, ferrous ions appear to accumulate in the nigrostriatal pathway, promoting catecholamine oxidation [8]. Fortunately, neurons have systems in place to combat this potential source for oxidative stress. Glutathione (GSH) is an important antioxidant that protects against the toxicity of quinones via conjugation reactions, and the resulting molecules may be incorporated into neuromelanin or released into the cerebral spinal fluid [9]. The cofactor, NADH is needed in the conjugation reaction between GSH and DA o-quinone. Unfortunately, GSH is competing with the cyclization product of DA o-quinone, aminochrome for NADH. Aminochrome uses NADH to form leukoaminochrome o-semiquinone radical and hydroxyl radicals (Figure 3.1) [10]. Therefore, excess quinone may deplete GSH as well as deplete the cofactor needed for the conjugation reaction. When GSH is depleted, neurons are vulnerable to oxidative stress and therefore, neurotoxicity. Additionally, the quinone forms thiol conjugates with sulfhydryl groups on cysteine residues that disrupt normal functioning of proteins including glucocerebrosidase and parkin, proteins known to be associated with Parkinson's disease [8]. In addition to damaging proteins, DA o-quinone and other reactive oxygen species are able to induce oxidative stress by lipid peroxidation and DNA oxidation leading to further damage of the neuron and eventually, apoptosis.

Some laboratories have investigated the potential for MPH to induce oxidative stress. Chronic MPH has been found to decrease GSH content in the several brain regions [9]. No studies have examined the production DA quinone and subsequent depletion of GSH in the STR following chronic MPH. Given the fact that the STR is rich in dopaminergic neurons and implicated in Parkinson's disease, we measured the amount of quinones and GSH in the STR following chronic MPH treatment.

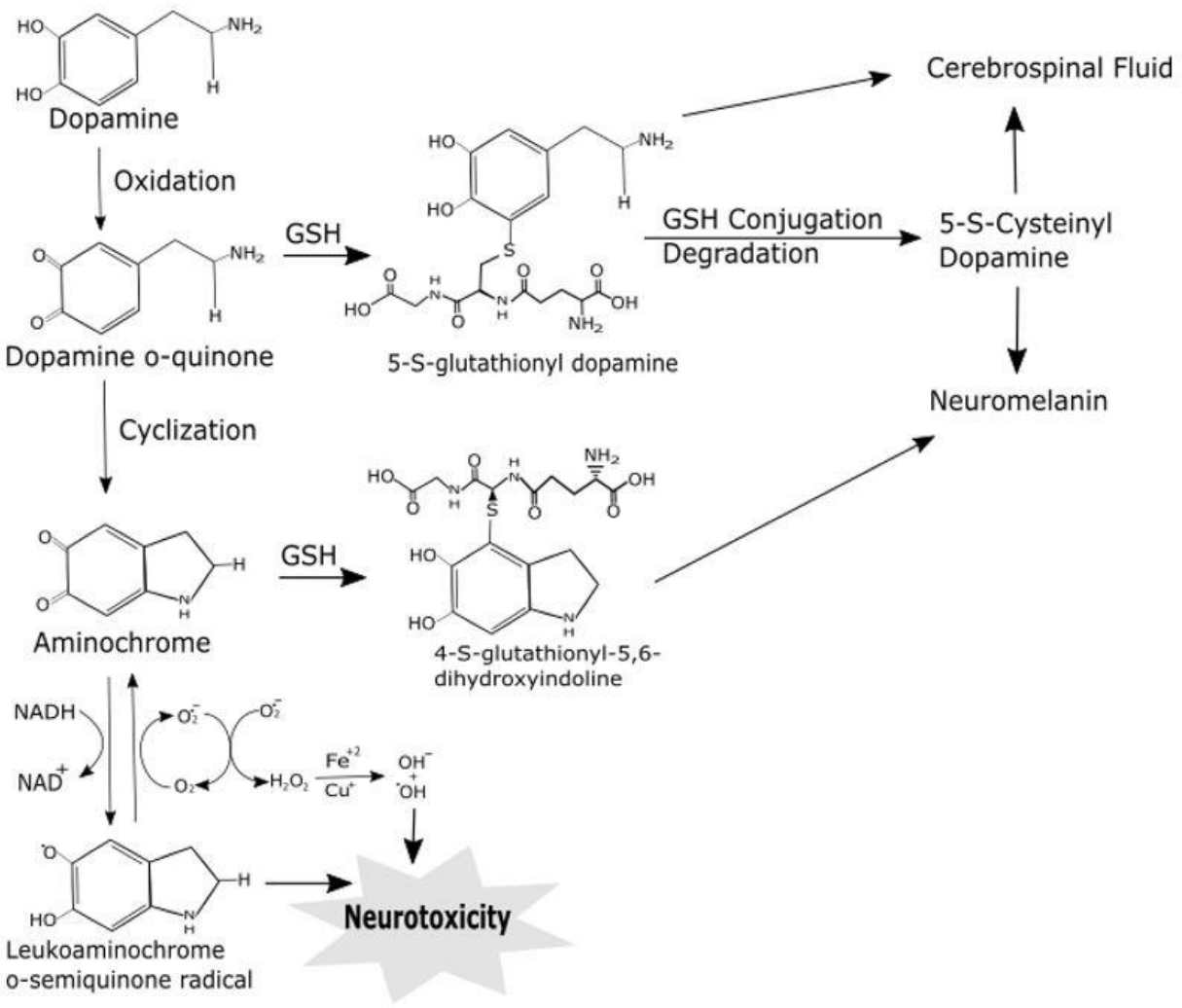

Figure 3.1 Dopamine oxidation pathway. Dopamine can be oxidized to dopamine o-quinone. Glutathione (GSH) then conjugates with dopamine o-quinone and degrades to form 5-S-cysteinyl dopamine. 5-S-cysteinyl dopamine is then incorporated into neuromelanin or released into the cerebral spinal fluid. GSH can also conjugate with aminochrome and is then incorporated into neuromelanin. Neurotoxicity results when superoxide and/or hydroxyl radicals are formed and is exacerbated when GSH is depleted.

Materials and Methods

Mice and Drug Treatment

All experiments and procedures with animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the University Committee on Animal Care (UCAC) at East Tennessee State University. Mice were acclimated to the animal facility for a week prior to treatment. Mice were allowed food and water ad libitum and were kept on a 12-hour light/dark cycle. Adolescent male Swiss-Webster mice received intraperitoneal injections twice daily of saline, 1, or 10 mg/kg MPH for 12 weeks using a school week (5 days/week) dosing schedule per the previous study [2]. Swiss-Webster mice were chosen due to their resistance to MPTP [11]. After 12 weeks, all animals were subjected to drug washout for 7 days to prevent MPH competing with MPTP at DA transporters. Then, half of each group was treated with MPTP (4 x 20 mg/kg, every 2 hours), while the other half was administered 4 injections of sterile saline. Seven days after the MPTP injection, mice were sacrificed by decapitation, and the striatum was removed, flash-frozen in liquid nitrogen, and stored at -70°C until analysis. An n of 4 mice was used for each group.

Quinone Assay

The amount of catechol-derived ortho-quinones in the STR was quantified using near-infrared fluorescence (nIRF) dot blots [12]. Samples were homogenized in 300 µL of cold PBS by sonication, centrifuged for 5 minutes at 4°C at 400 x g and supernatant discarded. Free and protein-bound ortho quinones were then extracted 3 times using the following procedure. Pellets were resuspended in cold lysis buffer then incubated for 30 minutes in an ice-water slurry.

Samples were then frozen and thawed 3 times and then centrifuged for 30 minutes at 4°C at 100,000 x g. Supernatant was saved as the triton-soluble extraction and the triton-pellet was used for the second extraction. The triton-pellet was resuspended in SDS lysis buffer, boiled for 10 minutes, sonicated and boiled for another 10 minutes and then centrifuged for 30 minutes at 22°C at 100,000 x g. Supernatant was saved as the SDS-soluble extraction and the SDS-pellet was used for the third extraction. The SDS-pellet was resuspended in 1 N sodium hydroxide (NaOH) and incubated overnight at 55°C. The samples were then speed-vacuumed and resuspended in base buffer and saved as NaOH-soluble extraction. A standard curve was prepared using DOPAC oxidized with equimolar sodium periodate. Each sample extraction and standard were dot blotted onto a membrane and allowed to dry in a fume hood. Each extraction was normalized for protein content following a BCA protein assay. Dot blots were scanned at 700 nm on the Odyssey Infrared Imaging System (li-Cor).

Glutathione Assay

GSH content in the STR was measured using a GSH assay kit (Cayman Chemical Co.). Briefly, tissues were homogenized in 50 mM phosphate buffer (10 ml/g of tissue) on ice and centrifuged (10,000 g for 15 minutes at 4°C). The supernatant was removed and incubated for 5 minutes at room temperature with 10% metaphosphoric acid (v/v) and then centrifuged at 2,000 g for 3 minutes. Supernatant was removed again and normalized for protein content following a Bradford protein assay. Samples were added to a 96-well plate and mixed with the reagent cocktail provided in the GSH assay kit and described in the protocol with no deviations, incubated for 25 minutes in the dark, and the absorbance was determined at 414 nm.

Statistics

Data are reported as means +/- SEM. All statistical analyses were performed using a multiple variable analysis of variance (ANOVA), followed by Fisher's multiple comparisons tests using Minitab 17 software. P values of less than 0.05 were considered significant.

Results and Discussion

Increased Quinone Formation after Chronic MPH

MPH dose-dependently increased ortho-quinone production in the STR (Figure 3.2). Notably, high dose MPH increased quinone production by nearly 1,700 fold compared to saline + saline and a 9-fold increase compared to 1 mg/kg MPH + saline. In animals treated with MPTP, there were no significant differences between drug groups; however, there was a trend towards increasing quinone formation with increasing doses of MPH (Figure 3.2). Interestingly, although MPTP increased quinone formation compared to saline-treated animals ($p < 0.05$ Saline + Saline vs. Saline + MPTP), there appears to be an overall decrease in quinone formation in MPH-MPTP-treated animals compared to MPH-saline-treated animals. Although MPTP is known to acutely increase DA release and eventually induce oxidative stress, the oxidative stress results in DA neurotoxicity in vulnerable animal models [13]. In a previous study using the same model, chronic MPH followed by MPTP resulted in a significant increase in sensitivity to MPTP, as evidenced by a decrease in dopaminergic cell bodies in the SN [2]. Thus, following long-term MPH, even in MPTP-resistant animals, it is likely that DA and thus, DA quinone will be depleted in the terminals 7 days following exposure. Given MPTP neurotoxicity is widely recognized as retrograde toxicity, it is likely that damage to terminals was even greater than the

cell body loss previously reported. While several studies have shown that MPH causes oxidative damage in the brain [9, 14, 15], to our knowledge this is the first study to show an increase in quinones with MPH exposure.

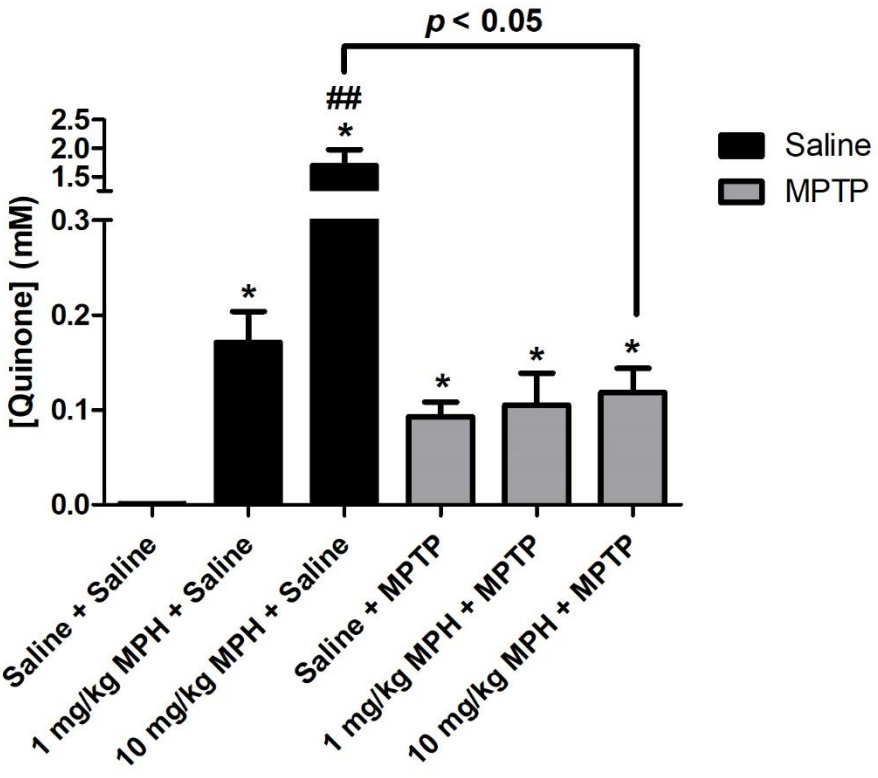

Figure 3.2 Total free and protein-bound ortho-quinones in the STR. Swiss-Webster mice received intraperitoneal injections of saline, 1, or 10 mg/kg MPH for 12 weeks followed by either saline or MPTP. Data are expressed as means ± SEM (n=4). One-way ANOVA followed by Fisher's post-hoc test. (n = 4) *$p < 0.05$ *vs.* Saline + Saline; ##$p < 0.05$ *vs.* 1 mg/kg MPH + Saline

Glutathione Depletion after Chronic MPH

GSH is an important antioxidant found throughout the CNS that has been shown to protect neurons from excess DA quinone formation [16-18]. GSH conjugates with quinones, preventing radical formation (Figure 3.1) [18]. In order to further elucidate if oxidative stress was related to DA oxidation, we measured the GSH content in the STR, as an increase in quinone formation should result in a depletion of GSH. Indeed, MPH dose-dependently depleted GSH in the STR (Figure 3.3). These data are consistent with studies that observed a decrease in GSH in the hippocampus, striatum, and prefrontal cortex with MPH exposure [9]. Additionally, MPTP caused GSH depletion in the STR, with the greatest depletion of GSH occurring in high dose (10 mg/kg) MPH animals exposed to MPTP (Figure 3.3). This is consistent with previous animal studies [9], and the discovery that GSH is depleted in post-mortem brains of Parkinson's disease patients [19]. Overall, the implications of GSH depletion are significant, because DA toxicity is enhanced when GSH is depleted [17].

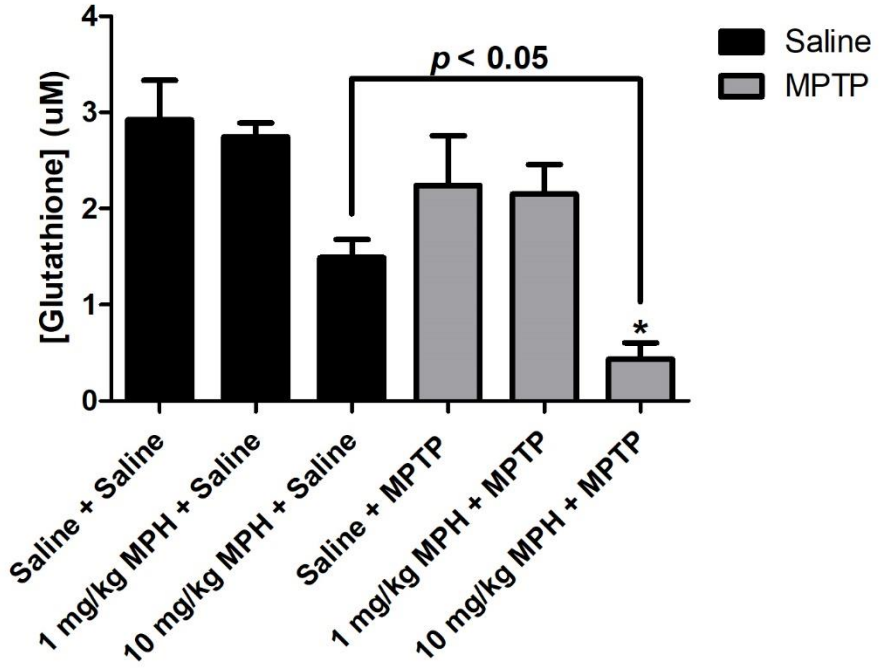

Figure 3.3 Glutathione content in the STR. Swiss-Webster mice received intraperitoneal injections of saline, 1, or 10 mg/kg MPH for 12 weeks followed by either saline or MPTP. Data are expressed as means ± SEM. One-way ANOVA followed by Fisher's post-hoc test (n = 4). *$p < 0.05$ *vs.* Saline + Saline

Conclusion

This study is the first to show a dose-dependent increase in striatal quinone production following chronic MPH exposure. The increased quinone formation with chronic MPH exposure is accompanied by a depletion in striatal GSH, with a greater depletion in animals exposed to the Parkinsonian toxin MPTP. Thus, it is likely that chronic elevations in DA resulting from long-term MPH result in the oxidation of DA to the DA quinone. In an effort to protect neurons from potential oxidative stress, the DA quinone is conjugated to GSH. However, over time, GSH

becomes depleted, leaving cells vulnerable to oxidative stress. Thus, following long-term MPH exposure, nigrostriatal neurons become more sensitive to the Parkinsonian toxin, MPTP. These findings lend mechanistic insight to epidemiological studies that indicate chronic psychostimulant use increases the risk of developing Parkinson's disease [3, 4, 7, 9, 14, 15]. Alarmingly, medications for ADHD are being prescribed for longer periods of time, sometimes from childhood into adulthood [20]. Unfortunately, chronic MPH exposure may predispose individuals who are treated with these psychostimulant medications to the development of Parkinson's disease later in life.

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CHAPTER 4. EFFECT OF CHRONIC METHYLPHENIDATE IN A FEMALE MODEL OF PARKINSONISM

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Abstract

Methylphenidate (MPH) is the most commonly prescribed drug for the treatment of ADHD in males and females. However, a majority of previous studies investigated the effect of MPH in only males, and little is known regarding consequences of female exposure to MPH. This is unfortunate because the few studies that have been conducted indicate females have a greater sensitivity to MPH. Previous research in male mice has shown that chronic exposure to MPH causes dopaminergic neurons within the nigrostriatal pathway to be more sensitive to the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, estrogen has been shown to protect dopaminergic neurons from MPTP neurotoxicity. Therefore, in this study, we test the hypothesis that chronic MPH exposure in female mice will render dopaminergic neurons in the nigrostriatal pathway more sensitive to MPTP, and that estrogen may play a protective role. Interestingly, proestrus females exhibited greater sensitivity to MPTP, with significantly reduced dopaminergic neurons in the SN and significant increases in DA quinone production. Chronic MPH exposure contributed to GSH depletion, but surprisingly, it did not increase dopamine quinone levels or dopaminergic cell loss. There were no significant differences in anestrus animals, with the exception of a depletion in GSH seen when animals received chronic high dose (10 mg/kg) MPH followed by MPTP. Thus, estrogen may actually sensitize neurons to MPTP in this model, and chronic MPH may contribute to GSH depletion within the striatum. This study provides insight into how chronic psychostimulant use may affect males and females differently.

Keywords: Methylphenidate, Dopamine, Oxidative stress, Glutathione, Dopamine quinone, Estrogen

Introduction

The Centers for Disease Control and Prevention (CDC) reports that as of 2016, 6.1 million children have been diagnosed with attention deficit hyperactivity disorder (ADHD) in the United States alone (Danielson et al. 2018). Methylphenidate (MPH) is the most commonly prescribed drug for the treatment of ADHD, and many children receive MPH from childhood to early adulthood; yet, most of the scientific literature focuses on understanding short-term consequences of MPH. As such, it is extremely important to examine the long-term consequences of MPH exposure. Additionally, the preponderance of previous studies investigated the effect of methylphenidate in only males, and little is known regarding consequences of female exposure to MPH. This is unfortunate due to the fact that the few studies that have been conducted indicate females have a greater sensitivity to MPH (Brown et al. 2012). Additionally, females have been shown to have higher brain concentrations of MPH than males (Bentley et al. 2015). The therapeutic effect of MPH is due to its ability to increase the amount of dopamine (DA) and norepinephrine (NE) in the synaptic cleft by blocking DA and NE transporters. Interestingly, there are sex differences reported in regard to dopaminergic tone (Cummins et al. 2014; Frolich et al. 2014). For example, in the striatum, females have a higher concentration of DA transporters, and higher concentrations of DA release when compared to males (Walker et al. 2006). Furthermore, females have a greater DA turnover rate than males. The increased DA turnover rate has been shown to be estrogen dependent, because an ovariectomy reduces the DA turnover rate and estrogen restores it (Dluzen et al. 1996). DA release in the striatum can be increased when ovariectomy mice are treated with estrogen (Dluzen et al. 1996). Moreover, natural fluctuations of estrogen in the estrous cycle are capable of altering the activity of DA in the striatum (Dluzen et al. 1996).

At a physiological pH, DA is capable of autoxidizing by dissociation of a hydroxyl group, creating a DA o-quinone. This unstable DA o-quinone then cyclizes to an aminochrome, which is reduced to leukoaminochrome o-semiquinone radicals, forming H_2O_2 and hydroxyl radicals. In addition to autoxidation, iron and copper, as well as enzymes with peroxidase activity are capable of catalyzing DA conversion to DA o-quinone, speeding up the reaction and increasing the levels of DA o-quinones (Klein et al. 2019). DA o-quinones are capable of disrupting normal cell function by forming adducts with cysteine residues and inducing oxidative stress throughout the cell (Monzani et al. 2018; Park et al. 2007). Interactions with cysteine residues can be particularly harmful to cell viability as cysteine residues are often found at the active site of enzymes. To prevent DA oxidation, excess DA is normally sequestered in vesicles with a lower pH (Klein et al. 2019). However, since MPH is capable of increasing the concentration of DA in the synaptic cleft, it may allow for excess free DA to autoxidize and produce reactive oxygen species. Furthermore, estrogen is thought to play a protective role against oxidative stress, as an increase in oxidative stress was seen in ovariectomy mice, and estrogen was able to decrease the oxidative stress (Gaignard et al. 2015).

Fortunately, our brains are capable of quickly removing small amounts of DA o-quinone by using a natural antioxidant, glutathione (GSH) (Motaghinejad et al. 2016). In fact, GSH has been shown to prevent DA-induced cell death in a number of models (Park et al. 2007; Stokes et al. 2000; Zhou and Lim 2009). GSH forms conjugation reactions with quinones or aminochromes, eventually leading to the formation of 5-S-cysteinyl DA or 4-S-glutathionyl-5,6-dihydroxyindoline, respectively. 5-S-cysteinyl DA and 4-S-glutathionyl-5,6-dihydroxyindoline may then be incorporated into neuromelanin (Zhou and Lim 2009), and some 5-S-cysteinyl DA is released into cerebral spinal fluid. However, GSH can become depleted when there is excess

quinone production. If GSH is depleted, there are more free quinones, and therefore more free radicals, leading to oxidative stress and eventually neurotoxicity via apoptosis (Stokes et al. 2000). An estimated 80% of the DA in the brain is found within the nigrostriatal pathway (Golan D 2011; Stahl 2008). The primary responsibility of neurons within this pathway is purposeful movement, and damage to this pathway can result in tremors, spasms, tardive dyskinesia, and Parkinson's disease (Klein et al. 2019). Therefore, drugs, such as MPH, that increase synaptic DA levels may have detrimental effects on the nigrostriatal pathway. In fact, MPH has been shown to induce oxidative stress by forming DA o-quinones and depleting GSH in the hippocampus and nigrostriatal pathway (Martins et al. 2006; Motaghinejad et al. 2016; Oakes et al. 2019).

Previous research in male mice has shown that chronic exposure to MPH causes dopaminergic neurons within the nigrostriatal pathway to be more sensitive to the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sadasivan et al. 2012). MPTP produces an experimental model of Parkinson's disease by causing a significant loss in DA neurons within this pathway. Furthermore, our lab has previously shown a dose-dependent increase in quinone formation in the striatum of male mice following chronic exposure to MPH (Oakes et al. 2019). Moreover, the increase in quinone formation in the striatum was accompanied by a depletion of GSH, and the depletion of GSH was enhanced when male mice were additionally exposed to MPTP (Oakes et al. 2019). Of note, estrogen has been found to be neuroprotective against MPTP, and it is thought that estrogen conveys neuroprotection by modulating the DA transporter (Dluzen et al. 1996). Interestingly, chronic use of psychostimulants appears to increase the risk of developing Parkinson's disease (Curtin et al. 2015; Moratalla et al. 2017; Perfeito et al. 2013). Although estrogen has been shown to be

protective against MPTP (Dluzen et al. 1996), human epidemiological studies have demonstrated that the use of stimulant drugs puts females at a greater risk of developing neurodegenerative disorders (Curtin et al. 2015). In contrast, estrogen is capable of working synergistically with psychostimulants, increasing the interactions between the psychostimulant and DA reward system (Curtin et al. 2015). Therefore, in this study, we test the hypothesis that chronic MPH exposure in female mice will render dopaminergic neurons in the nigrostriatal pathway more sensitive to MPTP, and that estrogen may play a protective role.

Materials and Methods

Mice and Drug Treatment

Experiments and procedures with the animals were performed following the regulations set forth by the NIH Guide for the Care and Use of Laboratory Animals. The protocols followed were approved by the University Committee on Animal Care (UCAC) at East Tennessee State University. Mice were allowed food and water ad libitum and were kept on a 12-hour light and dark cycle. Adolescent female Swiss-Webster (MPTP-resistant (Heikkila 1985)) mice received intraperitoneal (i.p.) injections of saline, 1, or 10 mg/kg MPH (Sigma Aldrich, dissolved in 0.9% sterile saline) for 12 weeks. A dose of 1 mg/kg MPH was used because doses of less than 5 mg/kg MPH i.p. in rodents may represent the clinical treatment of ADHD (Gerasimov et al. 2000; Koda et al. 2010). In contrast, a dose of 10 mg/kg MPH i.p. was chosen as it may represent recreational misuse or use of MPH treatment in narcolepsy (Valvassori et al. 2007). Injections were administered using a school week (5 days/week) dosing schedule to prevent weight loss (Martins et al. 2004; Roche et al. 1979). Female mice were allowed to enter the estrus cycle as

normal. The Whitten effect was used on the day of the last MPH injection by introducing male pheromones into half the cages prompting female mice to enter the estrus cycle at the same time (Gangrade and Dominic 1984; Whitten 1957). Half of the cages had no pheromones introduced keeping the female mice in anestrus via the Lee-Boot effect (Ma et al. 1998). Female mice were either in proestrus or anestrus 7 days after the last MPH injection, which was confirmed by vaginal smear (McLean et al. 2012).

MPTP Dosing in Female Swiss-Webster Mice

Previous work has indicated that different strains of mice can have varied sensitivity to MPTP (Boyd et al. 2007; Hamre et al. 1999; Heikkila 1985; Hoskins and Davis 1989; Sedelis et al. 2000; Sonsalla and Heikkila 1988; Sundström et al. 1987; Vidyadhara et al. 2017). Swiss-Webster mice have been found to be MPTP-resistant and thus, this strain is useful in examining changes in sensitivity to this Parkinsonian toxin. There is an established protocol for administration of MPTP (4 x 20 mg/kg, i.p.) in male Swiss-Webster mice (Hamre et al. 1999; Heikkila 1985). In addition, there appears to be differences in sensitivity to MPTP depending on gender (Alam et al. 2016; Disshon and Dluzen 2000; Dluzen et al. 1996; Sedelis et al. 2000). However, the use of the MPTP model for Parkinson's has been limited in female models, and none of these studies used female Swiss-Webster mice as a model for MPTP. Therefore, the appropriate MPTP dose for female Swiss-Webster mice was determined by analyzing the survival rate at 4 different doses of MPTP. Anestrus female mice were challenged with injections of MPTP 4 times every 2 hours. The MPTP doses given were 10 mg/kg, 12.5 mg/kg, 15 mg/kg, or 20 mg/kg. The probability of survival was calculated based on the number survived in a group

divided by the total of the group. Each of the 4 groups had an n of 5. After this preliminary experiment, a dose of 12.5 mg/kg was chosen and utilized for the remaining work. Briefly, after the 12 weeks of MPH treatment, all animals were subjected to drug washout for 7 days, and then half of each group were treated with MPTP 4 times every 2 hours (12.5 mg/kg), while the other half was administered 4 injections of sterile 0.9% saline. Seven days after the MPTP injection, mice were sacrificed.

Immunohistochemistry

Mice were sacrificed via transcardial perfusion with saline, followed by 4% paraformaldehyde. The brains were then removed, embedded in paraffin, and serially sectioned at 10 μm from the rostral hippocampus to the cerebellar-midbrain junction. Serial sections were mounted 5 sections per slide onto polyionic slides (Superfrost-plus, Fisher Scientific). Deparaffinized slides were incubated with an antibody against tyrosine hydroxylase to identify DA neurons (mouse monoclonal anti-tyrosine hydroxylase, Sigma-Aldrich;1:500 and biotinylated mouse IgG; 1:1000). A diaminobenzindine (DAB) reaction was used to yield a brown color to mark TH-expressing DA neurons. All tissue sections were counter stained with the nissl stain Neutral Red for landmark identification. TH-positive neurons and TH-negative, Nissl-positive cells within the pars compacta region of the substantia nigra (SNpc) that had the characteristics of dopaminergic neurons were counted using a 40x objective (total magnification 400x). Specifically, neurons from both left and right sides of the SNpc within one section per slide (chosen randomly and then maintained throughout all sections, (i.e. the 3rd section on each slide) were counted). TH positive neurons within the SNpc were counted and numbers estimated

using the Abercrombie correction factor as reported previously (Sadasivan et al. 2012). An n of 3 to 5 mice was used for each group.

Quinone Assay

Mice were sacrificed by decapitation, and the striata were removed, flash-frozen in liquid nitrogen, and stored at -70°C until analysis. An n of 4 to 5 mice was used for each group. The amount of free and protein bound ortho-quinones in the striatum was quantified using near-infrared fluorescence (nIRF) dot blots (Mazzulli et al. 2016). As reported previously samples were homogenized in 300 µL of cold PBS by sonication, centrifuged for 5 minutes at 4°C at 400 x g and supernatant discarded. Free and protein-bound ortho quinones were then extracted 3 times using the following procedure. Pellets were resuspended in cold lysis buffer then incubated for 30 minutes in an ice-water slurry. Samples were then frozen and thawed 3 times and then centrifuged for 30 minutes at 4°C at 100,000 x g. Supernatant was saved as the triton-soluble extraction and the triton-pellet was used for the second extraction. The triton-pellet was resuspended in SDS lysis buffer, boiled for 10 minutes, sonicated and boiled for another 10 minutes and then centrifuged for 30 minutes at 22°C at 100,000 x g. Supernatant was saved as the SDS-soluble extraction and the SDS-pellet was used for the third extraction. The SDS-pellet was resuspended in 1 N sodium hydroxide (NaOH) and incubated overnight at 55°C. The samples were then speed-vacuumed and resuspended in base buffer and saved as NaOH-soluble extraction. A standard curve was prepared using DOPAC oxidized with equimolar sodium periodate. Each sample extraction and standard were dot blotted onto a membrane and allowed to dry in a fume hood. Each extraction was normalized for protein content following a BCA protein

assay. Dot blots were scanned at 700 nm on the Odyssey Infrared Imaging System (li-Cor) (Oakes et al. 2019).

Glutathione Assay

GSH content in the striatum was measured as previously stated (Oakes et al. 2019). A GSH assay kit (Cayman Chemical Co.) was utilized. Briefly, tissues were homogenized in 50 mM phosphate buffer (10 ml/g of tissue) and centrifuged (10,000 g for 15 minutes at 4°C). The supernatant was removed and incubated for 5 minutes at room temperature with 10% metaphosphoric acid (v/v) and then centrifuged at 2,000 g for 3 minutes. Supernatant was removed again and normalized for protein content following a Bradford protein assay. Samples were added to a 96-well plate and mixed with the reagent cocktail provided in the GSH assay kit and described in the protocol with no deviations, incubated for 25 minutes in the dark, and the absorbance was determined at 414 nm.

Statistical Analysis

Data are reported as means +/- SEM and the statistical analyses were performed using multiple variable analysis of variance (two-way ANOVA), followed by Tukey's multiple comparisons test using GraphPad Prism software version 8. An n of 3 to 5 mice was used for each group. P values of < 0.05 were considered significant.

Results

MPTP Treatment in Female Swiss-Webster Mice

As mentioned previously, there is an established protocol for administration of MPTP (4 x 20 mg/kg, i.p.) in male Swiss-Webster mice (Hamre et al. 1999; Heikkila 1985). However, when we administered MPTP according to this protocol, 100% of the female Swiss-Webster mice died. Many died after the second and third injections, with the remaining mice dying shortly after the last injection. Previous work with other strains of mice (such as MPTP-sensitive C57 mice) have also shown complete lethality when female mice were given the same dose as males (4 x 20 mg/kg, i.p.) (Schwarting et al. 1999). While some peripheral toxicity is expected with MPTP, it is clear that female mice require MPTP dose adjustments. As such, we performed a preliminary experiment whereby we tested various doses of MPTP to determine the maximal dose we could use that would allow for at least 50% survival. As above, all doses were given 4 times, every two hours and included 20 mg/kg, 15 mg/kg, 12.5 mg/kg, and 10 mg/kg. The 20 mg/kg dose again produced a 100% death rate, and 15 mg/kg produced an 80% death rate. With a dose of 12.5 mg/kg, the death rate was 50%, and with 10 mg/kg, the death rate was 40%. Thus, for the remaining experiments, a dose of 12.5 mg/kg MPTP, every 2 hours for a total of 4 injections, was utilized.

Dopamine Cell Count in the Substantia Nigra

We conducted a stereological analysis of TH+ cells within the SNpc in order to determine how chronic MPH followed by saline/MPTP affected numbers of dopaminergic neurons within that brain region. Females were further subdivided based on the stage of the estrus cycle at the

time of MPTP exposure, as proestrus animals have high estrogen levels and anestrus animals have low estrogen levels. Interestingly, in proestrus females exposed to chronic saline treatment, MPTP caused a significant decrease in the number of dopaminergic neurons within the SNpc (Fig. 4.1m). This is surprising given the dose is lower than doses used in males, and these mice are typically MPTP-resistant (Hamre et al. 1999). Although this trend was observed in animals exposed to chronic MPH, there were no significant differences. Additionally, there were no significant differences observed in anestrus animals across any of the treatments (Fig. 4.1n).

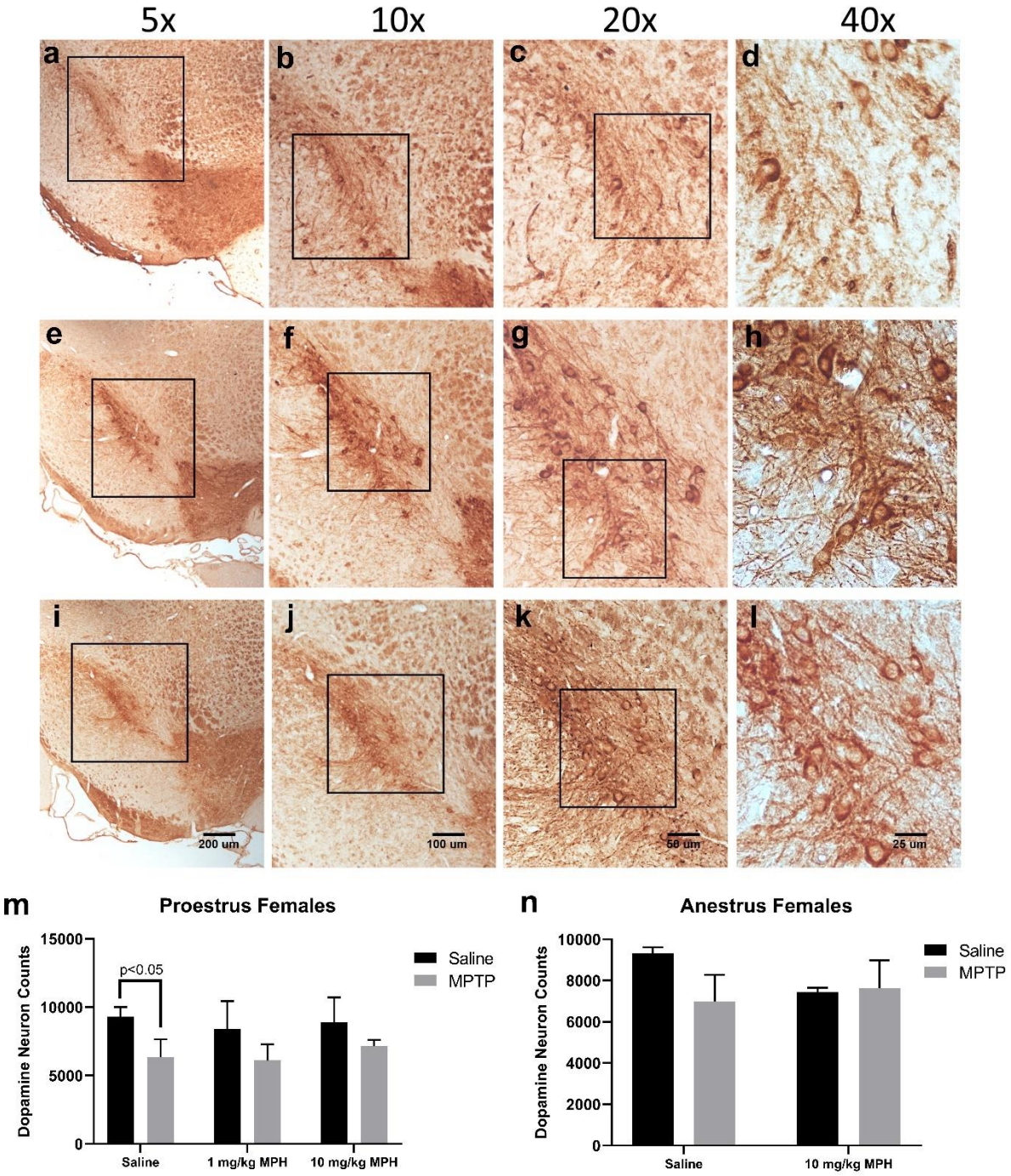

Fig. 4.1 Representative images of the substantia nigra par compacta (SNpc) of proestrus female mice treated with saline (a-d), 1 mg/kg (e-h), or 10 mg/kg (i-l) MPH for 12 weeks followed by MPTP. Stereological estimates of dopaminergic neuron numbers in the SNpc of proestrus (m) or

anestrus (n) female mice. Female Swiss-Webster mice received intraperitoneal injections of saline, 1, or 10 mg/kg MPH for 12 weeks (labeled at the bottom of the bar graph) followed by either saline (black bars) or MPTP (gray bars). Female mice were confirmed to be in proestrus or anestrus at the time of the injection, and tissue was collected 7 days later. Data are expressed as means ± SEM (n = 3 to 5). Two-way ANOVA followed by Tukey's post-hoc analyses

Dopamine O-Quinone Formation in the Striatum

Dopamine may contribute to neurotoxicity when it oxidizes to a DA o-quinone. As such, we quantified levels of quinones within the terminal region of the nigrostriatal pathway, the striatum. The production of DA o-quinones was significantly increased in proestrus mice that were exposed to chronic saline followed by MPTP compared to those exposed only to saline (Fig. 4.2a). Interestingly, again, no significant differences were observed in proestrus mice exposed to chronic MPH followed by MPTP or saline. Moreover, no significant differences were observed among anestrus subgroups (Fig. 4.2b). However, anestrus mice exposed to chronic saline followed by MPTP displayed significantly lower dopamine quinone production compared their proestrus counterparts (Fig. 4.2a and 4.2b)

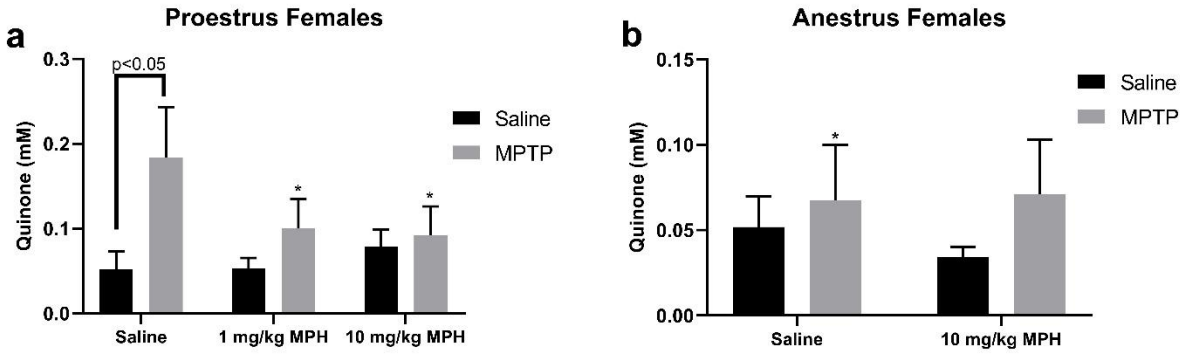

Fig. 4.2 The concentration of free and protein-bound ortho-quinones in the striata of proestrus (a) or anestrus (b) female mice. Female Swiss-Webster mice received intraperitoneal injections of saline, 1, or 10 mg/kg MPH for 12 weeks followed by either saline or MPTP. Female mice were confirmed to be in proestrus or anestrus at the time of the injection. Data are expressed as means ± SEM (n = 4 to 5). Two-way ANOVA followed by Tukey's post-hoc test. *$p<0.05$ vs. proestrus saline + MPTP

Glutathione Concentration in the Striatum

GSH is an important antioxidant within the CNS that can conjugate to DA ortho-quinones in an effort to prevent formation of free radicals. Thus, subsequently, we quantified striatal GSH levels in proestrus and anestrus subgroups following chronic saline or MPH exposure, followed by saline or MPTP. Interestingly, in proestrus females, a significant decrease in GSH levels was observed following chronic exposure to both low (1 mg/kg) and high (10 mg/kg) doses of MPH (Fig. 4.3a). Furthermore, mice exposed to chronic MPH followed by MPTP displayed even greater depletion in GSH levels, with mice receiving 10 mg/kg MPH followed by MPTP having the lowest levels. In anestrus females, chronic MPH did not deplete

GSH, but when chronic MPH animals also received MPTP, there was a significant depletion in GSH levels within the striatum (Fig. 4.3b)

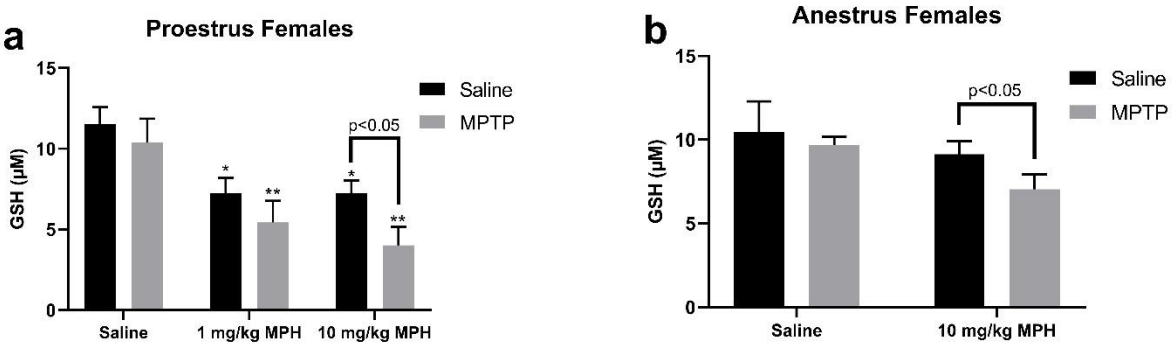

Fig. 4.3 Glutathione (GSH) concentration in the striata of proestrus (a) or anestrus (b) female mice. Female Swiss-Webster mice received intraperitoneal injections of saline, 1, or 10 mg/kg MPH for 12 weeks followed by either saline or MPTP. Female mice were confirmed to be in proestrus or anestrus at the time of the injection. Data are expressed as means ± SEM (n = 3 to 5). Two-way ANOVA followed by Tukey's post-hoc test. *$p<0.05$ vs. proestrus saline + saline, **$p<0.05$ vs. proestrus saline + MPTP

Discussion

Previously, we found that chronic MPH increased quinone formation and depleted GSH in the striatum of male Swiss-Webster mice, and MPTP caused an even greater depletion in GSH (Oakes et al. 2019). In this study, we examined the effect of chronic MPH exposure in female Swiss-Webster mice on dopaminergic neurons in the nigrostriatal pathway and whether or not

those dopaminergic neurons became sensitive to the neurotoxin, MPTP. We also investigated the role estrogen may play by utilizing the estrus cycle. Firstly, we elucidated the appropriate MPTP dose for female Swiss-Webster mice. Swiss-Webster mice are known to be MPTP-resistant (Heikkila 1985), and estrogen has been shown to be neuroprotective (Dluzen and Horstink 2003; McArthur and Gillies 2011). Unfortunately, when female Swiss-Webster mice are administered the typical acute MPTP regimen (20 mg/kg i.p. x 4 injections), it results in complete lethality, consistent with other mouse strains (Schwarting et al. 1999). This is likely due to peripheral toxicity, as female mice are particularly vulnerable to cardiovascular side effects in response to MPTP (Jackson-Lewis and Przedborski 2007). However, death due to MPTP-induced cardiovascular issues is unrelated to the loss of dopaminergic neurons in the nigrostriatal pathway; thus, female mice treated with MPTP may succumb and die due to cardiovascular events, before dopaminergic neuron loss in the SNpc may be observed. (Jackson-Lewis and Przedborski 2007). We found that a dose of 12.5 mg/kg MPTP given 4 times i.p. allowed for 50% or greater survival in female Swiss-Webster mice, and interestingly, it did produce some dopaminergic neuron loss within the SNpc.

Our data indicate that MPTP was capable of decreasing the number of dopaminergic neurons within the SNpc of proestrus females. In addition, increased quinone formation is seen in the striatum of proestrus female mice treated with MPTP. In contrast, anestrus females had no significant differences in the number of dopaminergic neurons in the SNpc. Following this trend, anestrus females showed no significant differences in quinone formation in the striatum. Taken together, these results suggest that high physiological levels of estrogen in proestrus females may actually sensitize female Swiss-Webster mice to MPTP. These data conflict with previous studies that have shown that physiological levels of estrogen can be neuroprotective against

neurotoxins such as MPTP, and proestrus females have less dopaminergic neuron loss when compared to diestrus females (Dluzen and Horstink 2003; Dluzen et al. 1996; Gomez-Mancilla and Bédard 1992; McArthur and Gillies 2011). While this may be true, these studies used different animal models and dosing regimens. For example, Gomez-Mancilla and Bédard utilized a female monkey model and administered MPTP immediately prior to administration of estrogen (Gomez-Mancilla and Bédard 1992). In studies that utilized mouse models, different strains were used, and the female mice were ovariectomized and then given a bolus of estrogen at the time of the MPTP injection (Dluzen and Horstink 2003; Dluzen et al. 1996; McArthur and Gillies 2011). However, it is also of note that levels of estrogen above physiological levels have been shown to worsen the dopaminergic neuron loss within the SNpc in response to MPTP (Bourque et al. 2009; McArthur and Gillies 2011). In general, studies have shown that female mice exhibit more variability in MPTP-induced neuronal damage to dopaminergic neurons when compared to males (Przedborski et al. 2001). Given the effect of MPTP on dopaminergic neuron number in the SNpc and quinone production in the striatum, it was surprising that GSH was not significantly depleted in proestrus females exposed to chronic saline followed by MPTP. However, females are known to have high concentrations of GSH, which may explain why significant depletions in GSH were not seen (Gaignard et al. 2015).

Another unexpected finding was that chronic MPH did not seem to sensitize female mice to SNpc dopaminergic cell loss in response to MPTP, as it does in males (Sadasivan et al. 2012). Although there was a trend towards a decreased neuron number within the SNpc in the proestrus groups exposed to MPH, there were no significant differences, unlike the mice treated with chronic saline. Additionally, although there was a trend towards an increase in DA quinone levels within the striatum, this was also not significantly changed in the chronic MPH mice.

Again, this is in contrast to results observed in males, although notably, males are able to tolerate and receive a higher dose of MPTP than could be utilized in females (Oakes et al. 2019). Furthermore, MPTP induces a retrograde cell death, where synaptic terminals die prior to cell bodies and may experience toxicity at lower doses of MPTP (Al Sweidi et al. 2012). Therefore, the MPTP dose of 12.5 mg/kg may have been insufficient to result in cell body toxicity but was sufficient in destroying the axon terminals. This could potentially lead to less DA release in the striatum, and therefore, decreased quinone production in the striatum as well.

Of note, chronic MPH did appear to induce a significant depletion in striatal GSH in proestrus females, and GSH was further reduced by MPTP. This suggests that the GSH available was capable of handling the extra quinone production that was induced by chronic MPH and MPTP. Of note, females are known to have increased concentration of GSH in mitochondria when compared to males (Gaignard et al. 2015; Turrens 2003). Therefore, it is possible that the increased concentrations of GSH were capable of dispatching any quinones that formed, preventing excess quinones and loss of dopaminergic cell bodies within the SNpc.

In conclusion, female Swiss-Webster mice show dopaminergic neuron loss within the SNpc at a MPTP dose of 12.5 mg/kg i.p. x 4 injections, when MPTP is administered in the proestrus period. Additionally, an increase in levels of quinones within the striatum was also observed when proestrus females were administered chronic saline followed by MPTP. Again, this is surprising given the Swiss-Webster mouse is traditionally more resistant to MPTP and the dose of MPTP utilized in females was less than that typically used in males. Taken together, these results demonstrate that estrogen may sensitize dopaminergic neurons within the SNpc to the Parkinsonian toxin, MPTP. Finally, although long-term MPH did not appear to increase SNpc neuron loss or quinone formation, it did produce a significant depletion in GSH levels

within the striatum, that was further reduced with MPTP. Thus, these data may provide insight into possible consequences of long-term MPH exposure in a female model.

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Compliance with Ethical Standards

Ethical Approval

All protocols followed were approved by the University Committee on Animal Care (UCAC) at East Tennessee State University. Experiments and procedures with the animals were performed following the regulations set forth by the NIH Guide for the Care and Use of Laboratory Animals.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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CHAPTER 5. SUMMARY

The number of individuals diagnosed with ADHD has steadily increased since the early 1990s (Danielson et al. 2018). Psychostimulants have remained first-line treatment, and the most commonly prescribed drug to treat ADHD is MPH (Gibson et al. 2006; Hanwella et al. 2011). Initially, ADHD was thought to be a childhood disorder and that medication was not needed once adulthood was reached; however, ADHD is now thought to be a life-long disorder. As such, individuals are taking MPH for many years. Unfortunately, a vast majority of the scientific literature focuses on the short-term consequences of MPH exposure. Furthermore, reports of abuse and misuse of MPH have increased. For example, in 2017, an estimated 12% of college students reported using ADHD as a "cognitive enhancer" (Abelman 2017). Additionally, other studies have found that individuals inject MPH to experience euphoria (Frauger et al. 2016). Finally, most studies investigating MPH have utilized only males; this is unfortunate as the few studies that have included females show that they respond differently to MPH. As such this dissertation endeavored to fill the gap in understanding long-term MPH exposure, the effects of both therapeutic and abusive doses, and the differential effects of the drug in males and females.

MPH acts by increasing the amounts of DA and NE in the synaptic cleft, and thus, chronic MPH may have long-term consequences on dopaminergic pathways within the brain. This study focused on MPH modulation of the mesolimbic and nigrostriatal pathways. The mesolimbic pathway is well-known for its role in reward, as neurons project from the ventral tegmental area to the nucleus accumbens. However, mesolimbic dopamine neurons also project to the hippocampus, an area of the brain that plays an important role in memory and recall. Dopaminergic neurons within the nigrostriatal pathway are important in motor function, and a loss of DA neurons in this pathway leads to the movement disorder, Parkinson's disease.

In chapter 2, we evaluated the effects of chronic exposure to low or high doses of MPH on neurogenesis in the hippocampus of the mesolimbic pathway. Additionally, these studies included an investigation of how proteins involved in neurogenesis are altered due to chronic MPH exposure. We proposed that treatment with chronic low or high doses of MPH may affect the proliferation and survival of granule cells within the dentate gyrus of the hippocampus and that MPH may influence hippocampal neurogenesis by altering the expression of proteins that influence neurogenesis, including BDNF, GDNF, VEGF, TrkB, and beta-catenin. In this study, mice received chronic low (1 mg/kg) or high (10 mg/kg) i.p. doses of MPH for 28 or 56 days. Interestingly, MPH, at both doses, increased neurogenesis. However, if MPH treatment was not continued, the newly generated cells did not survive after 28 days. If treatment was continued, the newly generated neurons survived only in the mice receiving low dose MPH. To investigate the mechanism for this effect, we examined levels of proteins linked to cell proliferation in the hippocampus. BDNF or GDNF levels were not significantly different between groups. However, hippocampal VEGF, TrkB, and beta-catenin were significantly increased in mice receiving low dose MPH for 28 days compared to controls. Interestingly, high dose MPH significantly decreased beta-catenin after 28 days and decreased VEGF, beta-catenin, and TrkB after 56 days compared to controls. Thus, low dose MPH appears to increase cell proliferation and cell survival in the hippocampus, and these effects may be mediated by increases in VEGF, TrkB, and beta-catenin. While high dose MPH may initially increase neuronal proliferation, newly-generated neurons are unable to survive long-term, possibly due to decreases in VEGF, TrkB and beta-catenin.

In chapter 3, we examined if chronic exposure to MPH will render dopaminergic neurons within the nigrostriatal pathway more sensitive to oxidative stress. We utilized the Parkinsonian

toxin, MPTP, in this model as it is known to produce oxidative stress and neurodegeneration of neurons within the substantia nigra. A previous study by my advisor, Brooks Pond, showed that chronic MPH rendered dopaminergic neurons in the nigrostriatal pathway to be more sensitive to MPTP (Sadasivan et al. 2012). We hypothesized that chronic MPH exposure leads to oxidative stress caused by the autoxidation of excess DA to a DA-o-quinone and that it is this oxidative stress that renders dopaminergic neurons within the striatum to be more sensitive to MPTP. Normally, the antioxidant GSH conjugates to the quinone, protecting neurons from oxidative stress; however, we proposed that MPH induced excess DA quinone production may lead to GSH depletion. To investigate this, male mice received chronic low or high doses of MPH and were exposed to saline or MPTP following a 1-week washout. Quinone formation in the striatum was examined via dot blot, and striatal GSH was quantified using a GSH assay. Our results showed that quinone formation increased with increasing doses of MPH. Additionally, MPH dose-dependently resulted in a depletion of GSH, which was further depleted following MPTP treatment. Thus, the increased sensitivity of DA neurons to MPTP toxicity following chronic methylphenidate exposure may be due to quinone production and subsequent depletion of GSH.

In chapter 4, we examined the interaction between chronic MPH and vulnerability to MPTP in a female model. Previously, studies have found that estrogen is neuroprotective against MPTP (Dluzen and Horstink 2003; Dluzen et al. 1996; Gomez-Mancilla and Bédard 1992; McArthur and Gillies 2011). Therefore, we proposed that estrogen may play a protective role when dopaminergic neurons within the nigrostriatal pathway are chronically exposed to MPH. Female mice received that same treatment as the male mice in chapter 3; however, the mice were either in anestrus (low estrogen) or proestrus (high estrogen) at the time of the MPTP injection. Interestingly, proestrus females exhibited greater sensitivity to MPTP, with significantly reduced

dopaminergic neurons in the SN and significant increases in DA quinone production. Chronic MPH exposure contributed to GSH depletion, but surprisingly, it did not increase DA quinone levels or dopaminergic cell loss. There were no significant differences in anestrus animals, with the exception of a depletion in GSH seen when animals received chronic high dose MPH followed by MPTP. Thus, estrogen may actually sensitize neurons to MPTP in this model, and chronic MPH may contribute to GSH depletion within the striatum. This study provides insight into how chronic psychostimulant use may affect males and females differently.

As more individuals are prescribed MPH and as the length of MPH treatment increases, the more important it becomes to study the chronic effects of this drug on important dopaminergic pathways. Altogether, this work has contributed to understanding how chronic MPH exposure may alter the mesolimbic and nigrostriatal dopaminergic pathways. Interestingly, MPH may increase neurogenesis within the hippocampus through mesolimbic modulation. However, this work supports epidemiological studies that indicate psychostimulant use may contribute to degenerative changes within the nigrostriatal pathway. Finally, this study highlights the need to include both males and females in MPH studies. Hopefully, the knowledge of how chronic MPH affects dopaminergic plasticity can be utilized when physicians and patients are making decisions about how best to utilize this drug.

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