NEUROPROTECTION AGAINST METHAMPHETAMINE INDUCED

NEUROTOXICITY: APPLICATIONS FOR PARKINSON'S DISEASE

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NEUROPROTECTION AGAINST METHAMPHETAMINE INDUCED NEUROTOXICITY: APPLICATIONS FOR PARKINSON'S DISEASE

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NEUROPROTECTION AGAINST METHAMPHETAMINE INDUCED

NEUROTOXICITY: APPLICATIONS FOR PARKINSON'S DISEASE

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VITA

Bessy Michelle Thrash, daughter of Michael Harmon Thrash and Kelly Nichols Thrash, was born on April 28, 1979. She is the third of four daughters born to Mr. and Mrs. Thrash, and was raised in the suburbs of Birmingham, Alabama. She attended the University of Alabama at Birmingham on a full academic scholarship, and earned her first baccalaureate degree in Criminal Justice and Chemistry in May of 2002. She earned her second baccalaureate degree in Biology in August of 2003 also from the University of Alabama at Birmingham. As an undergraduate, Ms. Thrash participated in the university's interdisciplinary Honors Program and as a result graduated with full university honors. She remained at the University of Alabama at Birmingham, joining the Masters Program in Forensic Sciences. She earned a masters degree in Forensic Sciences in May 2005. She worked for the Alabama Department of Forensic Sciences from 2003 to 2005. In August 2005 she joined the Auburn University doctoral program in Pharmacal Sciences, where she was funded as a graduate teaching and research assistant. Ms. Thrash has one son, John Carson Thrash, born on November 24, 1999.

DISSERTATION ABSTRACT

NEUROPROTECTION AGAINST METHAMPHETAMINE INDUCED

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Parkinson's disease is a progressive neurodegenerative disorder associated with selective degeneration of nigrostriatal dopaminergic neurons. It is the most common of the neurodegenerative movement disorders, affecting approximately 1% of the population over age 65. In Parkinson's disease, the shortage of dopamine in the striatum causes various motor abnormalities and complete immobility usually occurs, despite treatment. Studies have implicated exposure to toxins like methamphetamine as contributors to the development of Parkinson's disease. There is a significant degree of striatal dopamine depletion produced by methamphetamine, making the toxin useful in the creation of an animal model to study the disease. In this study a mouse model of Parkinson's disease was created by administering two intraperitoneal injections of methamphetamine (10mg/kg) two hours apart.

The neurotoxic effects of methamphetamine and neuroprotective effect of ramelteon, amantadine and salicylic acid were evaluated both *in vitro* and *in vivo*. Effects of reduced energy metabolism and oxidative stress were evaluated using biochemical assays. Changes in neurotransmitter levels (norepinepherine, serotonin and its metabolite, 5-HIAA, dopamine and its metabolites, DOPAC and HVA) were measured using high pressure liquid chromatography (HPLC)-electrochemical detection. In addition, behavioral analysis was performed on the treated mice to evaluate the effect of methamphetamine on movement (catalepsy, akinesia, swim score, straub tail and tremor). Results of these studies revealed that methamphetamine caused significant generation of reactive oxygen species, significantly increased superoxide dismutase activity and significantly decreased Complex I activity both *in vitro* and *in vivo*. Methamphetamine caused significant dopamine depletion in the striatum of treated mice and caused significant alterations in movement behaviors as compared to controls. Ramelteon did not block the neurotoxic effects of methamphetamine. Amantadine (1mg/kg) blocked the neurotoxic effects of methamphetamine and salicylic acid (50 & 100mg/kg) also blocked the neurotoxic effects of methamphetamine, causing a reduced amount of dopamine depletion in the striatum.

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1. INTRODUCTION

Methamphetamine is a psycostimulant and sympathomimetic drug and is a powerful central nervous system (CNS) stimulant. The increased use and abuse of the drug has become a major problem in the United States. The recent increase in methamphetamine use, dependance and/or addicition has become a major health concern worldwide. Despite the drug's addictive properties, it is sometimes prescribed to treat attention-deficit hyperactivity disorder (ADHD), narcolepsy and obesity (Reynolds, 1996). However, it is only used as a second line of treatment for these conditions, prescribed when amphetamine and methylphenidate cause the patient too many side effects. It is also used illegally for weight loss and to maintain alertness, focus, motivation, and mental clarity for extended periods of time, and for recreational purposes. Interestingly, there are medical histories of politician or leaders using methamphetamine intravenously by their personal physicians as a treatment for their depression and fatigue (Doyle, 2005). It is possible that their Parkinson-like symptoms which developed were related to the regular use of methamphetamine.

Methamphetamine can cross the blood-brain barrier, triggering a release of monoamines (dopamine, norephinephrine and seratonin). It can also act as a dopamine reuptake inhibitor and in high concentrations as a monoamine oxidase inhibitor. The major dopaminergic pathways in the brain are nigrostriatal, mesolimbic, mesocortical and tuberinfundicular pathways. Methamphetamine can significantly stimulate the

mesolimbic reward and nigrostriatal pathway. Stimulation of mesolimbic reward pathway by methamphetamine can cause euphoria and excitement, thus it is prone to abuse and addiction (McGregor et al., 2005). Methamphetamine affects biochemical mechanisms responsible for regulating heart rate, body temperature, blood pressure, appetite, attention, mood and responses associated with alertness or alarm conditions. Physical effects of the drug are similar to those seen in the fight-or-flight response induced by the release of epinephrine. This reaction includes increases heart rate and blood pressure, vasoconstriction, bronchodilation, and hyperglycemia. There is a decrease in appetite, a lowering of fatigue, and an increase in focus and mental alertness.

Methamphetamine can be neurotoxic and has been shown to cause dopaminergic degeneration in rodents and primates (Davidson et al., 2001; Dluzen et al., 2001; Itzhak et al., 2002). High doses or chronic administration of methamphetamine produce losses in several markers of brain dopamine and serotonin neurons. Dopamine and serotonin concentrations, dopamine and seratonin uptake sites, and tyrosine and tryptophan hydroxylase activities are reduced after the administration of methamphetamine. It has been proposed that dopamine plays a role in methamphetamine induced neurotoxicity because experiments which reduce dopamine production or block the release of dopamine decrease the toxic effects of methamphetamine administration. Dopamine metabolism generates reactive oxygen species such as hydrogen peroxide. Hence, there is a strong possibiliy that the oxidative stress that occurs after taking methamphetamine can mediate its neurotoxicity (Yamamoto and Zhu, 1998). The long lasting dopamine depletion in the striatum mimics the neurodegenerative process seen in Parkinson's

disease. This makes methamphetamine an ideal toxic candidate for producing an animal model for Parkinson's disease. Such a model could be used to study the mechanism of dopaminergic neurodegeneration and for aiding therapeutic interventions in Parkinson's disease (Lau et al., 2000; Gluck et al, 2003; Kita et al., 2003; LaVoie et al., 2004; Thomas et al., 2004; Riddle et al., 2006).

Animal models are utilized in order to gain an understanding of the pathogenesis of Parkinson's disease and the causes of the selective loss of dopaminergic neurons. Scientists want to mimic this pathogenesis as closely as possible. Therefore, these models are designed to reproduce the clinical and pathological features of Parkinson's disease, such as the progressive loss of dopaminergic neurons, and can be very helpful in dissecting the many different molecular and biochemical pathways in the phenotype of Parkinson's disease. As such, the methamphetamine animal model could help in gaining a better understanding of the neurodegeneration in the nigrostriatal system associated with Parkinson's disease and could be used to investigate pharmacological agents for any neuroprotective or neurorestorative effects they may exhibit. This could lead to the development of much needed novel therapeutic agents and even to a proactive treatment strategy in Parkinson's disease. With no proven neurorestorative or neuroprotective treatment strategies for this disease, these animal models are essential to discovering such treatments and to lowering the mortality rate of this disease.

With this study we examined the neurotoxicity of methamphetamine, its role in dopamine depletion (implications for Parkinson's disease) and possible neuroprotective treatments (ramelteon, amantadine and salicylic acid). Closing the mechanistic gap in

understanding could lead to proactive strategies to combat this disease. A more complete understanding of the progressive and selective loss of the nigrostriatal dopaminergic neurons that occurs in Parkinson's disease could advance treatment of this disease in numerous ways. Discovery of a pharmacological agent to provide neuroprotection in the substantia nigra from oxidative stressand the scavenging of hydroxyl radicals, or the identification of a therapy that could protect dopaminergic cells from apoptosis induced by neurotoxins, could improve the duration and quality of life for all Parkinson's disease sufferers.

Parkinson's disease causes a significant economic impact. It is estimated that medical costs alone in Parkinson's disease amount to around 25 billion dollars a year (Siderowf, 2003). Examination of the neurotoxicity of methamphetamine, its role in dopamine depletion, and implications for Parkinson's disease could aid in closing the mechanistic gap in understanding and could lead to proactive strategies to combat this disease. Abuse of drugs, like methamphetamine, also has a significant economic impact. The cost of drug abuse in America is estimated at around one trillion dollars a year (NIDA, 2002; Cadet et al., 2003). A better understanding of the mechanistic properties of this drug could also aid in strategies to combat addiction. Therefore, this study could have a two-fold beneficial effect for the American economy.

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2. REVIEW OF LITERATURE

2.1 Clinical and pathologic features of Parkinson's disease

Parkinson's disease is a progressive neurodegenerative disorder of the central nervous system. It is associated with a deficiency of striatal dopamine due to the selective degeneration of nigrostriatal dopaminergic neurons (Hornykiewicz, 1973). In Parkinson's disease, most of the neurons located in the substantia nigra (figure 2.1) that produce dopamine have undergone neurodegeneration, causing a severe shortage of dopamine in the striatum (Caine and Langston, 1983).

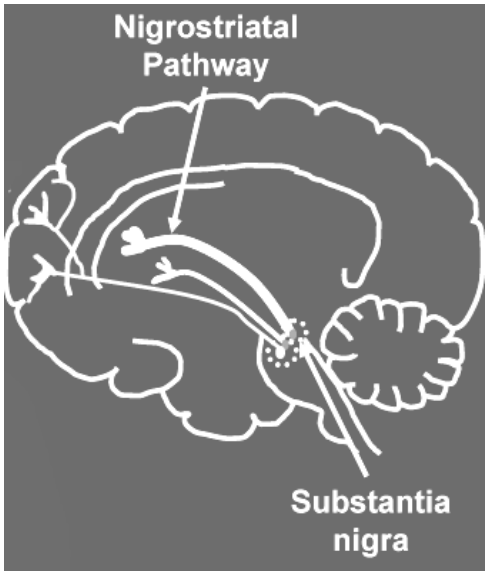

Figure 2.1. Dopamine deficiency of the nigrostriatal system: Dopamine neurons originate in the substantia nigra and terminate in the corpus striatum.

Dopamine from the substantia nigra activates neurons in the striatum that are important for initiation of movement. The loss of dopaminergic function leads to various motor symptoms including resting tremors, bradykinesia, muscular rigidity, and abnormalities in posture and gait (Kopin, 1992). Thus, one affected with Parkinson's disease would be expected to exhibit some or all of the following motor deficits: slowness in initiation and execution of voluntary movements, increased muscle tone, increased resistance to movement, shuffling of feet, stooped posture and equilibrium and righting reflex (Sethy et al., 1997). There are also other symptoms related to the parasympathetic and sympathetic peripheral nervous system seen in Parkinson's disease patients. In many cases of Parkinson's disease, complete immobility eventually occurs despite treatment (Kopin, 1992; Marsden, 1994; Sethy et al., 1997). Parkinson's disease is a slow-onset disease and the prevalence of Parkinson's disease increases with age. There are estimates of up to 800 affected per 100,000 individuals age 65 or older (Savitt, 2006). This makes it the most common of the neurodegenerative movement disorders, affecting approximately 1% of the population (Siderowf, 2003). The prolonged life span of the human population has brought about an increasing number of senior citizens in our society. The problems of neurodegenerative diseases have become a prevalent cause of disabilities in old age which poses an increasing problem.

2.2 Etiology and treatment of Parkinson's disease

The etiology of Parkinson's disease is not entirely clear. Though the specific cause of Parkinson's disease is unknown, studies have shown that age, genetics and

environmental factors (figure 2.2) all contribute to the disease (Fahn and Cohen, 1992; Marsden, 1994; Sethy et al., 1997; Patel t al., 2006)). Parkinson's disease can be sporadic and it also can be a familial form of disease. The genes that can cause the familial form of Parkinson's disease are alpha-synuclein, parkin, DJ-1, PINK-1 and LRRK2. Environmental toxins have repeatedly been shown as contributors to the development of Parkinson's disease (Ungerstedt, 1971; Davis et al., 1979; Burns et al., 1984; Di Monte et al., 2002; Sherer et al., 2002; Patel et al., 2006). These toxic substances have been suggested to contribute to Parkinson's disease by producing loss of dopaminergic neurons of the nigrostriatal pathway. Studies have demonstrated that exposures to the toxins can produce progressive, permanent and cumulative neurotoxicity of the nigrostriatal system. Among the environmental factors suspected to participate in the onset of Parkinson's disease are the synthetic heroin compound MPTP (l-methyl-4-phenyl tetrahydropyridinium) (Davis et al., 1979, Burns et al.,1984; Sherer et al., 2002, Di Monte et al., 2002), 6-hydroxydopamine (Ungerstedt, 1971) and the widely used and abused drug methamphetamine (Bondy et al., 2000), as well as environmental toxins like the herbicide paraquat (1, 1'-dimethyl-4, 4'-bipyridinium) (McCormack et al., 2005; Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b), and the fungicide maneb (manganese ethylene-bisdithiocarbamate) (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). Successful animal models for the study of Parkinson's disease have been created using these toxins alone (Langston et al., 1983; Di Monte et al., 2002), or in combinations (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b; Cory-Slechta et al., 2005).

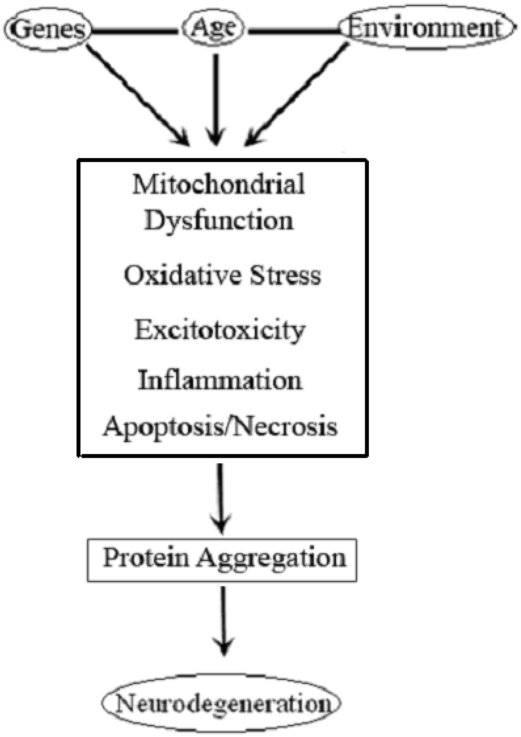

Figure 2.2. Markers for Neurodegeneration: Oxidative stress, excitotoxicity, mitochondrial dysfunction, and inflammation are seen in the early physiology of Parkinson's disease. Protein aggregation and cell death are seen in the later stages.

The current treatment strategy for Parkinson's disease remains primarily symptomatic, with no proven neuroprotective or neurorestorative treatments (Dawson and Dawson, 2003). Current treatments, including levodopa (a dopamine precursor), dopamine agonists, monoamine oxidase (MAO) inhibitors, anticholinergics and catechol *O*-methyltransferase (COMT) inhibitors, are used to alleviate symptoms and slow the progression of the disease, however such effects are temporary and these drugs often have debilitating side effects. Long-term drug therapy has been associated with motor complications that can be as disabling as the disease itself. There are also a few surgical

approaches that can be used as treatment. Howeve, despite advances in pharmacotherapy that have improved quality of life, the mortality rate among Parkinson's disease sufferers remains largely unchanged. There is need for a proactive treatment strategy that could provide neuroprotection or neurorestoration. Since evidence has shown that neurotoxins play an important role in niagral degeneration, there is obviously a need to take a closer look at such toxins and utilize them in animal models to study possible neuroprotection in Parkinson's disease. A mechanistic understanding of such toxicity could lead to proactive strategies to counteract the neurological damage.

2.3 Methamphetamine

Methamphetamine (N-methyl-1-phenyl-propan-2-amine-IUPAC; figure 2.3) is a white crystalline solid which is highly water soluble (1g/2ml) and has a melting point between 171 and 175 degrees Celsius. It is odorless, bitter tasting and slightly acidic in aqueous solution (Remington, 1985). A. Ogata, a Japanese chemist synthesized methamphetamine in 1919. His method of synthesis was based on the reduction of ephedrine. Methamphetamine can be synthetically prepared by catalytic hydrogenation of ephedrine or pseudoephedrine which is then converted to hydrochloride. It can also be prepared by reducing the condensation product of benzylmethyl ketone and methylamine. Methamphetamine shares its structurally similarily with other CNS stimulants like amphetamine and methcathinone, which are also produced from ephedrine and pseudoephedrine by chemical reduction. Methamphetamine is a synthetic stimulant and this is the major difference as compared to Cannabinoids and cocaine, which are obtained

from natural resources (canabinionids are dervided from *Cannabis sativa* and cocaine is derived from *Erythroxylon coca*).

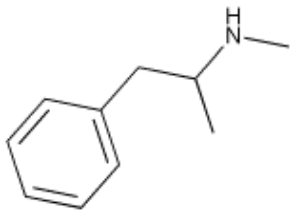

Figure 2.3. Methamphetamine chemical structure

The methyl group present in the structure of methamphetamine makes the substance lipid soluble, thus easing transport across the blood brain barrier. It also increases the ressitance against enzymatic degradation by monoamine oxidase. Methamphetamine causes the dopamine, norepinephrine and serotonin transporters to reverse their direction of flow. This inversion leads to a release of these transmitters from the vesicles to the cytoplasm and from the cytoplasm to the synapse causing increased stimulation of post-synaptic receptors. Methamphetamine also indirectly prevents the monoamine reuptake of these neurotransmitters, causing them to remain in the synaptic cleft for a prolonged period (Rothman et al., 2001).

Its effects on the body (figure 2.4) include acute central nervous system stimulation and cardiotoxicity, including induction of tachycardia, arrhythmias, hypertension and cardiovascular collapse. The drug has a high risk of dependency and abuse. Effects on the central nervous system include tremors, restlessness, agitation, insomnia, increased motor activity, headache, convulsions, and coma. Psychiatric side

effects include agitation, confusion, mood elevation, increased wakefulness, talkativeness, irritability and panic attacks. Chronic abuse can cause delusions and paranoia (Budavari, 1996).

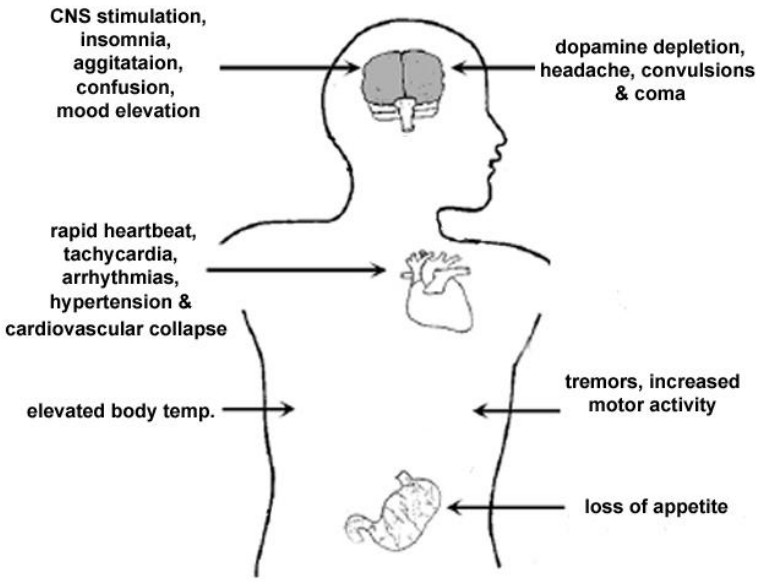

Figure 2.4. Methamphetamine toxicity

A withdrawal syndrome occurs after abrupt cessation following chronic use (Budavari, 1996). Moreover, withdrawal from methamphetamine dependence is distinguished by protracted anhedonia, dysphoria and severe craving. Withdrawal is also characterized by excessive sleeping, eating, and depression-like symptoms, often accompanied by anxiety and drug-craving (McGregor et al., 2005). Withdrawal symptoms of methamphetamine can be reduced by pharmacological or non-pharmacological approaches. Psychosocial behavioral therapy and Contingency reward

therapy have been successfully used in patients addicted to methamphetamine. Opioid receptor antagonists and antidepressants have been tested to reduce the severe withdrawal symptoms related to methamphetamine. Its widespread abuse has been a growing problem in recent years. It is a highly addictive stimulant with acute neurotoxic and powerful dopamine-depleting properties. Side effects of abuse of this drug include memory loss, aggression, psychotic symptoms and behavioral abnormalities, and potential heart and brain damage (NIDI Research Report, 2002).

2.4 Methamphetamine Toxicity: Revelance for Parkinson's disease

Major markers seen in Parkinson's disease are nigral neuronal loss and dopamine depletion in the striatum. Methamphetamine-induced neurotoxicity of the nigrostriatal dopaminergic system is commonly seen in lab studies (Asanuma et al., 2003). In experiments, repeated administration of the drug caused neurotoxicity in both rodents and non-human primates (Hashimoto et al., 2004; Zhang et al., 2006). It has been shown that methamphetamine treatment depletes dopamine as well as its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), in the striatum (Bondy et al., 2000). It has been further shown that repeated administration of methamphetamine reduces dopamine transporter (DAT) binding and, in extreme cases, causes cell apoptosis and persistent dopaminergic degeneration in the striatum (Davidson et al., 2001; Cadet et al., 2003; Kita et al., 2003). Examination of post-mortem brain tissue from methamphetamine users showed a significant decrease in dopamine and dopamine transporter density (Wilson et al. 1996).

Methamphetamine is highly lipid soluble and readily crosses the blood-brain barrier. It is a cationic lipophilic molecule that can diffuse into the mitochondria and be retained there. As such, it enters into the brain rapidly, interacts with transporters, and displaces dopamine in the vesicles releasing it into the synaptic cleft. Once there, the dopamine can be rapidly oxidized into quinones and semiquinones which are then converted through redox cycling to toxic radicals like superoxide, hydrogen peroxide and nitrogen radicals, creating oxidative stress (Halliwell, 1989; Stokes et al., 1999; Zhu et al., 2006). Oxidative stress causes an inhibition of mitochondrial ATP production, a major determinant of lethal cell injury. A recent study showed that cells can be protected from injury as long as ATP levels can be maintained. In this study, the use of fructose to maintain ATP increased cell survival. This study, therefore, indicates that the loss of ATP is a central mechanism of oxidative stress (Imberti et al., 1993). Mitochondrial DNA is sensitive to reactive oxygen species because mitochondria have only a limited arsenal of DNA repair processes. Therefore, mitochondrial DNA mutations would cause defects in the respiratory chain function leading to severe cellular damage. Free radicals can interact with sugars, proteins and lipids creating a number of modifications which could then lead to mitochondrial dysfunction, apoptosis and necrosis. The damage to the striatal terminals and the nigral cell bodies observed in methamphetamine users seems indicative of all of these toxic mechanisms, the same mechanisms indicated in Parkinson's disease (Imberti et al., 1993; Jones and Vale, 2000).

Toxicological studies on the brain tissue of human methamphetamine users have shown long term and possibly irreversible damage to dopaminergic neurons and loss of

striatal dopamine transporters even after as much as three years of abstinence from the drug (Cadet et al., 2003). Researchers have found dopaminergic toxicity in mice after a single dose of methamphetamine (25 mg/kg) (Hayashi et al., 2001) or after multiple doses of methamphetamine varying from 2.5 to 10 mg/kg given at 2 hour intervals (Sonsalla and Heillila, 1988; Chan et al., 1994; O'Callaghan and Miller, 1994). After such doses of methamphetamine there was seen a long lasting decrease in nigrostriatal dopamine similar to that seen in Parkinson's disease (Sonsalla, et al., 1996).

Though the exact mechanism of the neurotoxicity caused by methamphetamine use is unclear, mitochondrial dysfunction, as well as *N*-methyl-D-aspartic acid (NMDA) receptor-mediated excitotoxicity and neuroinflammation are all mechanisms commonly implicated in its toxicity. In addition, the long lasting damage to striatal neurons mimics that which is seen in the neurodegenerative process in Parkinson's disease. This makes the methamphetamine animal model ideal to use for replication of Parkinson's disease (Lau et al., 2000; Gluck et al, 2001; Kita et al., 2003; LaVoie et al., 2004; Thomas et al., 2004; Riddle et al., 2006).

2.5 Oxidative Stress in Parkinson's disease: Relevance to Methamphetamine

Oxidative stress (figure 2.5) has been strongly implicated in the pathogenesis of Parkinson's disease. Brains from patients with Parkinson's disease have shown elevated markers of oxidative damage, indicating that oxidative stress may play a key role in the disease (Hensley et al., 1998; Sherer et al., 2002). Oxidative species are important in cellular respiration and aid in the regulation of signal transduction pathways. However,

they can be converted into free radicals, containing unpaired electrons, which are collectively referred to as reactive oxygen species (ROS). Oxidative stress involves the excessive formation of reactive oxygen species, like the hydroxyl radicals (OH., OH-) and superoxide (O_2.), among others, which are formed when molecular oxygen is reduced non-enzymatically (Fahn and Cohen, 1992; Jenner et al., 1992). The brain has been shown to contain low to moderate levels of enzymes such as catalase, superoxide dismutase and glutathione peroxidase that play an important role in the metabolism of reactive oxygen species (Cohen, 1988). Superoxide dismutase (SOD) converts the superoxide to hydrogen peroxide, and hydrogen peroxide is typically detoxified by glutathione peroxidase, and catalase. However, oxidative stress occurs when free radicals are in excess of these antioxidant defenses. Free radicals are capable of reacting with DNA, lipids and proteins to alter their structure and function (Turrens and Boveris, 1980; Hasegawa et al., 1990). These alterations would be evidenced by an accumulation of oxidized products which serve as markers of oxidation, such as aldehydes and isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation and base adducts from DNA oxidation (Olanow, 1993).

Because methamphetamine displaces the dopamine in the vesicles, large amounts of it are released into the synaptic cleft and cytosol. There is an established relationship between the oxidative process of dopamine itself and nigrostriatal degeneration. During the auto-oxidation, synthesis and metabolism of dopamine, hydrogen peroxide (H_2O_2) is produced as a byproduct by tyrosine hydroxylase (TSH) and monoamine oxidase (MAO) (Lotharius and O'Malley, 2000). The hydrogen peroxide, which can react

nonenzymatically to form highly reactive hydroxyl radicals, can then take part in a positive feedback loop causing further progression of oxidative damage (Jenner, 1998). This demonstrates that dopaminergic neurons exist in a constant state of oxidative stress, and this could also explain the selective toxicity on dopaminergic neurons observed in Parkinson's disease. All current evidence suggests that oxidizing species exert a toxic effect that is significantly dangerous to the dopaminergic system, causing the death of dopaminergic neurons (Di Monte, 2001).

2.6 Free Radical Damage

The brain and nervous system are particularly vulnerable to free radical damage for a number of reasons. The membrane lipids in the brain contain high levels of polyunsaturated fatty acid side chains, which are prone to free radical attack, and are readily peroxidizable. The brain also consumes large quantities of total oxygen for its relatively small weight, contributing further to the formation of reactive oxygen species. It has been estimated that up to 2% of the oxygen consumed by healthy mitochondria is converted to superoxide, and this amount is higher in damaged and aged mitochondria. Evidence seems to suggest that the neurons in the substantia nigra are particularly prone to oxidative stress, which would explain the selective degeneration in this region (Halliwell, 1989).

Another possible contributing factor to the neurotoxicity and selective neurodegeneration seen in the substantia nigra is the presence of iron in this area, which can also contribute to the production of reactive oxygen species. In the presence of

transition metals, hydrogen peroxide can be converted by the Fenton reaction, an iron mediated reaction that requires iron in the free ferrous form, to the highly reactive hydroxyl radical. Seen as further evidence that oxidative stress plays an important role in the development of Parkinson's disease was the discovery of higher than normal levels of iron in the substantia nigra of Parkinson's disease patients (Bharath et al., 2002) and lower than normal concentrations of reduced glutathione. Glutathione, which removes hydrogen peroxide, is present in lower levels in the brains of patients with Parkinson's disease. Reduced glutathione is decreased in Parkinson's disease substantia nigra by approximately 50% (Perry et al., 1986). Because of the deficiency in glutathione, there is higher than normal levels of hydrogen peroxide in the neurons. This hydrogen peroxide is further broken down into hydroxyl radicals by the Fenton reaction (Bharath et al., 2002; Foley and Riederer, 2000). Thus, the higher than normal iron levels in Parkinson's disease patients is another strong indicator of the involvement of oxidative stress in the development of Parkinson's disease. In addition, further evidence in favor of using methamphetamine to create an animal model for Parkinson's disease is that the striatum has been shown to be the primary site of methamphetamine toxicity, just as it is the primary site of dopamine depletion in Parkinson's disease (Kupperman et al., 1997), and by the finding of increased reactive oxygen species in methamphetamine intoxication (Huang et al., 1997; Yamamoto and Zhu, 1998; Kita et al., 1999).

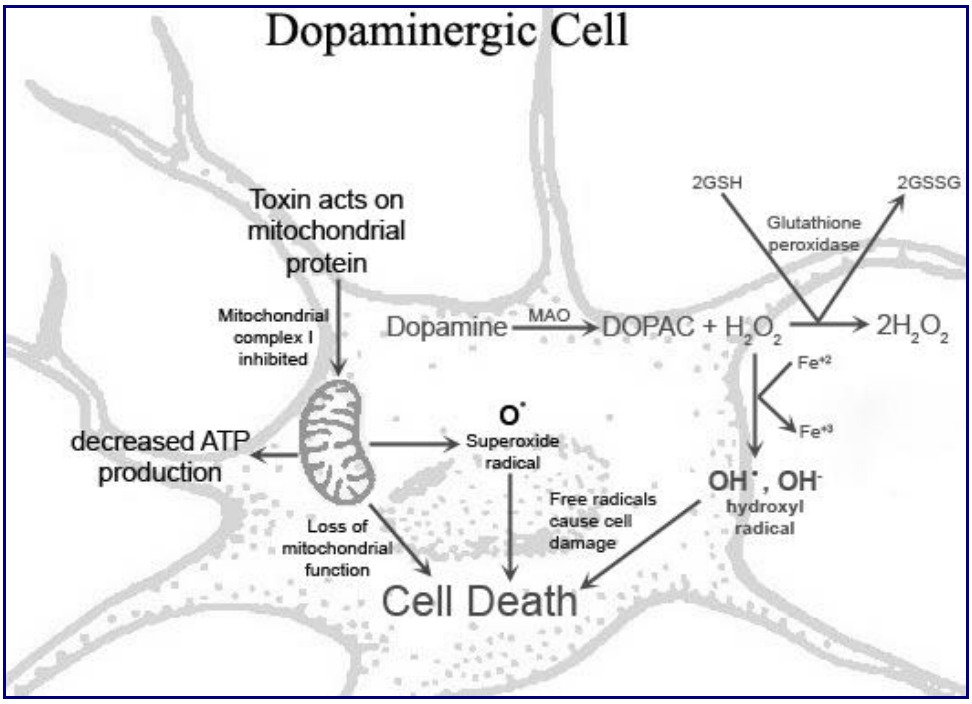

Figure 2.5. Mitochondrial Dysfunction and Oxidative Stress

2.7 Mitochondrial Dysfunction: Relevance to Parkinson's Disease

Mitochondrial dysfunction (figure 2.5) has long been thought to play a significant role in depletion of nigral neurons, leading to dopaminergic depletion. Evidence seems to suggest that the neurons in the substantia nigra are particularly prone to mitochondrial energy depletion, which would explain the selective degeneration in this region. The central dopaminergic system that plays such an important role in motor activity is comprised of a surprisingly small number of neurons. Because of this, it is especially vulnerable and even minor insults may lead to irreparable functional deficits (Fariello et al., 1988).

The exact mode of cell death in the substantia nigra in Parkinson's disease is unknown but early researchers suspected it involved some deficiency in mitochondrial

complex I (NADH:ubiquinone oxidoreductase). There is a tendency for free radicals to be more easily formed and less easily scavenged in the brain of Parkinson's disease patients (Thiruchelvam et al., 2005). Along the mitochondrial electron transport chain are sites where electrons can "leak" out and combine with molecular oxygen to form oxidizing species. Of particular relevance in Parkinson's disease, mitochondrial complex I is one of the sites of electron leak along the electron transport chain (Ranjita et al., 2002). An inhibition of complex I leads to an increased production of reactive oxygen species, resulting in a feed-forward cycle where complex I is further damaged and more reactive oxygen species are produced (Hensley et al., 1998; Sherer et al., 2002).

Researchers observed a deficiency in complex I activity in Parkinson's disease patients, including significantly reduced activity in the substantia nigra (Janetzky et al., 1994) and platelets (Krige et al., 1992; Yoshino et al., 1992). In another study there was seen as much as a 30% decrease in complex I activity in the substantia nigra, striatum, skeletal muscle and platelets, with no detectable structural or mitochondrial DNA changes (Yoshino et al., 1992; Foley and Riederer, 2000). In addition to reduced complex I, mitochondria in patients with early, untreated Parkinson's disease show decreased complex II (succinate-ubiquinone oxidoreductase)/ III (ubiquinol:cytochrome c oxidoreductase) activity (Shults 2005). Mitochondria play an important role in both cell survival and death, as the ATP produced there is critical in the mitochondrial respiratory chain providing protection to dopaminergic neurons (Tipton and Singer, 1993). Inhibition of mitochondrial respiration is sufficient to cause nigral cell death and is a common step

in the signal transduction pathways of apoptosis and necrosis inducing cell death (Hartley et al., 1994).

2.8 Neurotoxins and Mitochondrial Dysfunction

Animal studies with other neurotoxins like MPTP shed light on the role of mitochondrial dysfunction in Parkinson's disease. MPTP is a precursor neurotoxin and its oxidized form, MPP+, generated by monoamine oxidase type B, is the active neurotoxin. The acute neurodegenerative action of this substance was discovered as a result of fatal poisonings in 'street heroin' users. After the neurotoxic effect of MPTP was found to induce parkinsonian symptoms in humans (Langston, 1985; Tetrud and Langston, 1989; Di Monte et al., 2002), laboratory studies showed that it also selectively damaged the nigrostriatal dopaminergic system in animals producing similar parkinsonian symptoms (Langston et al., 1983).

The biochemical changes following MTPT induced toxicity reinforced researchers beliefs that Parkinson's disease could involve a disorder of the mitochondrial electron transport chain (Strijks et al., 1997). MPTP inhibits mitochondrial functions by causing a blockade of complex I, interrupting the electron transport chain and leading to reduction in mitochondrial ATP production. The inhibition of mitochondrial complex I disrupts electron transport from NAHD dehydrogenase to ubiquinone (Schapira, 1995). Since the substantia nigra has the lowest concentration of ubiquinone within the brain, this is further evidence that this region is more susceptible to mitochondrial energy depletion (Strijks et al., 1997).

The theory that mitochondrial dysfunction plays a role in Parkinson's disease has been strengthened by research demonstrating that chronic administration of rotenone, a complex I inhibitor, produced an animal model of Parkinson's disease in rats (Betarbet et al., 2000). Further support for this theory came from studies showing that complex I defects from Parkinson's disease platelets are transferable into mitochondrial deficient cell lines (Gu et al., 1998; Swerdlow et al., 1996). Also supporting the mitochondrial dysfunction theory, are recent studies on the antioxidant coenzyme Q_{10}, which plays an important role in the mitochondrial electron transport chain as the electron acceptor for complex I. Levels of Q_{10} were found to be reduced in Parkinson's disease patients. Coenzyme Q_{10} has provided protection against both MPTP (Akaneya et al., 1995) and rotenone (Sherer et al., 2003). Also, clinical studies have shown that Q_{10} can provide some protection for the nigrostriatal dopaminergic system and can slow the progressive disability of the disease (Shults, 2005).

Methamphetamine is thought to cause mitochondrial dysfunction by disrupting the electron transfer chain. Because of its physical properties, it can diffuse into the mitochondria and the buildup of positively charged particles in the mitochondria interferes with the chemical gradient needed by the electron transport chain. This interferes both with the mitochondrial membrane and with ATP synthase which then causes the initiation of the apoptotic process (Chance and Williams, 1956, Davidson et al., 2001). A second proposed mechanism for methamphetamine induced mitochondrial dysfunction is by the displacement of Ca^{2+} molecules stored in the endoplasmic reticulum causing an influx into the mitochondria. The release of these molecules results in

activation of proaptotic factors like caspase-3 and cytochrome-c and disrupts cellular metabolism leading to necrosis (Davidson et al., 2001; Cadet et al., 2003).

2.9 Excitotoxicity

Excitotoxicity plays an important role in both Parkinson's disease and methamphetamine toxicity. Excitotoxicity is caused when excessive glutamate is released to activate receptors of the NMDA subtype. Normal stimulation of these receptors has been shown to enhance dopamine release and synthesis within the striatum. These excitatory interactions are important for normal striatal function. Glutamate is the most abundant excitatory neurotransmitter; however, it becomes cytotoxic if released in excess, causing a calcium influx into the cell (Schwarcz and Meldrum, 1985). Methamphetamine causes a release of excess glutamate into the striatum via a direct pathway. Mitochondrial dysfunction, as seen in both Parkinson's disease and methamphetamine toxicity, can lead to excitotoxicity, as a reduction in ATP would result in a loss of the ATP-dependent Mg-blockade of NMDA receptors, causing physiological concentrations of glutamate to mediate a calcium influx into the cell (Beal et al., 1993). The resulting rise in calcium increases nitric oxide synthase (NOS). This increases the production of nitric oxide (.NO) which reacts with the superoxide radical to form peroxynitrite and the hydroxyl radical, both powerful oxidizing agents (Dawson et al., 1991). Thus, the end product of excitotoxicity is the formation of reactive oxygen species as well as reduced intracellular glutathione synthesis, two of the indicators that are often seen in Parkinson's disease (Murphy et al., 1989). If mitochondrial dysfunction and

oxidative stress are caused by methamphatamine, it may also act to increase excitotoxicity. This has been seen in other toxins used to create aminal models for Parkinson's disease. For example, it has been shown that excessive MPP^+ concentrations promote excitotoxicity, by enhancing glutamate release (Carboni et al., 1990). Dopaminergic neurons in the substantia nigra are rich in glutamate receptors, and thus highly susceptible to excitotoxicity. This emphasizes the importance of a better understanding of the role of excitotoxicity in Parkinson's disease.

2.10 Neuroinflammation

The neurodegeneration seen with acute administration of methamphetamine has been suggested to be associated with the induction of cyclooxygenase (COX) which causes a neuroinflammatory process resulting in deleterious events in the cell (Zhang et al.,2007). Similarly, there is increasing evidence that one contributor to the later development of Parkinson's disease is inflammation of neuronal tissue, caused by exposure to microorganisms, toxicants, or environmental factors in early life. This inflammation involves the activation of brain immune cells, microglia. Activation of these cells can be beneficial in the protection of the cells, but they can also release neurotoxic cytokines which could have a deleterious effect and produce neurodegeneration (Liu et al., 2003). The substantia nigra has the highest density of microglia in the brain, and studies have shown that Parkinson's disease patients have an even higher number of reactive glial cells than do patients without the disease (McGreer et al, 1988). Researchers have examined this as a risk factor for Parkinson's disease for a

long time, and it has even been said that the influenza pandemic of 1918 caused an increase in the risk of developing the disease (Landrigan et al., 2005). Studies in animals have shown that dopaminergic neurodegeneration resulted from exposure to bacterial endotoxin lipopolysacchide while the animal was *in utero* (Gao et al., 2002). Evidence of the involvement of inflammation came from studies showing that animals exposed to rotenone showed enhanced neurodegeneration in the presence of glia. Further studies showed synergistic effects when rotenone was combined with lipopolysaccharide (Thiruchelvam et al., 2000b). This would support the use of anti-inflammatory therapeutic agents for Parkinson's disease.

2.11 Lipid Peroxidation and Protein Aggregation: Further Markers of Neurotoxicity

Also significant in Parkinson's disease, studies have shown increased levels of markers for lipid peroxidation (cholesterol lipid hydroperoxides) (Dexter et al., 1998; Dexter et al., 1994), markers for oxidative DNA damage (8-hydroxy-2-deoxyguanosine) (Alam et al., 1997a), and increased protein carbonyls (Alam et al., 1997b; Sanchez-Ramos et al., 1994) in post mortem brain tissue of Parkinson's disease patients. Reactive oxygen species cause lipid peroxidation by interacting with unsaturated lipids of membranes. This destroys organelles and causes cell death (Cicchetti et al., 2005). Further studies have demonstrated a link between oxidative damage and protein aggregates, which are characteristic features of Parkinson's disease (Jenner, 2003). Evidence shows that oxidative damage impairs ubiquitination and degradation of proteins by the proteasome. This may aid in the aggregation of α-synuclei. This is the main

protein forming eosinophilic inclusions known as Lewy bodies, a pathological hallmark of Parkinson's disease. The formation of these Lewy bodies is a key determinant that differentiates this disorder from other neurodegenerative diseases (Manning-Bog et al., 2002). An up-regulation and increased aggregation of α-synuclein was also seen after administration of neurotoxins (Bus and Gibson 1984).

2.12 Apoptosis/Necrosis

There is some debate about the notion of whether cell death in Parkinson's disease occurs by way of apoptosis or necrosis. These two pathways are not, however, mutually exclusive as apoptosis and necrosis can occur alone, in combination, or as sequential events. Both have been put forth as pathways for cell death in both Parkinson's disease and methamphetamine toxicity. Apoptosis or programmed cell death is a gradual, progressive cell death. Necrosis, on the other hand, is a rapid form of cell death. Apoptosis is characterized by marked cell shrinkage, fragmentation of nuclear DNA by endonucleases, and chromatin condensation with the formation of nuclear vesicles, which are phagocytosed by nearby cells (Nijhawan et al., 2000; Zimmermanet al., 2001). Necrosis is characterized by a massive ionic influx, cellular swelling with disruption of subcellular organelles and rupture of plasma membrane. It does not, however, involve extensive damage to nuclear DNA. Oxidative stress and mitochondrial dysfunction have been shown to cause both necrotic and apoptotic cell death.

Because of its central position in the energetics of the cell, the mitochondrion plays a central role in apoptosis. Mitochondria influence apoptosis by maintenance of

ATP levels, maintenance of the mitochondrial membrane potential, and the release of proapoptotic factors. Mitochondrial dysfunction leads to cessation of electron transport, which brings about an energy crisis followed by apoptosis/necrosis. The depletion of ATP initiates an apoptotic cell death mechanism, which may be initiated by exposure to one or more of the neurotoxins discussed in this paper (Langston et al., 1983).

2.13 Possible Neuroprotective Treatments

In summary, the previous sections provide literature support for the neurotoxic effects of methamphetamine and discuss the mechanisms implicated in causing damage to dopaminergic cells. Thus, methamphetamine's use can increase the risk for nigrostriatal neurodegeneration significantly. The mechanistic similarity of methamphetamine toxicity to the dopaminergic neurodegeneration seen in Parkinson's disease indicates the usefulness of this model to test pharmacological agents for neuroprotective properties against this neurodegeneration. The literature supports the idea that, in Parkinson's disease, oxidative stress and mitochondrial dysfunction are primary mediators of excitotoxicity, neuroinflammation, apoptosis and necrosis (Nam et al., 2005). Thus, if a pharmacological agent can disrupt these neurotoxic mechanisms, it could provide protection from the resulting neurodegeneration.

2.13.1 Ramelteon

Ramelteon, (S)-N-[2-(1,6,7,8-tetrahydro2H-indeno-[5,4-b]furan-8-yl)ethyl] propionamide, is an orally active sleep agent (for insomnia) and a hypnotic substance. It

has a molecular weight of 259.34 and an empirical formula of $C_{16}H_{21}NO_2$. In its pure form, it is a white powder. It is freely soluble in organic solvents, such as methanol, ethanol, and dimethyl sulfoxide; soluble in 1-octanol and acetonitrile; and very slightly soluble in water and in aqueous buffers (Takeda, 2008a). Ramelteon (figure 2.6) is available commercially as *Rozerem* by Takeda Pharmaceuticals North America. The 8 mg tablets are round, pale orange-yellow and film-coated, with "TAK" and "RAM-8" printed on one side. This is the first in a new class of sleep agents that selectively binds to MT_1 and MT_2 receptors versus binding to GABA $_A$ receptors, such as with other hypnotic drugs like zolpidem, eszopiclone, and zaleplon. Ramelteon is approved by the FDA for long-term use in the treatment of insomnia.

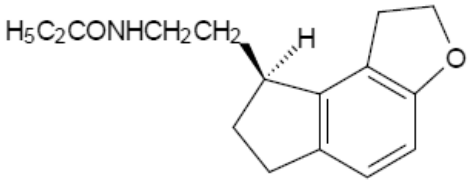

Figure 2.6. Chemical Structure of Ramelteon

Ramelteon is a melatonin receptor agonist with both high affinity for melatonin MT_1 and MT_2 receptors and selectivity over the MT_3 receptor (Owen, 2006). This is believed to contribute to its sleep-promoting properties, as these receptors, acted upon by endogenous melatonin, are thought to be involved in the maintenance of the circadian rhythm underlying the normal sleep-wake cycle (Miyamoto et al., 2004). The significance of ramelteon's lack of affinity for the MT_3 receptor is not clear. The MT_3

receptor appears almost exclusively in the gut and might not have any relationship to sleep or wakefulness.

Ramelteon does not show any appreciable binding to $GABA_A$ receptors, which are associated with anxiolytic, myorelaxant, and amnesic effects (Wang et al., 2003). It also shows no appreciable affinity for receptors that bind neuropeptides, cytokines, serotonin, dopamine, noradrenaline, acetylcholine or opiates. The major metabolite of ramelteon, M-II, has weak affinity for the serotonin $5\text{-}HT_{2B}$ receptor, but no appreciable affinity for other receptors or enzymes. All other known metabolites of ramelteon are inactive (Erman et al., 2006; Zammit et al., 2007). Thus, ramelteon has not been shown to produce dependence and has shown no potential for abuse, and the withdrawal and rebound insomnia that is typical with other GABA modulators is not present in ramelteon. It is currently the only non-scheduled prescription drug for the treatment of insomnia available in the United States (Kato et al., 2005; Takeda, 2008b).

It is not well studied whether ramelteon has other functional similarities with melatonin, besides induction of sleep. Besides its primary function of regulating the sleep cycle, melatonin may exert a powerful antioxidant activity (Tan et al., 2007). Melatonin is an antioxidant that can easily cross cell membranes and the blood brain barrier (Hardeland, 2005). Melatonin is a direct scavenger of OH, O_2^-, and NO (Poeggeler et al., 1994). Unlike other antioxidants, melatonin does not undergo redox cycling, the ability of a molecule to undergo reduction and oxidation repeatedly. Redox cycling may allow other antioxidants (such as vitamin C) to regain their antioxidant properties. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms

several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan et al., 2000). In animal models, melatonin has been demonstrated to prevent the damage to DNA by some carcinogens, stopping the mechanism by which they cause cancer (Karbownik et al., 2001). It also has been found to be effective in protecting against brain injury caused by reactive oxygen species release in experimental hypoxic brain damage in newborn rats (Tutunculer et al., 2005). Melatonin's antioxidant activity may reduce neuronal damage caused in Parkinson's disease (Oaknin-Bendahan et al., 1995). This raises the interesting question of whether ramelteon might also have such beneficial effects. If it posesses the same free radical scavenging properties as melatonin (Reiter et al., 2001), it could prove to be a neuroprotective drug.

2.13.2 Amantadine

Amantadine (figure 2.7), 1-aminoadamantane, is an organic compound that is sold commercially as Amantadine hydrochloride, under the name *Symmetrel*, for use both as an antiviral and an antiparkinsonian drug. Its molecular weight is 187.71 with a molecular formula of $C_{10}H_{18}NCl$. Amantadine hydrochloride is a stable white or nearly white crystalline powder, freely soluble in water and soluble in alcohol and in chloroform (Sandoz, 2008).

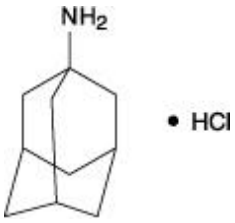

Figure 2.7. Chemical Structure of Amantadine

Amantadine was approved by the U.S. Food and Drug Administration in October 1966 as a prophylactic agent against Asian influenza and eventually received approval for the treatment of Influenzavirus A in adults (Maugh, 1976; Maugh 1979). For the mechanism of its antiviral properties, amantadine interferes with a viral protein, M2 (an ion channel), which is required for the viral particle to become "uncoated" once it is taken inside the cell by endocytosis (Wang et al., 1993). In 1969 the drug was also discovered by accident to help reduce symptoms of Parkinson's disease. The mechanism for its antiviral and antiparkinsonian effects seems to be unrelated. The mechanism of its antiparkinsonian effect is poorly understood. The drug appears to induce release of dopamine from the nerve endings of the brain cells, together with stimulation of norepinephrine response. Furthermore, it appears to be a weak NMDA receptor antagonist and an anticholinergic (Blanpied et al., 2005). As an antiparkinsonian it can be used as monotherapy; or together with levodopa to treat levodopa-related motor fluctuations, or shortening of levodopa duration of clinical effect, and levodopa-related dyskinesias, such as choreiform movements associated with long-term levodopa use (Sandoz, 2008).

Amantadine also has several off label uses. It is frequently used to treat the characteristic fatigue often experienced by patients with multiple sclerosis (Cohen and Fisher, 1989). Additionally, there have been anecdotal reports that low-dose amantadine has been successfully used to treat ADHD (Hallowell and Ratey, 2005). Amantadine has also been shown to relieve SSRI-induced sexual dysfunction (Balogh et al., 1992; Shrivastava et al., 1995; Keller et al., 1997). It is amantadine's effectiveness as an NMDA receptor antagonist that led to its inclusion in this study as possibly neuroprotective. Since NMDA receptors are one of the most harmful factors in excitotoxicity, antagonists of the receptors have held much promise for the treatment of conditions that involve excitotoxicity, including traumatic brain injury, stroke, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Maas, 2001). Excitotoxicity is commonly seen in Parkinson's disease. The defective mitochondrial energy metabolism seen in Parkinson's disease patients may predispose them to excessive glutamate-evoked calcium influx. This could cause toxicity to the cell (Beal, 1992; Blandini, 1996). MPTP-induced parkinsonism is attenuated by a blockade of NMDA receptors (Lange, 1993; Nash, 2000). Amantadine is the only NMDA receptor antagonist that is actively used to treat Parkinson's disease. If it proved to be neuroprotective, with its therapeutic use already well established, it would prove cost effective as well as ground breaking.

2.13.3 Salicylic Acid

Salicylic acid (figure 2.8), 2-Hydroxy-benzoic acid, is found in several plants, notably in wintergreen leaves and the bark of sweet birch. It was made synthetically by heating sodium phenolyate with carbon dioxide under pressure and microbial oxidation of naphthalene (Windholz, 1983). Its molecular weight is 138.1 with a molecular formula $C_7H_6O_3$. It belongs to a class of drugs known as analgesics or antipyretics. Salicylic acid is a metabolite produced in the body following ingestion of Aspirin, acetylsalicylic acid, a commonly used nonsteroidal anti-inflammatory drug (Hardman, 1996). Salicylic acid is a white crystalline powder with a sweetish acrid taste. If prepared from natural methyl salicylate, it may have a faint mint like odour. It is available in forms of powder, ointments, cream, gel, liquids and plaster. Salicylic acid is soluble 1 in 460 to 550 of water, 1 in 15 of boiling water, 1 in 3 to 4 in alcohol, 1 in 3 in ether and 1 in 45 in chloroform (Reynolds, 1996).

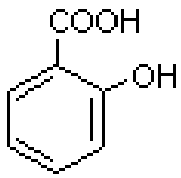

Figure 2.8. Chemical Structure of Salicylic Acid

Aspirin was the first-discovered member of the class of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs), not all of which are salicylates, although they all have similar effects and most have inhibition of the enzyme cyclooxygenase as

their mechanism of action. Today, Aspirin is one of the most widely used medications in the world, with an estimated 40,000 metric tons of it being consumed each year. In countries where Aspirin is a registered trademark owned by Bayer, the generic term is acetylsalicylic acid (Warner and Mitchell, 2002).

Medicines containing derivatives of salicylic acid, structurally similar to Aspirin, have been in medical use since ancient times. Salicylate-rich willow bark extract became recognized for its specific effects on fever, pain and inflammation in the mid-eighteenth century. By the nineteenth century pharmacists were experimenting with and prescribing a variety of chemicals related to salicylic acid, the active component of willow extract. A French chemist, Charles Frederic Gerhardt, was the first to prepare acetylsalicylic acid (named Aspirin in 1899) in 1853. In the course of his work on the synthesis and properties of various acid anhydrides, he mixed acetyl chloride with a sodium salt of salicylic acid (sodium salicylate). A vigorous reaction ensued, and the resulting melt soon solidified. In 1897, scientists at the drug and dye firm Bayer began investigating acetylsalicylic acid as a less-irritating replacement for standard common salicylate medicines. By 1899, Bayer had dubbed this drug Aspirin and was selling it around the world. Aspirin's popularity grew over the first half of the twentieth century, spurred by its effectiveness in the wake of the Spanish flu pandemic of 1918, and aspirin's profitability led to fierce competition and the proliferation of Aspirin brands and products, especially after the American patent held by Bayer expired in 1917 (Jeffreys, 2005).

Aspirin suppresses the production of prostaglandins and thromboxanes. Prostaglandins are local hormones produced in the body and have diverse effects in the

body, including the transmission of pain information to the brain, modulation of the hypothalamic thermostat, and inflammation. Thromboxanes are responsible for the aggregation of platelets that form blood clots. Heart attacks are primarily caused by blood clots, and low doses of Aspirin are seen as an effective medical intervention for acute myocardial infarction. The major side-effect of this is that because the ability of blood to clot is reduced, excessive bleeding may result from the use of aspirin. Aspirin's ability to suppress the production of prostaglandins and thromboxanes is due to its irreversible inactivation of the cyclooxygenase (COX) enzyme. Cyclooxygenase is required for prostaglandin and thromboxane synthesis. Aspirin acts as an acetylating agent where an acetyl group is covalently attached to a serine residue in the active site of the COX enzyme. This makes aspirin different from other NSAIDs, such as ibuprofen, which are reversible inhibitors. There are at least two different types of cyclooxygenase: COX-1 and COX-2. Aspirin irreversibly inhibits COX-1 and modifies the enzymatic activity of COX-2. Normally COX-2 produces prostanoids, most of which are pro-inflammatory. Aspirin-modified COX-2 produces lipoxins, most of which are anti-inflammatory (Vane, 1971; Tohgi et al., 1992; Vane and Botting, 2003).

Salicylic Acid is a known free radical scavenger (Kataoka et al., 1997). It has been shown to provide neuroprotection against MPTP induced neurotoxicity (Mohanakumar et al., 2000). Thus, it could provide much needed neuroprotection against oxidative stress which leads to neurodegeneration. With the ability of salicylic acid for free radical scavenging, it could play a much needed neuroprotetive role in not only Parkinson’s disease, but also in numerous other neurodegenerative disorders.

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3. STATEMENT OF RESEARCH OBJECTIVES

Overall, the conducted study seeks evidence that intraperitoneal methamphetamine administration in mice causes significantly reduced levels of dopamine in the brain, thus providing a relevant model for Parkinson's disease. In addition, this research evaluates the neuroprotective mechanisms of ramelteon, amantadine and salicylic acid using the methamphetamine lesioned mice model of Parkinson's disease. The long-term goal is to facilitate and improve the care of patients with Parkinson's disease by developing a cost-effective anti-Parkinson's drug that has the added benefit of a neuroprotective effect. This model could successfully further our understanding of the neurodegenerative process (i.e. dopamine depletion) that targets the nigrostriatal system. Studies on dopaminergic toxicants which are also widely used drugs of abuse could have added preventative effects by helping identify and increase awareness of the risks involved in such drug abuse.

The hypothesis of this study is that methamphetamine will cause significant dopaminergic depletion and that the neuroprotective effects of ramelteon, amantadine and salicylic acid will directly correlate with augmented behavioral, neurochemical and biochemical paremeters in the brain of methamphetamine treated mice and will render neuroprotection by reducing neuronal dopamine depletion.

4. CHAPTER 1: NEUROTOXIC EFFECTS OF METHAMPHETAMINE

4.1 Overview

Methamphetamine can cause striatal dopamine depletion and could be useful in making an animal model to study Parkinson's disease. Since the neurotoxic mechanism of methamphetamine is not well understood, this study was undertaken to gain greater mechanistic understanding of the drug. In this study, we examined the neurotoxic effects of methamphetamine. With regard to biochemical assays, we evaluated the effect of methamphetamine on mitochondrial reactive oxygen species (ROS) generation, monoamine oxidase (MAO) activity, superoxide dismutase (SOD) activity and complex I & IV activities. Behavioral analysis evaluated the effect on movement (tremor, straub tail, catalepsy, akinesia and swim score). In addition, neurotransmitter levels were evaluated using high pressure liquid chromatography (HPLC)-electrochemical detection. Results showed that methamphetamine caused significant dopamine depletion in the striatum and also caused significant generation of reactive oxygen species and decreased complex I activity in the mitochondria. Behavioral analysis showed a significant effect on catalepsy, akinesia and reduced swim score.

4.2 Introduction

Nigrostriatal dopaminergic neurons undergo selective degeneration in Parkinson's disease leading to a shortage of dopamine in the striatum (Hornykiewicz, 1973; Caine and Langston, 1983). Neurons in the striatum require dopamine to effectively control movement. Thus, the nigral dopaminergic neurodegeneration in Parkinson's disease causes motor deficits like slowness in initiation and execution of voluntary movements, increased muscle tone, increased resistance to movement, equilibrium and righting reflex, shuffling of feet and stooped posture (Sethy et al., 1997). The dopamine depletion is also evidenced by various motor symptoms including resting tremors, bradykinesia, muscular rigidity, and abnormalities in posture and gait. Parkinson's disease is a progressive disorder and complete immobility usually occurs, despite treatment (Kopin, 1992; Marsden, 1994; Sethy et al., 1997). Oxidative stress, mitochondrial dysfunction, apoptosis, neuroinflammation and N-methyl-D-aspartic acid (NMDA) receptor-mediated excitotoxicity are mechanisms commonly implicated in dopaminergic neurotoxicity (Lau et al., 2000; Dluzen et al., 2001; Gluck et al., 2001; Dawson and Dawson, 2003; Kita et al., 2003; Sherer et al., 2003; LaVoie et al., 2004; Thomas et al., 2004; Riddle et al., 2006). The above mentioned mechanisms are also indicated in Parkinson's disease. It is well known that a significant degree of striatal dopamine depletion, similar to that seen in Parkinson's disease, can be produced by endogenous and exogenous neurotoxins (Dluzen et al., 2001; Dawson and Dawson, 2003; Sherer et al., 2003). Studies have implicated exposure to toxins like 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), paraquat, maneb, rotenone, manganese and iron as contributors to the development of

Parkinson's disease (Dawson and Dawson, 2003; Sherer et al., 2003). These toxins are useful in the creation of animal models to study the different aspects of Parkinson's disease. With no proven neurorestorative or neuroprotective treatment strategies for this disease, these animal models are essential for discovering novel and efficient treatments that are proactive, not merely symptomatic, and to lowering the disease progression and mortality rate (Dawson and Dawson, 2003).

Methamphetamine is a highly addictive drug that is widely abused in the United States (NIDA, 2002). Methamphetamine is also used clinically in the treatment of obesity and depression, but this psycho-stimulatory drug has highly addictive properties and neurotoxic effects (NIDA, 2002; McMillen, 1983). It can cross the blood brain barrier and is possibly capable of causing damage to dopaminergic neurons. Methamphetamine administration results in long lasting dopamine depletion in humans and animals (Davidson et al., 2001; Cadet et al., 2003; Kita et al., 2003). Examination of post-mortem brain tissue from methamphetamine users showed a significant decrease in dopamine content and dopamine transporter density (Wilson et al., 1996). Methamphetamine has been shown to induce striatal dopamine depletion in mice striatum, hence it can be ideal to study the disease progression and for evaluating neuroprotective drugs (Bondy et al., 2000; Dluzen et al., 2001). It has been further shown that repeated administration of methamphetamine reduces dopamine transporter (DAT) binding and causes persistent dopaminergic degeneration in the striatum (Davidson et al., 2001; Cadet et al., 2003; Kita et al., 2003).

There is a lack of exact mechanistic understanding of the neurotoxicity of methamphetamine. There are several possible mechanisms such as generation of reactive radicals, mitochondrial dysfunction and reduced energy metabolism and DNA damage leading to eventual cell death (Fibiger and Mogeer, 1971; Imam et al., 2001; Pubill et al., 2005; Riddle et al., 2006). Mitochondria are vital organelles present in the mammalian cell that not only generate adenosine triphosphate but also perform such critical functions as hosting essential biosynthetic pathways, calcium buffering, and apoptotic signaling. The incomparable and excessive energy requirements of neurons require proper functioning and maintenance of mitochondria. Mitochondrial abnormalities have been associated to several neurodegenerative diseases, but the evidence is particularly strong, and continuously accumulating, in Parkinson's disease and Alzheimer's disease (Dawson and Dawson, 2003).

In this study we examined in vitro effect of the methamphetamine using mouse brain mitochondria and for the in vivo experiments C57/Bl6 mice were used. We analyzed the effect of methamphetamine on various behavioral, biochemical and neurochemical parameters relating to nigral dopaminergic damage. Methamphetamine like other dopaminergic neurotoxins induced significant behavioral changes similar to that seen in Parkinson's disease, caused oxidative stress, mitochondrial dysfunction and induced significant dopamine depletion in the striatum.

4.3 Materials and Methods

4.3.1 Animals

Male C57/Bl6 mice (4-6 weeks old) weighing 20-30 g were purchased from Charles Rivers. They were housed in a temperature controlled room with a 12h day and night cycle with free access to food and water. They were housed for 2-4 days prior to experiments. All the experimental procedures were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Auburn University.

4.3.2 Methamphetamine administration

C57BL/6 mice were separated into 3 groups (control, low dose methamphetamine & high dose methamphetamine) and treated with saline, methamphetamine (10mg/kg i.p., twice, 2hr apart) and methamphetamine (20mg/kg i.p., twice, 2hr apart), respectively. The animals were sacrificed 5 days after the last injection.

4.3.3 Behavioral Studies

Following methamphetamine administration, two examiners who were trained in evaluating different animal behaviors continuously observed mice. Tremor, straub tails, akinesia and catalepsy were monitored during this period following standard scoring procedures as described below. Animals were subjected to swim test on the 4^{th} day after methamphetamine administration.

Straub tail: Straub tail was defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as whether straub tail phenomenon was detected or not detected (Mohanakumar and Sood, 1986).

Akinesia: Akinesia usually refers to the impaired ability to initiate movements. It was measured by noting the latency in second(s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180s (Weihmuller et al., 1988; Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term implies the inability of an animal to correct an externally imposed posture. Catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Weihmuller et al., 1988; Murlikrishnan and Mohanakumar, 1998).

Swim test: Swim test was carried out in water tubs (40 l x 25 w x 12 h, in cm). The depth of water was kept at 8 cm and the temperature was maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were: 0 = hind part sinks with head floating, 1 = occasional swimming using hind limbs while floating on one side, 2 = occasional floating/swimming only, 3 = continuous swimming (Donnan et al., 1987).

4.3.4 Tissue Preparation for *in vivo* biochemical assays

Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001).

4.3.5 Measurement of Neurotransmitters

To determine dopamine depletion in the striatum, methamphetamine injected animals were sacrificed on the 5th day and the striata was dissected out and analyzed for DA content employing HPLC-electrochemistry. Dopamine and its metabolites were separated in high pressure liquid chromatography. An electrochemical detector was used to quantify dopamine and its metabolites. The C18 octadecyl silica column (8 cm X 4.6 mm i.d.) was used for the separation of the monoamines. Brain tissue was sonicated in 0.4 N $HClO_4$. These samples were then centrifuged and the 20 µl of supernatant used was injected in HPLC. The results were expressed as picomoles per milligram of tissue (Uthayathas et al., 2007).

4.3.6 Mouse Mitochondrial P_2 Fraction for *in vitro* biochemical assays

Mitochondrial (P_2) fraction was prepared from the mouse brain. The brain was homogenized in 0.1M phosphate buffer, pH 7.8, using a glass teflon homogenizer. Tissue homogenates were centrifuged at different rpm to get the mitochondrial P_2 fraction.

Different concentrations of methamphetamine (1, 2.5, 5 & 10µM) were incubated for 60 minutes with mitochondrial fraction obtained from the mouse brain. The *in vitro* effect of methamphetamine on complex I & IV activity, ROS generation, SOD activity and MAO activity in the mitochondria was evaluated using the following procedures:

4.3.7 Protein estimation

Protein was assayed using the coomassie plus protein assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

4.3.8 Mitochondrial Complex I Activity

Mitochondrial complex-I activity (NADH dehydrogenase activity) is based on the NADH oxidation. Oxidation of NADH by the NADH-dehydrogenase enzyme present in the tissue homogenate was measured spectrophotometrically at 340 nm. The mitochondrial P_2 suspension was added to the reaction mixture containing NADH, coenzyme Q_0 and phosphate buffered saline, to analyze its effect on NADH oxidation by monitoring the decrease in absorbance at 340 nm. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The change in absorbance indicates the amount of NADH oxidized. The mitochondrial complex-I activity is expressed as the amount of NADH oxidized/mg protein (Wharton and Tzagotoff, 1967; Ramsay et al., 1986).

4.3.9 Mitochondrial Complex IV Activity

Complex IV activity was based on the cytochrome-C oxidation. The mitochondrial P_2 suspension was added to the reaction mixture containing cytochrome-C and phosphate buffered saline. The oxidation was measured spectrophotometrically. The absorbance was measured at 550nm for 2 min and the enzyme activity was expressed as cytochrome-C oxidized/mg protein (Wharton and Tzagotoff, 1967; Ramsay et al., 1986).

4.3.10 Assay of MAO activity

Total MAO activity was based on the amount of 4-hydroxyquinoline formed by the oxidation of kynuramine (Morinan and Garratt, 1985). 4-hydroxyquinoline was measured fluorimetrically and the enzyme activity was expressed as 4-hyroxyquinoline formed/hour/mg protein (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001).

4.3.11 Assay of SOD activity

SOD activity was analyzed following the method of Marklund and Marklund (1974). Pyrogallol oxidation was measured at 420nm.

4.3.12 Assay of ROS production

Conversion of nonfluorescent chloromethyl-DCF-DA (2′,7′- dichlorofluorescin diacetate) to fluorescent DCF was used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission

wavelength of 527 nm. The generation of ROS was measured and reported as relative fluorescence intensity (Dhanasekaran et al., 2008).

4.3.13 Statistical Analysis

Results are expressed as the means±SEM. The statistical significance was evaluated by the one-way analysis of variance (ANOVA) using SigmaStat version 3. Values of *p* less than or equal to 0.05 were considered significant.

4.4 Results

4.4.1 Behavioral Analysis

Methamphetamine caused a significant motor impairment in the C57/BL6 mice. Intraperitoneal administration of methamphetamine (10 & 20 mg/kg) in mice induced significant dose dependant behavioral abnormalities including catalepsy (figure 4.1), akinesia (figure 4.2) and reduced swim time (figure 4.3) as compared to the controls. Straub tails and tremor were also observed (table 4.1).

4.4.2 Effect of methamphetamine on neurotransmitters

Administration of methamphetamine (10 & 20mg/kg) caused a significant reduction in the levels of dopamine in the striatum (figure 4.4). Methamphetamine (10 & 20mg/kg) treated animals showed significant dopamine depletion (n=12, $p<0.05$) as compared to control animals. Methamphetamine also affected levels of other striatal

neurotransmitters (NE, 5HT, 5HIAA) and dopamine metabolites (DOPAC, HVA) as shown in table 4.2.

4.4.3 *In vivo* biochemical analysis

Intraperitoneal administration of methamphetamine (10 & 20mg/kg) in mice significantly decreased complex I activity (n=12, $p<0.05$; figure 4.5) while having no significant effect on complex IV activity (figure 4.6). Administration of methamphetamine had no significant effect on the total mitochondrial monoamine oxidase (MAO) activity (figure 4.7). However, the administration of methamphetamine (10 & 20mg/kg) increased superoxide dismutase (SOD) activity (n=12, $p<0.05$; figure 4.8) and caused significant generation of reactive oxygen species (n=12, $p<0.001$; figure 4.9).

4.4.4 *In vitro* biochemical analysis

Incubation of mouse mitochondrial P_2 homogenate for 60 minutes with methamphetamine (1, 2.5, 5 & 10µM) significantly decreased complex I activity (n=6, $p<0.05$; figure 4.10) while having no significant effect on complex IV activity (figure 4.11). Incubation with methamphetamine at the same concentration had no significant effect on the total mitochondrial monoamine oxidase (MAO) activity (figure 4.12). However, the incubation with methamphetamine (1, 2.5, 5 & 10µM) increased superoxide dismutase (SOD) activity (n=6, $p<0.05$; figure 4.13) and caused significant generation of reactive oxygen species (n = 6, $p<0.001$; figure 4.14).

4.5 Discussion

Results of this study show that injection with methamphetamine caused a significant amount of dopamine depletion, as well as creating a striatal environment ripe for oxidative damage and mitochondrial dysfunction. Both the *in vitro* and *in vivo* results showed that methamphetamine caused significant generation of reactive oxygen species and increased superoxide dismutase activity. These results indicate an environment of oxidative stress. Methamphetamine also causes a significant decrease in complex I activity. Complex I mitochondrial dysfunction is specific to Parkinson's disease. Similarly there are numerous markers in the nigrostriatal pathway to indicate the role of oxidative stress in nigral dopaminergic neurodegeneration. The neurochemical data showed that methamphetamine caused significant dopamine depletion in the striatum of greater than 50% for both the 10mg/kg and the 20mg/kg doses. Behavioral analysis showed a significant motor impairment (catalepsy, akinesia, straub tail, tremor and swim score). These motor abnormalities are comparable to those seen with dopaminergic neurodegeneration.

Methamphetamine is a cationic and lipophilic compound capable of crossing the blood-brain barrier and acts as a potent stimulant. In the central nervous system, it displaces monoamines like dopamine, norepinephrine and serotonin from their vesicles. Once released, the dopamine is metabolized producing proxidant hydrogen peroxide and it can also be oxidized to reactive oxygen species. The combination of oxidative stress and mitochondrial complex I dysfunction leads to apoptosis and creates toxicity of striatal

nerve terminals. This leads to long lasting dopamine depletion (Stokes et al., 1999; Zhu et al., 2006).

The brain and nervous system are particularly vulnerable to free radical damage for a number of reasons. The membrane lipids in the brain contain high levels of polyunsaturated fatty acid side chains, which are prone to free radical attack and are readily peroxidizable. The brain also consumes large quantities of total oxygen for its relatively small weight, contributing further to the formation of reactive oxygen species. It has been estimated that up to 2% of the oxygen consumed by healthy mitochondria is converted to superoxide, and this amount is higher in damaged and aged mitochondria. Oxidative species are important in cellular respiration and aid in the regulation of signal transduction pathways. However, they can be converted into free radicals, containing unpaired electrons, which are collectively referred to as reactive oxygen species (ROS). Oxidative stress involves the excessive formation of reactive oxygen species, like the hydroxyl radicals (OH., OH-) and superoxide (O2.), among others, which are formed when molecular oxygen is reduced non-enzymatically (Fahn and Cohen, 1992; Jenner et al., 1992). The brain has been shown to contain low to moderate levels of enzymes such as catalase, superoxide dismutase and glutathione peroxidase that play an important role in the metabolism of reactive oxygen species (Cohen, 1988). Superoxide dismutase (SOD) converts the superoxide to hydrogen peroxide, and hydrogen peroxide is typically detoxified by glutathione peroxidase and catalase. However, oxidative stress occurs when free radicals are in excess of these antioxidant defenses. Neurotoxins damaging the dopaminergic neurons have been shown to generate hydroxyl radicals and significantly

alter the antioxidant enzyme activities in the mouse brain (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001). Neurotoxins such as MPTP, 6-OHDA, paraquat, maneb, iron and manganese have been shown to exhibit dopamine depletion in the mouse striatum.

Oxidative stress causes an inhibition of mitochondrial ATP production, a major determinant of lethal cell injury. Mitochondrial DNA is sensitive to the toxicity caused by reactive oxygen species because mitochondria have only a limited arsenal of DNA repair processes. Therefore, mitochondrial DNA mutations would cause defects in the respiratory chain function leading to severe cellular damage. Free radicals can interact with sugars, proteins and lipids creating a number of modifications which could then lead to mitochondrial dysfunction, apoptosis and even necrosis. The damage to the striatal terminals and the nigral cell bodies observed in methamphetamine users is indicative of all of these toxic mechanisms, the same mechanisms indicated in Parkinson's disease (Imberti et al., 1993; Jones and Vale, 2000).

There are studies lending evidence to support the theory that oxidative stress and mitochondrial dysfunction play a key role in neurodegeneration. Recent studies were conducted on the antioxidant coenzyme Q_{10}, which plays an important role in the mitochondrial electron transport chain as the electron acceptor for complex I. Levels of Q_{10} were found to be reduced in Parkinson's disease patients. Coenzyme Q_{10} has provided protection against both MPTP (Akaneya et al., 1995) and rotenone (Sherer et al., 2003). Also, clinical studies have shown that Q_{10} can provide some protection for the

nigrostriatal dopaminergic system and can slow the progressive disability of the disease (Shults, 2005).

Mitochondrial dysfunction has long been thought to play a significant role in depletion of nigral neurons, leading to dopaminergic depletion. Evidence seems to suggest that the neurons in the substantia nigra are particularly prone to oxidative stress, which would explain the selective degeneration in this region (Halliwell, 1998). The central dopaminergic system that plays such an important role in motor activity is comprised of a surprisingly small number of neurons. Because of this, it is especially vulnerable and even minor insults may lead to irreparable functional deficits (Fariello et al., 1988). Studies have implicated exposure to toxins like methamphetamine as contributors to the development of Parkinson's disease. This study shows the significant degree of dopamine depletion produced by methamphetamine, evidenced by both behavioral experiments and HPLC data. It also offers biochemical evidence of oxidative stress and mitochondrial dysfunction. These results indicate the usefulness of methamphetamine in making an animal model to study Parkinson's disease. The current treatment strategy for Parkinson's disease remains primarily symptomatic, with no proven neuroprotective or neurorestorative treatments. An animal model with a Parkinson disease phenotype could be utilized to gain a better understanding of the neurodegeneration in the nigrostriatal system and to investigate pharmacological agents for any neuroprotective or neurorestorative effects they may exhibit.

4.6 Refrences

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4.7 Figures

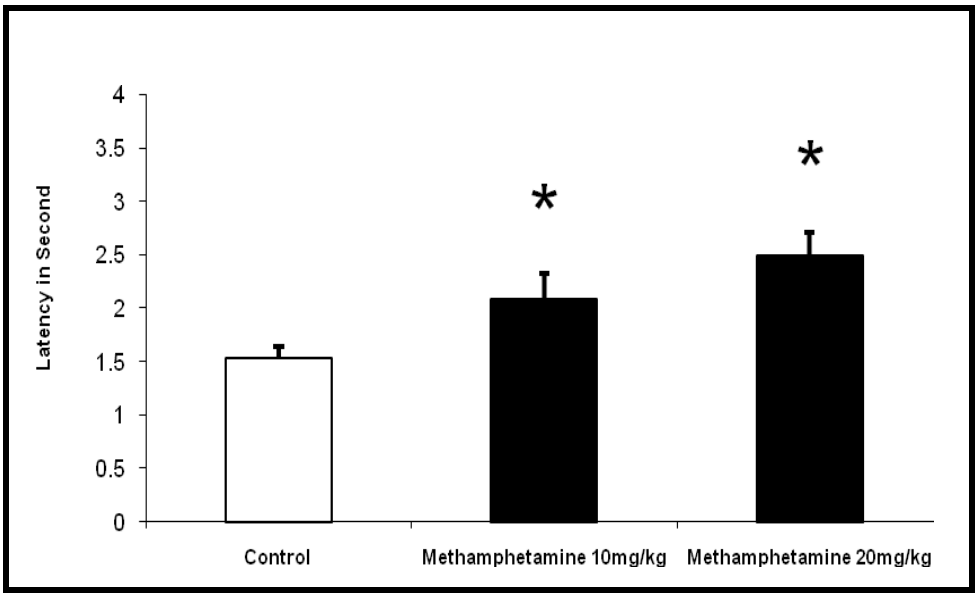

Figure 4.1. Effect of methamphetamine on catalepsy: Methamphetamine (10 & 20 mg/kg) significantly increased catalepsy (n=12, p*<0.05) as compared to control animals.

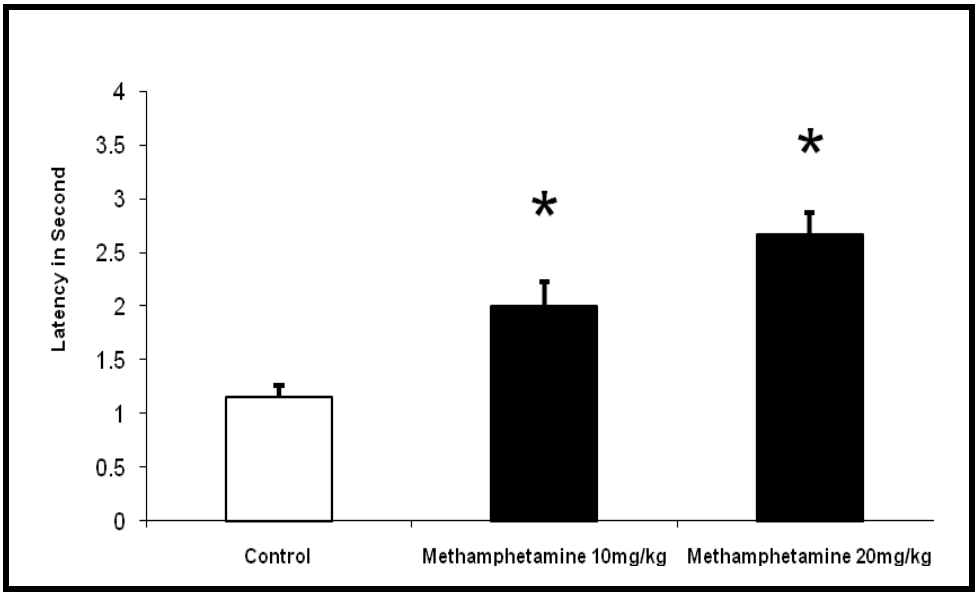

Figure 4.2. Effect of methamphetamine on akinesia: Methamphetamine (10 & 20 mg/kg) significantly increased akinesia (n=12, p*<0.05) as compared to control animals.

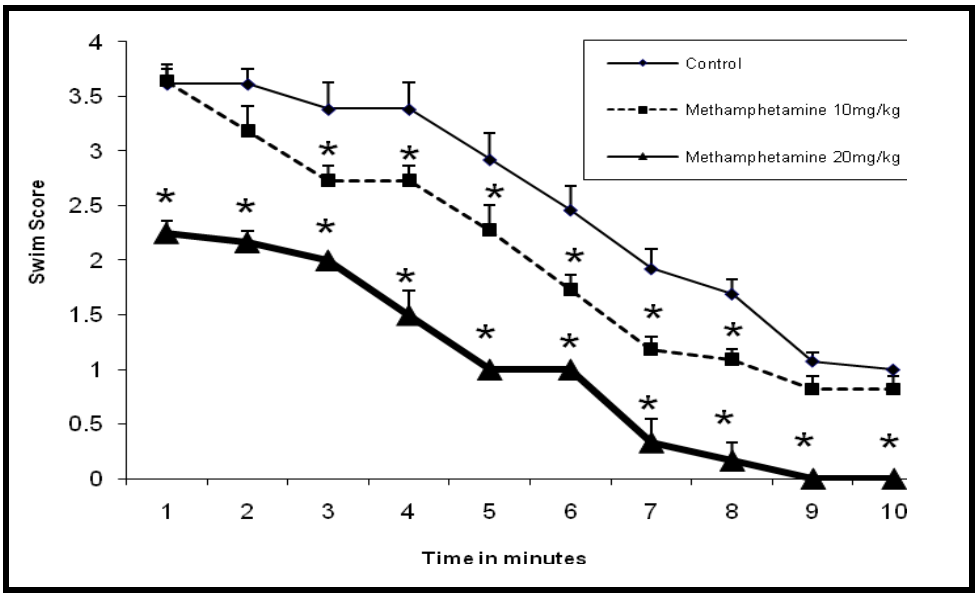

Figure 4.3. Effect of methamphetamine on swim score: Methamphetamine (10 & 20 mg/kg) significantly decreased swim score (n=12, $p^*<0.05$) as compared to control animals. Scored by immobility time.

	average weight	straub tail	tremor
Control	24.3±0.6	0	0
Methamphetamine 10mg/kg	24.2±0.5	n=12	4.0±0.1
Methamphetamine 20mg/kg	23.6±0.3	n=12	4.0±0.1

Table 4.1. Effects of methamphetamine on straub tail and tremor: Methamphetamine (10 & 20 mg/kg) administration induced straub tail and tremor.

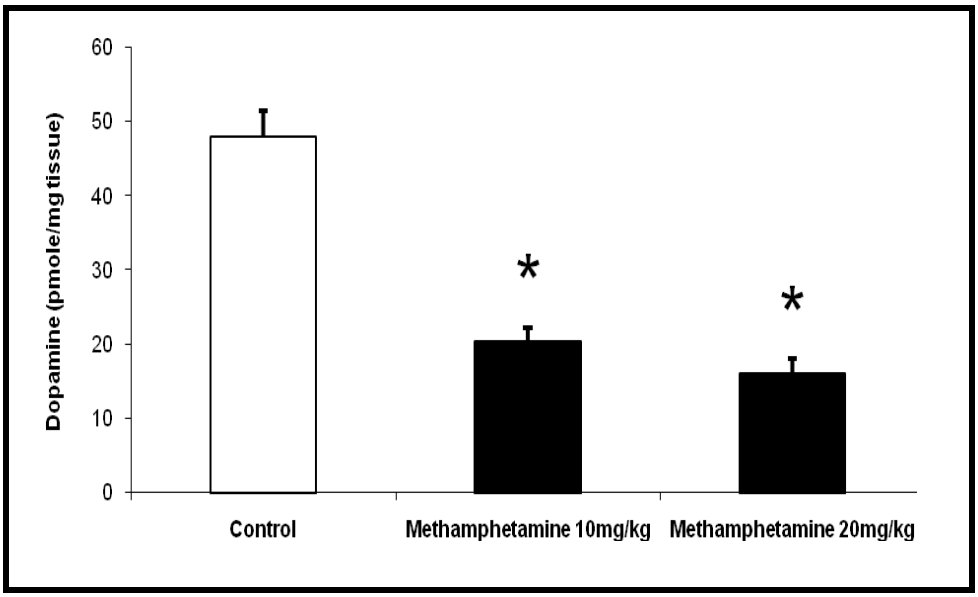

Figure 4.4. Effect of methamphetamine on striatal dopamine content: Methamphetamine (10 & 20mg/kg) treated animals showed significant dopamine depletion (n=12, p*<.05) as compared to control animals.

	Control	Methamphetamine 10mg/kg	Methamphetamine 20mg/kg
DOPAC	49.12±11.6	19.45±3.8 *	24.98±4.5 *
HVA	24.73±1.7	18.10±1.7 *	14.60±1.7 *
5-HT	2.99±1.6	2.69±0.2	4.28±0.7
5-HIAA	11.37±1.7	9.86±0.5	24.76±3.1 *
NE	15.21±0.9	14.49±0.9	12.91±0.9 *

Table 4.2. Effect of Methamphetamine on striatal neurotransmitter and metabolite levels (expresed as average pmole/mg tissue ± SEM). * significant (n=12, p*<.05) as compared to control animals.

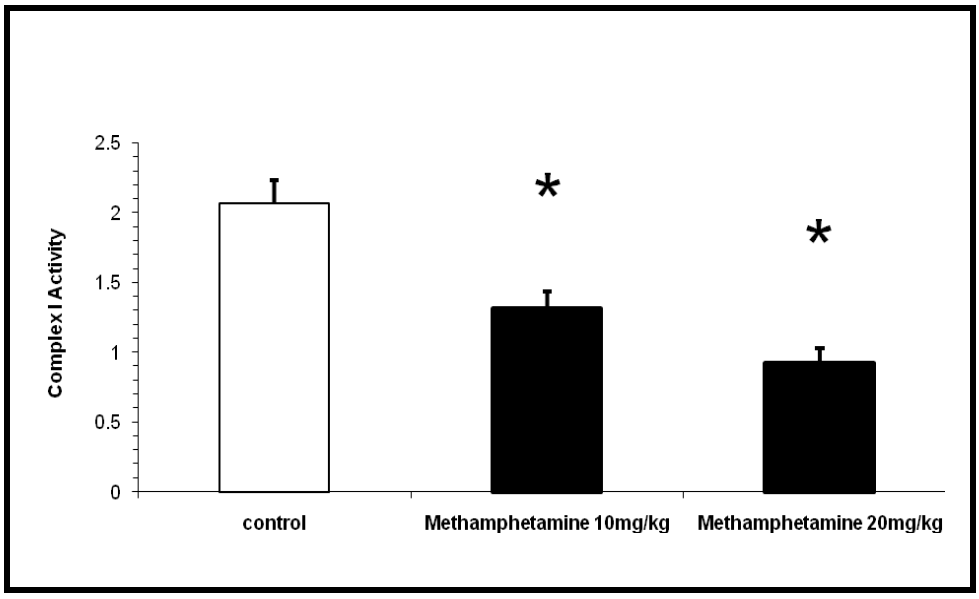

Figure 4.5. Effect of methamphetamine on mitochondrial Complex I activity (*in vivo*): Methamphetamine (10 & 20 mg/kg) significantly decreased complex I activity as compared to controls (n = 12, $p^*<0.05$). Activity expressed as µmol/min/mg protein.

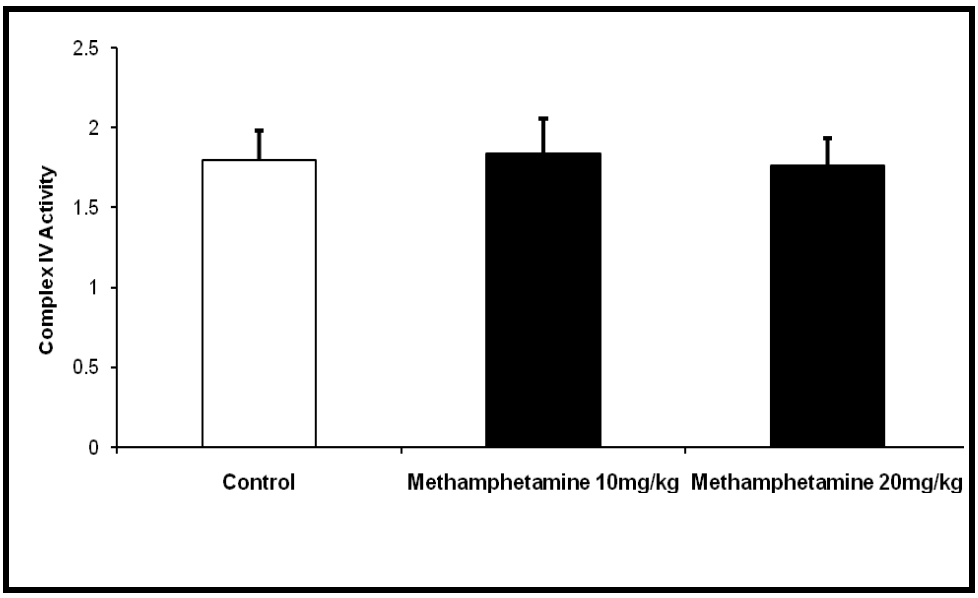

Figure 4.6. Effect of methamphetamine on mitochondrial Complex IV activity (*in vivo*): Methamphetamine (10 & 20 mg/kg) caused no significant change in complex IV activity (n=12). Activity expressed as µmol/min/mg protein.

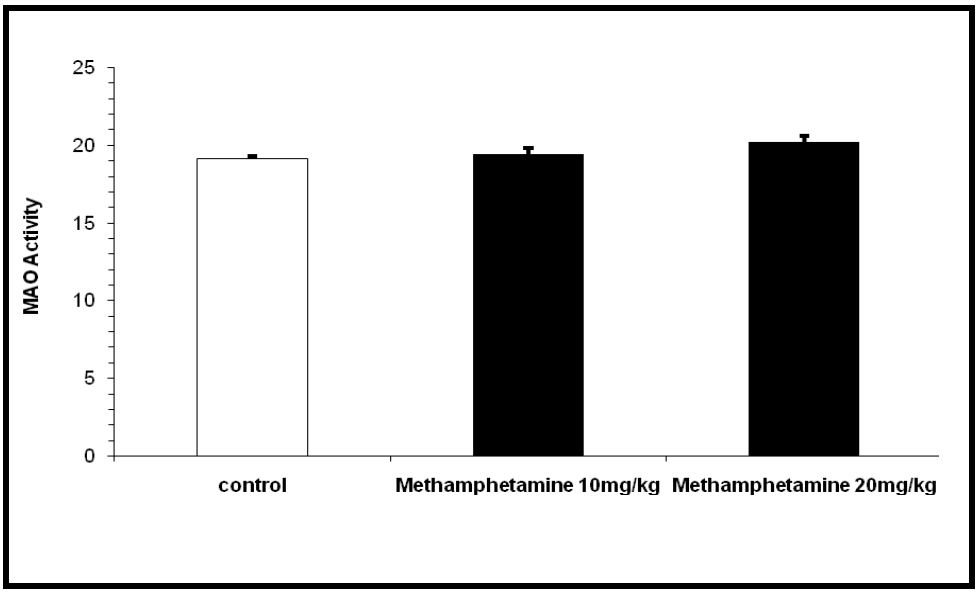

Figure 4.7. Effect of methamphetamine on mitochondrial MAO activity (*in vivo*): Methamphetamine (10 & 20 mg/kg) caused no significant change in MAO activity (n=12). Activity expressed as 4-OHQ/mg protein.

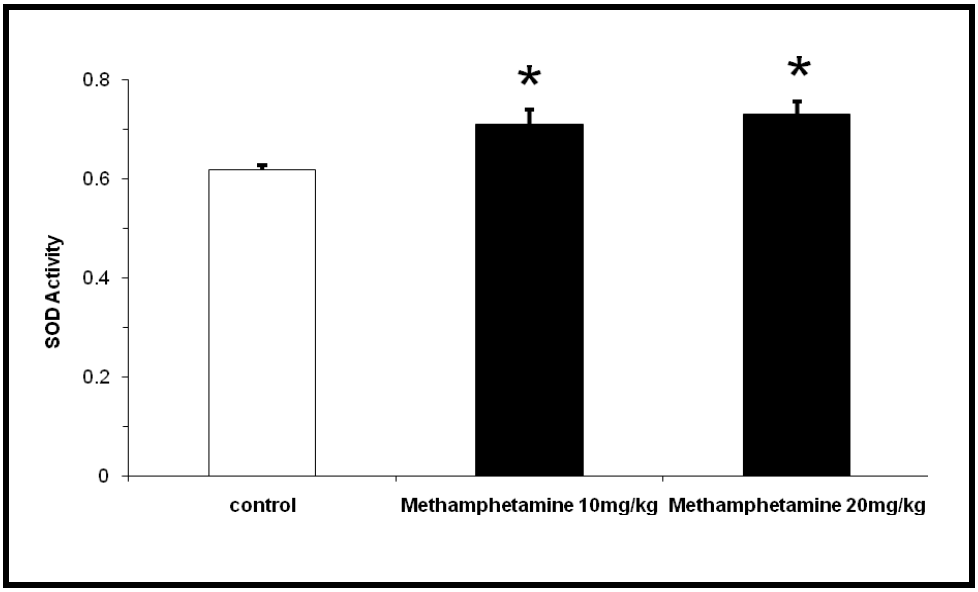

Figure 4.8. Effect of methamphetamine on mitochondrial SOD (*in vivo*): Methamphetamine (10 & 20 mg/kg) significantly increased SOD activity (n = 12, $p^*<0.05$) as compared to control animals.

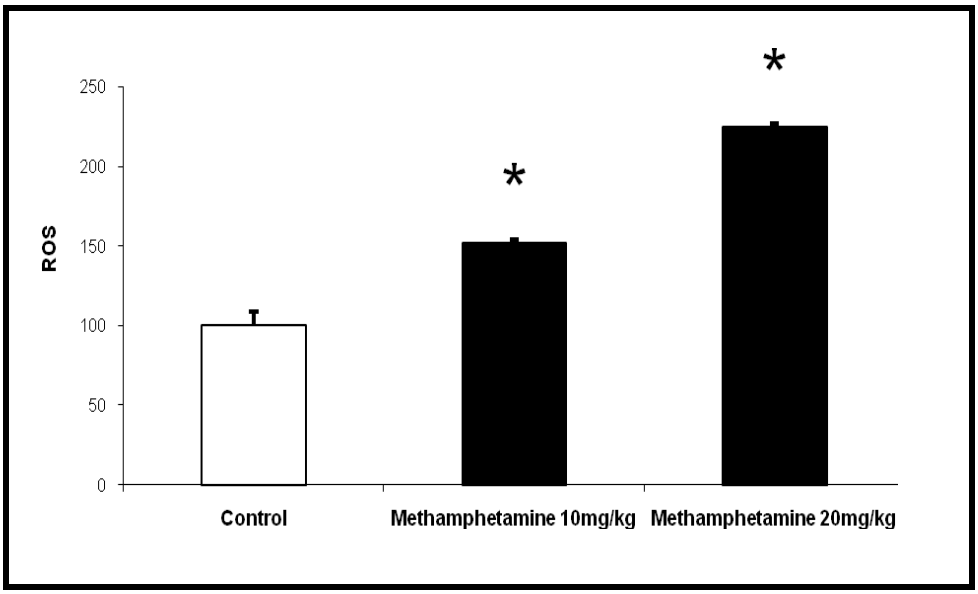

Figure 4.9. Effect of methamphetamine on mitochondrial ROS (*in vivo*): Methamphetamine (10 & 20 mg/kg) caused significant generation of ROS (n = 12, $p^{*}<0.001$) as compared to control animals.

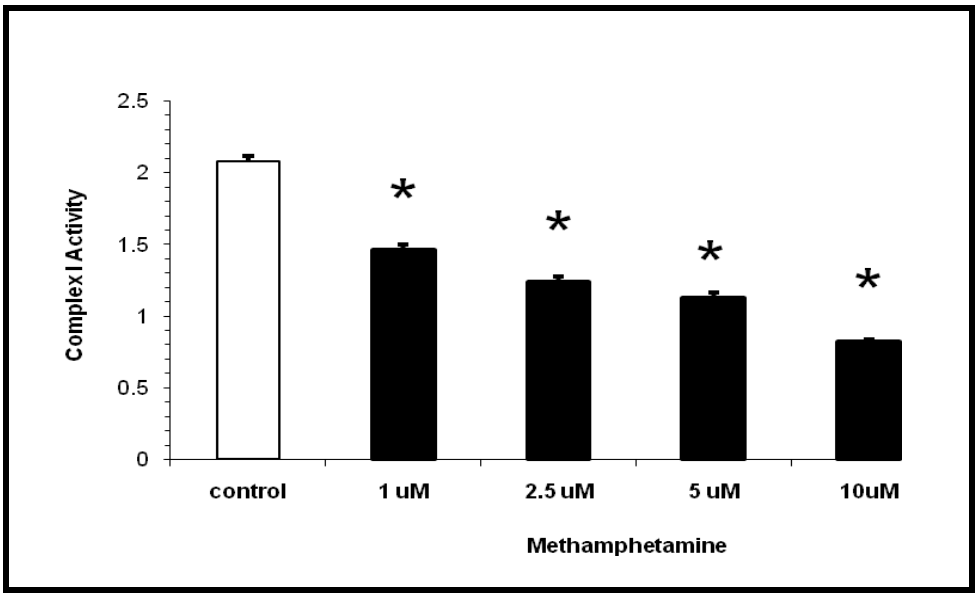

Figure 4.10. Effect of methamphetamine on mitochondrial Complex I activity (*in vitro*): Methamphetamine (1, 2.5, 5 & 10µM) dose dependently decreased complex I activity, expressed as µmol/min/mg protein (n = 6, $p^*<0.05$) as compared to controls.

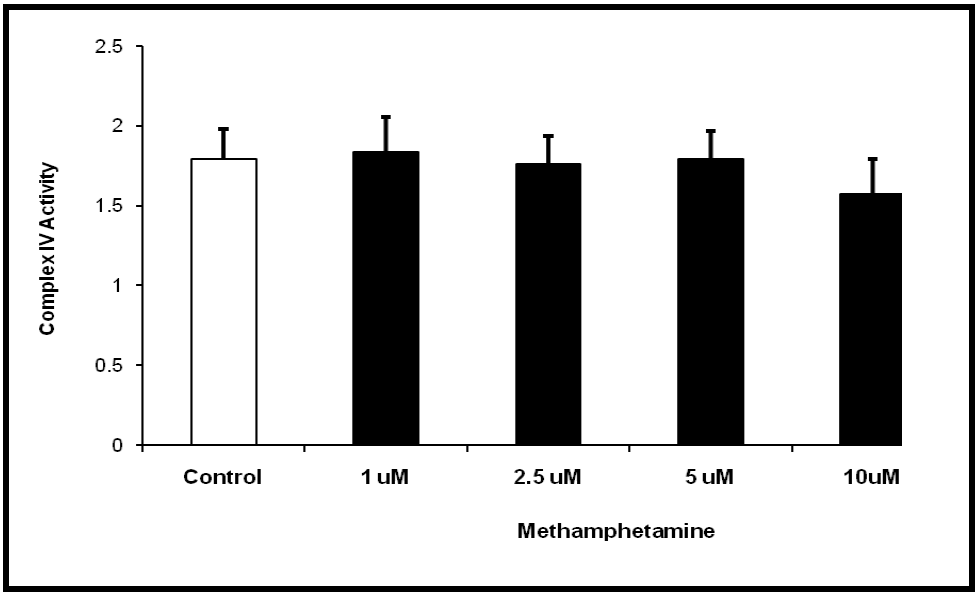

Figure 4.11. Effect of methamphetamine on mitochondrial Complex IV activity (*in vitro*): Methamphetamine (1, 2.5, 5 & 10µM) had no significant effect on complex IV activity (n=6). Activity expressed as µmol/min/mg protein.

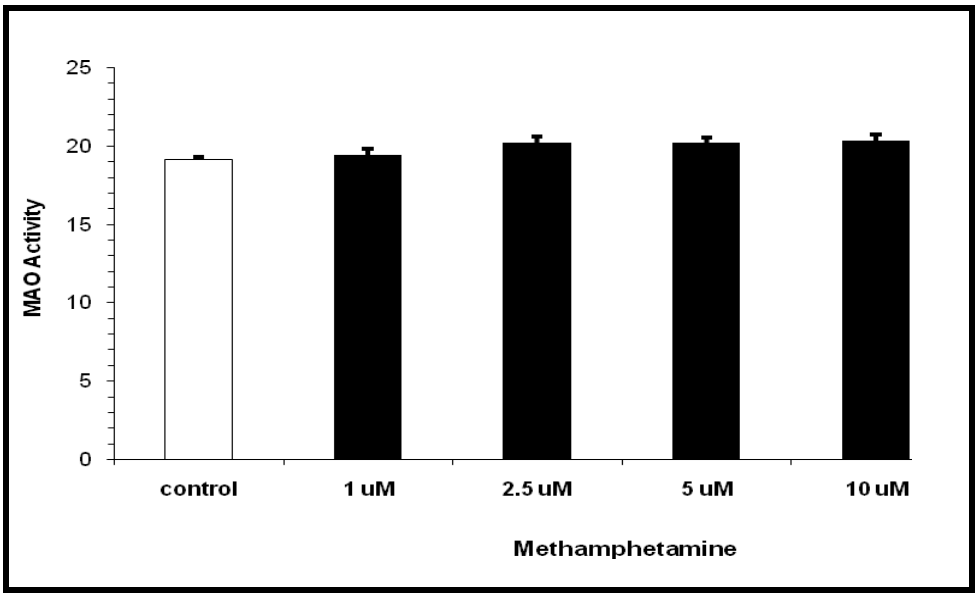

Figure 4.12. Effect of methamphetamine on mitochondrial MAO activity (*in vitro*): Methamphetamine (1, 2.5, 5 & 10 µM) had no significant effect on MAO activity (n=6). Activity expressed as 4-OHQ/mg protein.

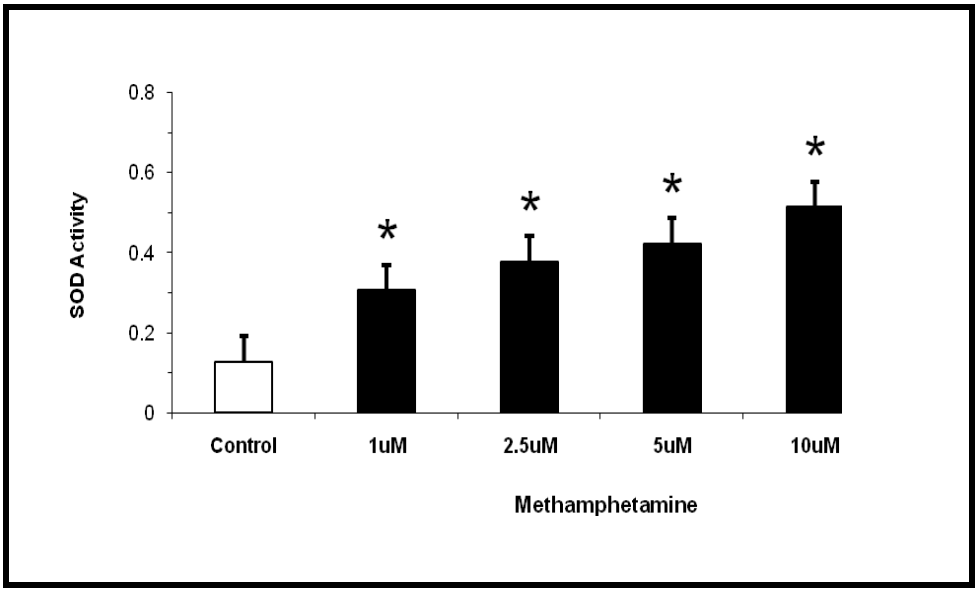

Figure 4.13. Effect of methamphetamine on mitochondrial SOD (*in vitro*): Methamphetamine (1, 2.5, 5 & 10 µM) significantly and dose dependently increased SOD activity (n = 6, $p^*<0.05$) as compared to controls.

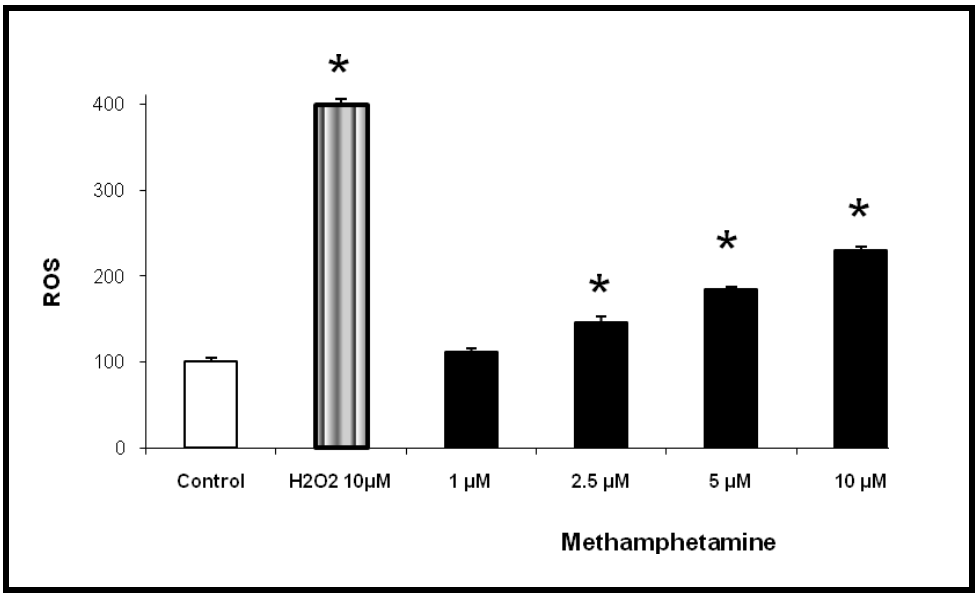

Figure 4.14. Effect of methamphetamine on mitochondrial ROS (*in vitro*): Methamphetamine (2.5, 5 & 10 µM) caused significant generation of reactive oxygen species (n = 6, $p^*<0.001$) as compared to controls. H_2O_2 10µM served as a positive control.

5. CHAPTER 2: NEUROPROTECTION OF RAMELTEON AGAINST NEUROTOXIC EFFECTS OF METHAMPHETAMINE

5.1 Overview

In this study we examined the neuroprotective effect of ramelteon against methamphetamine toxicity. Ramelteon is a novel melatonin receptor agonist that is used to treat insomnia. Melatonin itself is known to have neuroprotective effects such as antioxidant and antiapoptotic properties. If ramelteon possesses the same properties, it may reduce oxidative damage seen in neurodegenerative disorders like Parkinson's disease. Discovery of a neuroprotective pharmacological agent could lead to proactive strategies to prevent neurological damage. We examined the effect of ramelteon and methamphetamine on reactive oxygen species generation, superoxide dismutase, monoamine oxidaseactivity and complex I & IV activities. Behavioral analysis evaluated the effect on movement. Neurotransmitter levels were evaluated using HPLC-ECD. Results showed that ramelteon alone did not cause neurotoxicity to the cells. It showed no effect on behavior, biochemical or neurochemical parameters. Methamphetamine caused significant dopamine depletion in the striatum and also caused significant generation of reactive oxygen species and decreased complex I activity in the mitochondria. Behavioral analysis showed that methamphetamine had a significant effect on movement. Ramelteon did not block the neurotoxic effects of methamphetamine.

5.2 Introduction

Ramelteon is a novel melatonin (MT) receptor agonist that binds to MT1 and MT2 receptors selectively over MT3 receptors. Ramelteon is approved by the FDA for long-term use in the treatment of insomnia and is available commercially as Rozerem by Takeda Pharmaceuticals North America. Other drugs used in the treatment of insomnia are GABA modulators and have been shown to produce dependence, withdrawal and rebound insomnia. Ramelteon, however, has not been shown to produce dependence and has shown no potential for abuse. In addition, it does not appear to cause learning, memory or motor function impairment. It is currently the only non-scheduled prescription drug for the treatment of insomnia available in the United States (Kato et al., 2005).

Ramelteon possess structural similarity with melatonin and hence shares lot of similar physiological effects. Melatonin has been established in research as a direct scavenger of reactive oxygen species (ROS), like OH, $O2^-$, and NO (Poeggeler et al., 1994). It also has been found to be effective in protecting against brain injury caused by ROS release in experimental hypoxic brain damage in newborn rats (Tutunculer et al., 2005). Melatonin's antioxidant activity may reduce damage caused Parkinson's disease (Oaknin-Bendahan et al., 1995). If ramelteon possesses the same free radical scavenging properties as melatonin (Reiter et al., 2001) it could prove to be neuroprotective.

Oxidative stress has been strongly implicated in the pathogenesis of Parkinson’s disease. Brains from patients with Parkinson’s disease have shown elevated markers of oxidative damage, indicating that oxidative stress may play a key role in the disease (Hensley et al., 1998; Sherer et al., 2002). Oxidative stress involves the excessive

formation of reactive oxygen species, like the hydroxyl radicals (OH., OH-) and superoxide (O_2.), among others, which are formed when molecular oxygen is reduced non-enzymatically (Fahn and Cohen, 1992; Jenner et al., 1992). Oxidative stress occurs when free radicals are in excess of any antioxidant defense. There is a tendency for free radicals to be more easily formed and less easily scavenged in the brain of Parkinson's disease patients (Thiruchelvam et al., 2005). Free radicals are capable of reacting with DNA, lipids, and proteins to alter their structure and function (Turrens and Boveris, 1980; Hasegawa et al., 1990). Melatonin's antioxidant activity may reduce neuronal damage caused in Parkinson's disease (Oaknin-Bendahan et al., 1995).

One of the proposed mechanisms of this toxicity in Parkinson's disease is oxidative stress. Oxidative stress causes an inhibition of mitochondrial ATP production, a major determinant of lethal cell injury. The damage to the striatal terminals and the nigral cell bodies observed in methamphetamine users is also indicative of oxidative stress, the same mechanisms indicated in Parkinson's disease (Imberti et al., 1993; Jones and Vale, 2000). Methamphetamine is a highly neurotoxic substance capable of causing damage to dopaminergic neurons. Its mechanism of toxicity is thought to include generation of reactive radicals which are capable of causing mitochondrial dysfunction and reduced energy metabolism and eventual cell death (Riddle et al., 2006; Fibiger and Mogeer, 1971; Imam et al., 2001; Pubill et al., 2005).

In Parkinson's disease, most of the neurons located in the substantia nigra that produce dopamine undergo neurodegeneration, causing a severe shortage of dopamine in the striatum (Caine and Langston, 1983). Thus, one affected with Parkinson's disease

would be expected to exhibit some or all of the following motor deficits: slowness in initiation and execution of voluntary movements, increased muscle tone, increased resistance to movement, shuffling of feet, stooped posture and equilibrium and righting reflex (Sethy et al., 1997). In many cases of Parkinson's disease, complete immobility eventually occurs despite treatment (Marsden, 1994). Parkinson's disease is a slow-onset disease and the prevalence of Parkinson's disease increases with age. There are estimates of up to 800 affected per 100,000 individuals age 65 or older (Savitt, 2006). The current treatment strategy for Parkinson's disease remains primarily symptomatic, with no proven neuroprotective or neurorestorative treatments (Dawson and Dawson, 2003).With this study we examined the neuroprotective effect of ramelteon against methamphetamine toxicity, with implications for Parkinson's disease. Discovery of a neuroprotective pharmacological agent could lead to proactive strategies to counteract the neurological damage caused by the disease.

5.3 Materials and Methods

5.3.1 Animals

Male C57/Bl6 mice (4-6 weeks old) weighing 20-30 g were purchased from Charles Rivers. They were housed in a temperature controlled room with a 12h day and night cycle with free access to food and water. They were housed for 2-4 days prior to experiments. All the experimental procedures were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Auburn University.

5.3.2 Drug administration

C57/Bl6 mice were separated into 5 groups (control, methamphetamine only, high dose ramelteon only, ramelteon high dose + methamphetamine and ramelteon low dose + methamphetamine). The groups were given i.p. injections once daily for 1 week with a mixture of polyethelyne glycol (PEG) and sterile water (control & methamphetamine groups), 30mg/kg ramelteon, dissolved in PEG and diluted to appropriate concentration with sterile water (ramelteon high dose), 30mg/kg ramelteon, dissolved in PEG and diluted to appropriate concentration with sterile water (ramelteon high dose + methamphetamine group), and 3mg/kg ramelteon, dissolved in PEG and diluted to appropriate concentration with sterile water (ramelteon low dose + methamphetamine group). On day 7 the methamphetamine, ramelteon high dose + methamphetamine and ramelteon low dose + methamphetamine groups were injected with methamphetamine (10mg/kg i.p., twice, 2hr apart). The animals were sacrificed 5 days after the last injection.

5.3.3 Behavioral Studies

Following methamphetamine administration, two examiners who were trained in evaluating different animal behaviors continuously observed mice. Tremor, akinesia, catalepsy, and straub tails were monitored during this period following standard scoring procedures as described below. Animals were subjected to swim test on the 4^{th} day after methamphetamine.

Straub tail: Straub tail was defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as Straub tail phenomenon was detected or not detected (Mohanakumar and Sood, 1986).

Akinesia: Akinesia usually refers to the impaired ability to initiate movements. It was measured by noting the latency in second(s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180s (Weihmuller et al., 1988; Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term implies the inability of an animal to correct an externally imposed posture. Catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Weihmuller et al., 1988; Murlikrishnan and Mohanakumar, 1998).

Swim test: Swim test was carried out in water tubs (40 l x 25 w x 12 h, in cm). The depth of water was kept at 8 cm and the temperature was maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were: 0 = hind part sinks with head floating, 1 = occasional swimming using hind limbs while floating on one side, 2 = occasional floating/swimming only, 3 = continuous swimming (Donnan et al., 1987).

5.3.4 Measurement of Neurotransmitters

To determine dopamine depletion in the striatum, methamphetamine injected animals were sacrificed on the 5th day and the striata was dissected out and analyzed for DA content employing HPLC-electrochemistry. Dopamine and its metabolites were separated in high pressure liquid chromatography. An electrochemical detector was used to quantify dopamine and its metabolites. The C18 octadecyl silica column (8 cm X 4.6 mm i.d.) was used for the separation of the monoamines. Brain tissue was sonicated in 0.4 N $HClO_4$. These samples were then centrifuged and the 20 µl of supernatant used was injected in HPLC. The results were expressed as picomoles per milligram of tissue (Uthayathas et al., 2007).

5.3.5 Tissue Preparation for *In vivo* biochemical assays

Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi 2001).

5.3.6 Protein estimation

Protein was assayed using the coomassie plus protein assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

5.3.7 Mitochondrial Complex I Activity

Mitochondrial complex-I activity (NADH dehydrogenase activity) is based on the NADH oxidation. Oxidation of NADH by the NADH-dehydrogenase enzyme present in the tissue homogenate was measured spectrophotometrically at 340 nm. The mitochondrial P_2 suspension was added to the reaction mixture containing NADH, coenzyme Q_0 and phosphate buffered saline, to analyze its effect on NADH oxidation by monitoring the decrease in absorbance at 340 nm. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The change in absorbance indicates the amount of NADH oxidized. The mitochondrial complex-I activity is expressed as the amount of NADH oxidized/min/mg protein (Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

5.3.8 Mitochondrial Complex IV Activity

Complex IV activity was based on the cytochrome-C oxidation. The mitochondrial P_2 suspension was added to the reaction mixture containing cytochrome-C and phosphate buffered saline. The oxidation was measured spectrophotometrically. The absorbance was measured at 550nm for 2 min and the enzyme activity was expressed as cytochrome-C oxidized/mg protein (Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

5.3.9 Assay of MAO activity

Total MAO activity was based on the amount of 4-hydroxyquinoline formed by the oxidation of kynuramine (Morinan and Garratt, 1985). 4-hydroxyquinoline was

measured fluorimetrically and the enzyme activity was expressed as 4-hyroxyquinoline formed/hour/mg protein (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001).

5.3.10 Assay of ROS production

Conversion of nonfluorescent chloromethyl-DCF-DA (2′,7′- dichlorofluorescin diacetate) to fluorescent DCF was used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. The generation of ROS was measured and reported as relative fluorescence intensity (Dhanasekaran et al., 2008).

5.3.11 Statistical Analysis

Results are expressed as the means±SEM. The statistical significance was evaluated by the one-way analysis of variance (ANOVA) using SigmaStat version 3. Values of *p* less than or equal to 0.05 were considered significant.

5.4 Results

5.4.1 Behavioral Analysis

Methamphetamine (10mg/kg) caused a significant motor impairment in the C57/BL6 mice. Intraperitoneal administration of ramelteon (3 & 30mg/kg) did not block methamphetamine induced behavioral abnormalities including catalepsy (figure 5.1),

akinesia (figure 5.2), reduced swim score (figure 5.3), straub tails or tremor (table 5.1) (n=6). Ramelteon (30mg/kg) alone did not cause any behavioral abnormalities (n=6).

5.4.2 Effect of ramelteon and methamphetamine on neurotransmitters

Administration of methamphetamine (10mg/kg) caused a significant reduction in the levels of dopamine in the striatum. Ramelteon (3 & 30mg/kg) did not block methamphetamine induced dopamine depletion (n=6; figure 5.4). Methamphetamine and ramelteon affected levels of other striatal neurotransmitters (NE, 5HT, 5HIAA) and dopamine metabolites (DOPAC, HVA). The effect of ramelteon and methamphetamine on these neurotransmitters is shown in table 5.2. Ramelteon (30mg/kg) alone did not cause a significant reduction in the amount of striatal dopamine (n=6).

5.4.3 *In vivo* biochemical analysis

Intraperitoneal administration of methamphetamine (10mg/kg) in mice significantly decreased complex I activity. Ramelteon did not block methamphetamine induced decrease in complex I activity (n = 6; figure 5.5). Neither ramelteon nor methamphetamine had a significant effect on complex IV activity (figure 5.6). Ramelteon and methamphetamine also had no significant effect on the total mitochondrial monoamine oxidase (MAO) activity (figure 5.7). Methamphetamine caused significant generation of reactive oxygen species and ramelteon also did not provide neuroprotection from this effect (n = 6; figure 5.8). Ramelteon (30mg/kg) alone did not cause any

significant generation of reactive oxygen species nor any significant decrease in mitochondrial complex I (n=6).

5.5 Discussion

Our previous research showed that methamphetamine's mechanism of toxicity involves generation of reactive radicals leading to oxidative stress, mitochondrial disfunction, and damage to dopaminergic neurons. A combination of oxidative stress and mitochondrial dysfunction creates toxicity of dopaminergic neurons in the nigrostriatal tract. This leads to long lasting dopamine depletion (Stokes et al., 1999; Zhu et al., 2006).

Oxidative stress causes an inhibition of mitochondrial ATP production, a major determinant of lethal cell injury. Mitochondrial DNA is sensitive to the toxicity caused by reactive oxygen species because mitochondria have only a limited arsenal of DNA repair processes. Therefore, mitochondrial DNA mutations would cause defects in the respiratory chain function leading to severe cellular damage. Free radicals can interact with sugars, proteins and lipids creating a number of modifications which could then lead to mitochondrial dysfunction, apoptosis and even necrosis. The damage to the striatal terminals and the nigral cell bodies observed in methamphetamine users is indicative of all of these toxic mechanisms, the same mechanisms indicated in Parkinson's disease (Imberti et al., 1993; Jones and Vale, 2000). There are studies lending evidence to support the theory that oxidative stress and mitochondrial dysfunction play a key role in neurodegeneration. Research on antioxidants has shown that they could possibly be

neuroprotective against the neurodegeneration seen in Parkinson's disease as well as other neurodegenerative diseases (Shults, 2005).

It is not well studied whether ramelteon has other functional similarities with melatonin, besides induction of sleep. However, besides its primary function of regulating the sleep cycle, melatonin may exert a powerful antioxidant activity (Tan et al., 2007). Melatonin is an antioxidant that can easily cross cell membranes and the blood brain barrier (Hardeland, 2005). Melatonin is a direct scavenger of free radicals (Poeggeler et al., 1994). Unlike other antioxidants, melatonin does not undergo redox cycling, the ability of a molecule to undergo reduction and oxidation repeatedly. Redox cycling may allow other antioxidants (such as vitamin C) to regain their antioxidant properties. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan et al., 2000).

In animal models, melatonin has been demonstrated to prevent the damage to DNA by some carcinogens, stopping the mechanism by which they cause cancer (Karbownik et al., 2001). It also has been found to be effective in protecting against brain injury caused by reactive oxygen species release in experimental hypoxic brain damage in newborn rats (Tutunculer et al., 2005). Melatonin's antioxidant activity may reduce neuronal damage caused in Parkinson's disease (Oaknin-Bendahan et al., 1995). This raised the interesting question of whether ramelteon might also have such beneficial effects. Free radical scavenging properties, such as those seen with melatonin (Reiter et al., 2001), could add neuroprotective abilities to the drug.

Ramelteon alone did not induce any behavioral changes. Ramelteon (30mg/kg) did not induce tremor, akinesia, catalepsy or any other abnormality with regard to the movement or behavior. Ramelteon also had no effect on the body weight. With regard to the biochemical activities, ramelteon had no effect of the reactive oxygen species. Unlike, many other drugs that affect the mitochondrial respiration, ramelteon had no effect on the complex-I or complex-IV activity. Monoamine oxidase is an important target in the treatment of Parkinson's disease, however, ramelteon had no effect on this enzyme activity. Ramelteon also did not affect the dopamine content or dopaminergic turnover in the striatum. With regard to the effect of ramelteon against methamphetamine induced toxicity, results in this study show a significant amount of dopamine depletion due to administration of methamphetamine, as well of the creation of a striatal environment ripe for oxidative damage and mitochondrial dysfunction. However, the behavioral, HPLC-ECD, and *in vivo* biochemical results showed that ramelteon did not provide significant neuroprotection from the insults of oxidative stress. Thus, ramelteon does not appear to exibit the antioxidant properties observed in melatonin. However, ramelteon alone did not cause any neurotoxicity to the cells.

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5.7 Figures

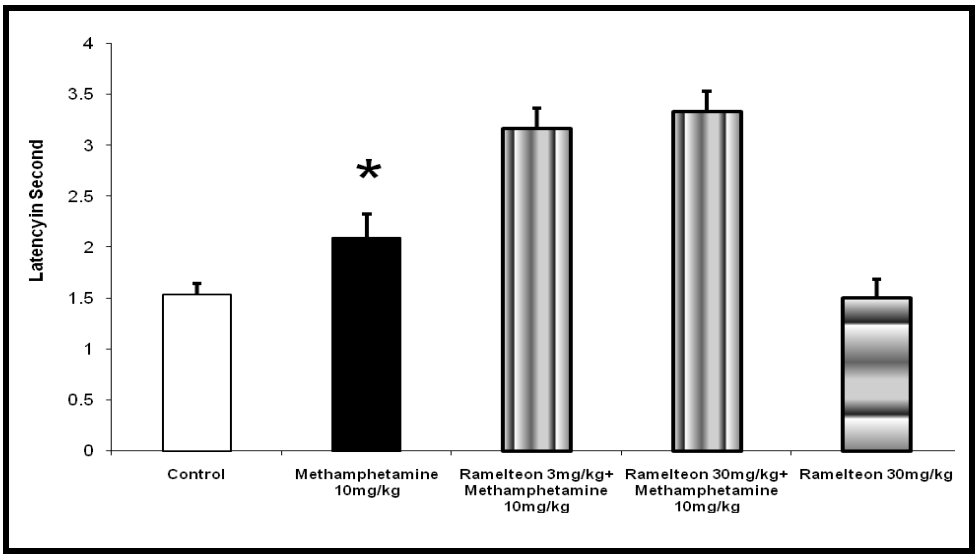

Figure 5.1. Effect of Ramelteon on catalepsy: Ramelteon (3 & 30mg/kg) did not block methamphetamine induced catalepsy (n=6). *significant (n=6, p*<.05) as compared to control animals.

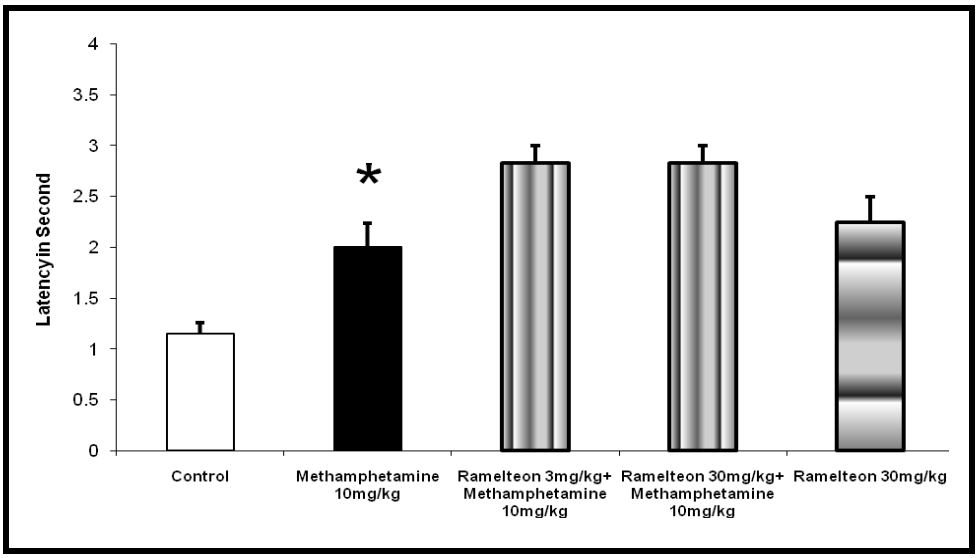

Figure 5.2. Effect of Ramelteon on akinesia: Ramelteon (3 & 30mg/kg) did not block methamphetamine induced akinesia (n=6). *significant (n=6, p*<.05) as compared to control animals.

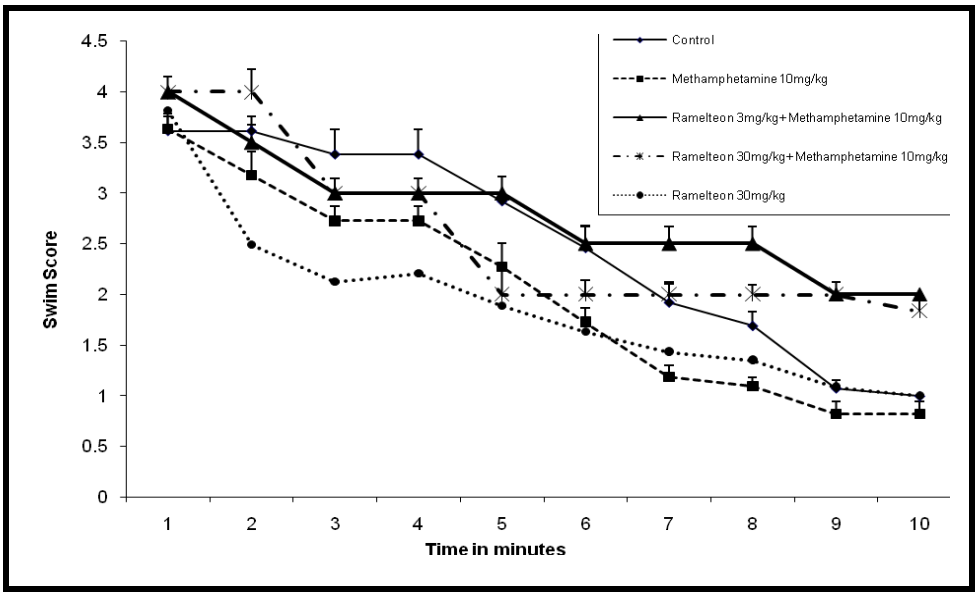

Figure 5.3. Effect of Ramelteon on swim score: Ramelteon (3 & 30mg/kg) did not block methamphetamine induced decrease in swim score (n=6). Scored by immobility time.

	average weight	straub tail	tremor
Control	24.3±0.6	0	0
Methamphetamine 10mg/kg	24.2±0.5	n=6	4.0±0.1
Ramelteon 3mg/kg+ Methamphetamine 10mg/kg	24.3±1.3	n=6	4.0±0.1
Ramelteon 30mg/kg+ Methamphetamine 10mg/kg	25.3±0.4	n=6	4.0±0.1
Ramelteon 30mg/kg	25.5±0.5	0	0

Table 5.1. Effect of Ramelteon on straub tail and tremor: Ramelteon alone did not induce tremor or straub tail. Ramelteon (3 & 30mg/kg) did not block/reduce methamphetamine induced straub tail and tremor (n=6).

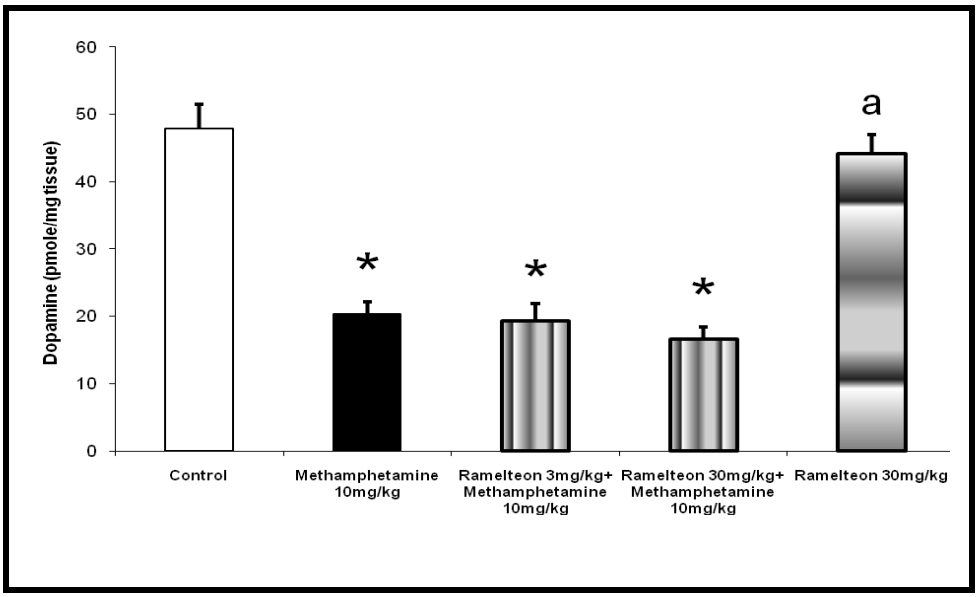

Figure 5.4. Effect of Ramelteon on striatal dopamine content: Ramelteon (3 & 30mg/kg) did not block methamphetamine mediated dopamine depletion (n=6). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

	Control	Methamphetamine 10mg/kg	Ramelteon 3mg/kg+ Methamphetamine 10mg/kg	Ramelteon 30mg/kg+ Methamphetamine 10mg/kg	Ramelteon 30mg/kg
DOPAC	49.12±11.6	19.45±3.8	47.55±7.6 [a]	40.20±7.0 [a]	84.46±8.0 * [a]
HVA	24.73±1.7	18.10±1.7	18.17±1.5 *	17.11±2.2 *	27.09±3.1 * [a]
5-HT	2.10±1.6	2.69±0.2	2.66±0.2	2.53±0.1	3.48±0.3
5-HIAA	11.37±1.7	9.86±0.5	47.55±7.6 * [a]	40.20±7.0 * [a]	84.47±8.0 * [a]
NE	15.21±0.9	14.49±0.9	12.71±1.8	14.93±1.0	17.94±0.8

Table 5.2. Effect of Ramelteon on striatal neurotransmitter and metabolite levels (expresed as average pmole/mg tissue ± SEM). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

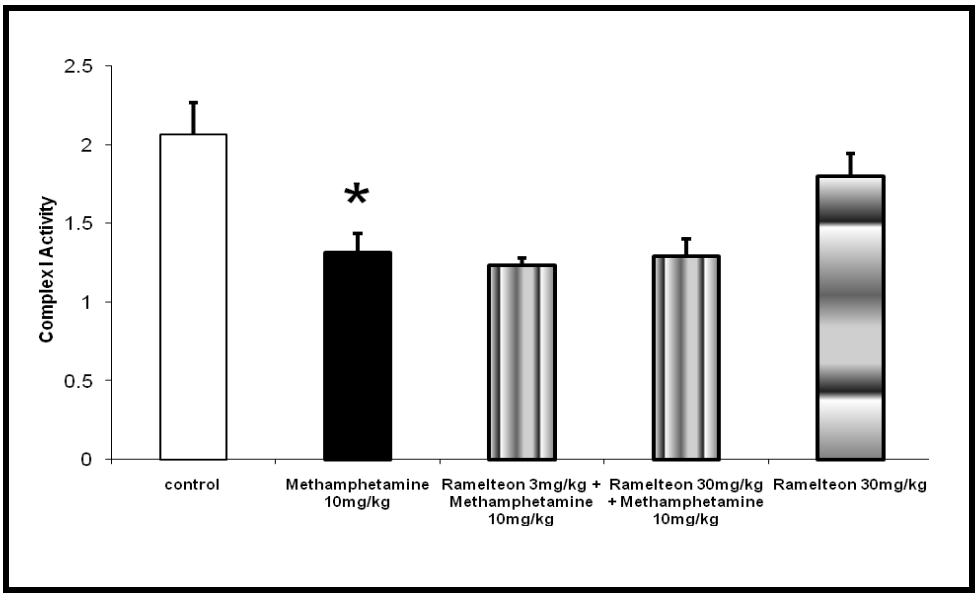

Figure 5.5. Effect of Ramelteon on mitochondrial Complex I activity (*in vivo*): Ramelteon (3 & 30mg/kg) did not block methamphetamine mediated decrease in complex I activity (n = 6). Activity expressed as µmol/min/mg protein. *significant (n=6, *p**<.05) as compared to control animals.

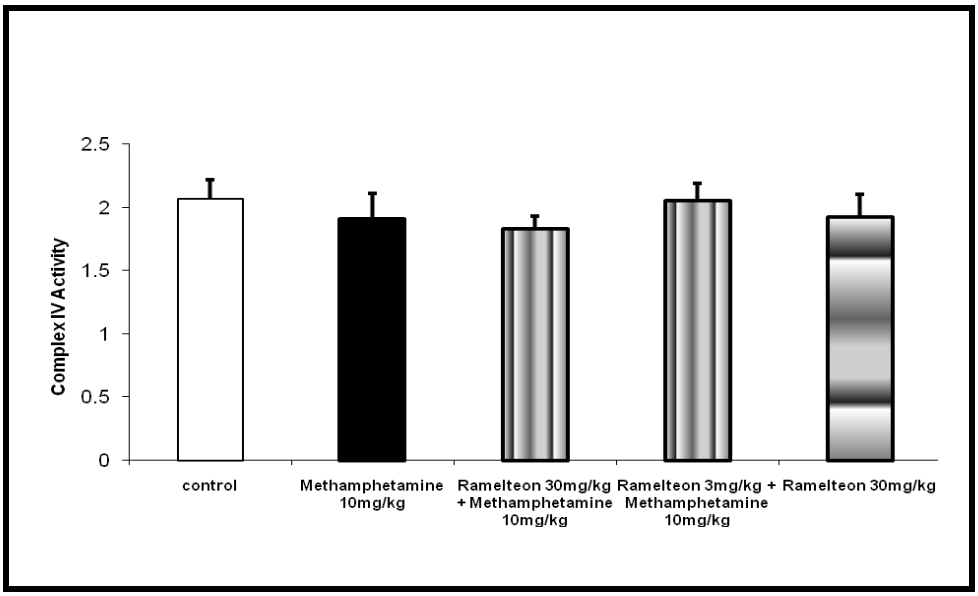

Figure 5.6. Effect of Ramelteon on mitochondrial Complex IV activity (*in vivo*): Ramelteon (3 & 30mg/kg) caused no significant change in complex IV activity (n=6). Activity expressed as µmol/min/mg protein.

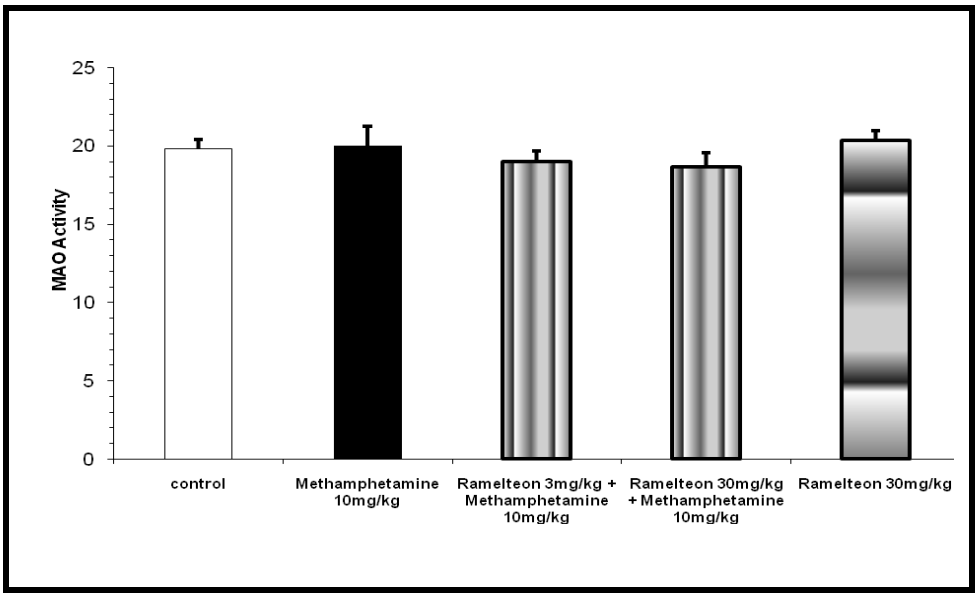

Figure 5.7. Effect of Ramelteon on mitochondrial MAO activity (*in vivo*): Ramelteon (3 & 30mg/kg) caused no significant change in MAO activity (n=6). Activity expressed as 4-OHQ/mg protein.

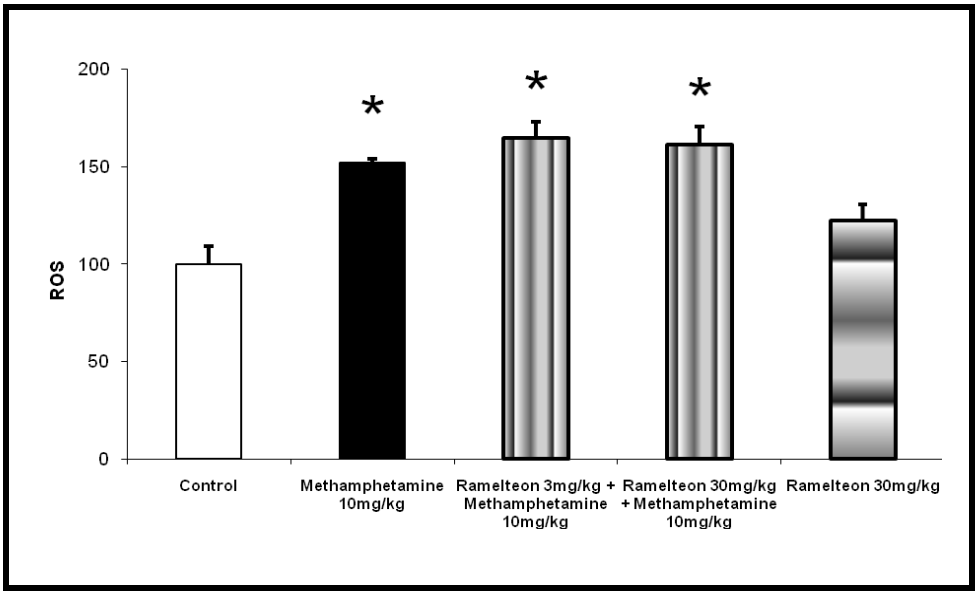

Figure 5.8. Effect of Ramelteon on mitochondrial ROS (*in vivo*): Ramelteon (3 & 30mg/kg) did not block methamphetamine mediated generation of ROS (n = 6). *significant (n=6, *p**<.05) as compared to control animals.

6. CHAPTER 3: NEUROPROTECTION OF AMANTADINE AGAINST NEUROTOXIC EFFECTS OF METHAMPHETAMINE

6.1 Overview

Amantadine is a drug used to help reduce the symptoms of Parkinson's disease. It releases dopamine from the nerve terminals and it appears to be a weak NMDA receptor antagonist. Excitotoxicity is involved in the pathogenesis of Parkinson's disease and NMDA receptors are thought to be one of the most harmful factors in excitotoxicity. Research has shown that excitotoxic effects can be blocked by NMDA receptor antagonists. Thus, amantadine could prove to be neuroprotective against the type of toxicity often seen in Parkinson's dsease. We examined the effect of amantadine and methamphetamine on reactive oxygen species generation, monoamine oxidase activity and complex I & IV activities. Behavioral analysis evaluated the effect on movement. Neurotransmitter levels were evaluated using high pressure liquid chromatography (HPLC)-electrochemical detection. Results showed that methamphetamine caused significant dopamine depletion in the striatum and also caused significant generation of reactive oxygen species and decreased complex I activity in the mitochondria. Amantadine (1mg/kg) significantly blocked methamphetamine mediated dopamine depletion in the striatum and significantly blocked generation of reactive oxygen species.

6.2 Introduction

Amantadine is a drug commonly used in the treatment of Parkinson's disease. Amantadine works, in part, by releasing dopamine from nigrostriatal neurons. However, it is amantadine's effectiveness as an NMDA receptor antagonist that is of interest to this study (Blanpied et al., 2005). Since NMDA receptors are one of the most harmful factors in excitotoxicity, antagonists of the receptors have held much promise for the treatment of conditions that involve excitotoxicity, including traumatic brain injury, stroke, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Maas, 2001).

Excitotoxicity is a process whereby a prolonged or excessive activation of excitatory amino acid receptors leads to an irreversible process of cell death. The high level of oxidative stress in Parkinson's disease sets off a chain of events leading to deficits in mitochondrial function, disruption of the electron transport chain and initiation of excitotoxicity. This leads to cell damage and death (Doble 1999). Excitatory interactions are important for normal striatal function. Glutamate is the most abundant excitatory neurotransmitter; however, it becomes cytotoxic if released in excess, causing a calcium influx into the cell (Schwarcz and Meldrum, 1985). Methamphetamine causes a release of excess glutamate into the striatum via a direct pathway. Also, mitochondrial dysfunction, as seen in both Parkinson's disease and methamphetamine toxicity, can lead to excitotoxicity, as a reduction in ATP would result in a loss of the ATP-dependent Mg-blockade of NMDA receptors, causing physiological concentrations of glutamate to mediate a calcium influx into the cell (Beal et al., 1993). The resulting rise in calcium

increases nitric oxide synthase (NOS). This increases the production of nitric oxide (.NO) which reacts with the superoxide radical to form peroxynitrite and the hydroxyl radical, both powerful oxidizing agents (Dawson et al., 1991). Thus, the end product of excitotoxicity is the formation of reactive oxygen species as well as reduced intracellular glutathione synthesis, two of the indicators that are often see in Parkinson's disease (Murphy et al., 1989).

Dopaminergic neurons in the substantia nigra are rich in glutamate receptors, and thus highly susceptible to excitotoxicity. Studies of excitotoxicity have implicated the NMDA receptor subtype as being the principal vehicle of excitotoxic damage, and research has shown that excitotoxic effects of glutamic acid can be blocked by NMDA receptor antagonists (Choi et al., 1988). In experiments using MPTP, NMDA receptor antagonists protected rats from the toxic effects of MPP1 (Turski et al., 1991). The NMDA receptor antagonist memantine is proposed to counteract cellular damage due to pathological activation of NMDA receptors by glutamate (Doraiswamy, 2002).

Our earlier studies show that administration of methamphetamine to mice produces a loss of dopaminergic neurons and a syndrome that behaviorally, biochemically, and neurochemically resembles Parkinson's disease. In this study, we use this model to study whether amantadine exhibits neuroprotection against the toxicity caused by methamphetamine. If amantadine proved to be a neuroprotectant substance, preventing the neuronal cell damage from excitotoxicity (Gualtieri et al., 1989), it could slow the progression of Parkinson's disease.

6.3 Materials and Methods

6.3.1 Animals

Male C57/Bl6 mice (4-6 weeks old) weighing 20-30 g were purchased from Charles Rivers. They were housed in a temperature controlled room with a 12h day and night cycle with free access to food and water. They were housed for 2-4 days prior to experiments. All the experimental procedures were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Auburn University.

6.3.2 Drug administration

C57/Bl6 mice were separated into 5 groups (control, methamphetamine only, high dose amantadine only, amantadine high dose + methamphetamine and amantadine low dose + methamphetamine). The groups were given i.p. injections once daily for one week with sterile water (control & methamphetamine groups), 10mg/kg amantadine, dissolved in sterile water (amantadine high dose), 10mg/kg amantadine, dissolved in sterile water (amantadine high dose + methamphetamine group), and 1mg/kg amantadine, dissolved in sterile water (amantadine low dose + methamphetamine group). On day 7 the methamphetamine, amantadine high dose + methamphetamine and amantadine low dose + methamphetamine groups were injected with methamphetamine (10mg/kg i.p., twice, 2hr apart). The animals were sacrificed 5 days after the last injection.

6.3.3 Behavioral Studies

Following methamphetamine administration, two examiners who were trained in evaluating different animal behaviors continuously observed mice. Tremor, akinesia, catalepsy, and straub tails were monitored during this period following standard scoring procedures as described below. Animals were subjected to swim test on the 4^{th} day after methamphetamine.

Straub tail: Straub tail was defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as Straub tail phenomenon was detected or not detected (Mohanakumar and Sood, 1986).

Akinesia: Akinesia usually refers to the impaired ability to initiate movements. It was measured by noting the latency in second(s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180s (Weihmuller et al., 1988; Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term implies the inability of an animal to correct an externally imposed posture. Catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Weihmuller et al., 1988; Murlikrishnan and Mohanakumar, 1998).

Swim test: Swim test was carried out in water tubs (40 l x 25 w x 12 h, in cm). The depth of water was kept at 8 cm and the temperature was maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were: 0 = hind part sinks with head floating, 1 = occasional swimming using hind

limbs while floating on one side, 2 = occasional floating/swimming only, 3 = continuous swimming (Donnan et al., 1987).

6.3.4 Measurement of Neurotransmitters

To determine dopamine depletion in the striatum, methamphetamine injected animals were sacrificed on the 5th day and the striata was dissected out and analyzed for DA content employing HPLC-electrochemistry. Dopamine and its metabolites were separated in high pressure liquid chromatography. An electrochemical detector was used to quantify dopamine and its metabolites. The C18 octadecyl silica column (8 cm X 4.6 mm i.d.) was used for the separation of the monoamines. Brain tissue was sonicated in 0.4 N $HClO_4$. These samples were then centrifuged and the 20 µl of supernatant used was injected in HPLC. The results were expressed as picomoles per milligram of tissue (Uthayathas et al., 2007).

6.3.5 Tissue Preparation for *In vivo* biochemical assays

Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi 2001).

6.3.6 Protein estimation

Protein was assayed using the coomassie plus protein assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

6.3.7 Mitochondrial Complex I Activity

Mitochondrial complex-I activity (NADH dehydrogenase activity) is based on the NADH oxidation. Oxidation of NADH by the NADH-dehydrogenase enzyme present in the tissue homogenate was measured spectrophotometrically at 340 nm. The mitochondrial P_2 suspension was added to the reaction mixture containing NADH, coenzyme Q_0 and phosphate buffered saline, to analyze its effect on NADH oxidation by monitoring the decrease in absorbance at 340 nm. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The change in absorbance indicates the amount of NADH oxidized. The mitochondrial complex-I activity is expressed as the amount of NADH oxidized/min/mg protein (Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

6.3.8 Mitochondrial Complex IV Activity

Complex IV activity was based on the cytochrome-C oxidation. The mitochondrial P_2 suspension was added to the reaction mixture containing cytochrome-C and phosphate buffered saline. The oxidation was measured spectrophotometrically. The

absorbance was measured at 550nm for 2 min and the enzyme activity was expressed as cytochrome-C oxidized/mg protein (Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

6.3.9 Assay of MAO activity

Total MAO activity was based on the amount of 4-hydroxyquinoline formed by the oxidation of kynuramine (Morinan and Garratt, 1985). 4-hydroxyquinoline was measured fluorimetrically and the enzyme activity was expressed as 4-hyroxyquinoline formed/hour/mg protein (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001).

6.3.10 Assay of ROS production

Conversion of nonfluorescent chloromethyl-DCF-DA (2′,7′- dichlorofluorescin diacetate) to fluorescent DCF was used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. The generation of ROS was measured and reported as relative fluorescence intensity (Dhanasekaran et al., 2008).

6.3.11 Statistical Analysis

Results are expressed as the means±SEM. The statistical significance was evaluated by the one-way analysis of variance (ANOVA) using SigmaStat version 3. Values of *p* less than or equal to 0.05 were considered significant.

6.4 Results

6.4.1 Behavioral Analysis

Methamphetamine (10mg/kg) caused a significant motor impairment in the C57/BL6 mice. Intraperitoneal administration of amantadine (1 & 10mg/kg) showed no significant neuroprotection against methamphetamine induced behavioral abnormalities including catalepsy (figure 6.1), akinesia (figure 6.2), reduced swim time (figure 6.3), straub tails or tremor (table 6.1) (n=6).

6.4.2 Effect of amantadine and methamphetamine on neurotransmitters

Administration of methamphetamine (10mg/kg) caused a significant reduction in the levels of dopamine in the striatum. Amantadine (1mg/kg) significantly blocked methamphetamine induced dopamine depletion (n=6, p=<0.05), amantadine (10mg/kg) did not significantly block methamphetamine induced dopamine depletion (n=6) as compared to control animals (figure 6.4). Methamphetamine and amantadine also affected levels of other striatal neurotransmitters (NE, 5HT, 5HIAA) and dopamine metabolites (DOPAC, HVA). The effect of amantadine and methamphetamine on these neurotransmitters is shown in table 6.2.

6.4.3 *In vivo* biochemical analysis

Intraperitoneal administration of methamphetamine (10mg/kg) in mice significantly decreased complex I activity. Amantadine did not block methamphetamine mediated decrease in complex I activity (n = 6; figure 6.5). Neither amantadine nor methamphetamine had a significant effect on complex IV activity (figure 6.6). Amantadine and methamphetamine also had no significant effect on the total mitochondrial monoamine oxidase (MAO) activity (figure 6.7). Methamphetamine caused significant generation of reactive oxygen species and amantadine (1mg/kg) did significantly block methamphetamine mediated generation of ROS (n = 6, p=<0.05). Amantadine (10mg/kg) did not block this effect (n = 6; figure 6.8)

6.5 Discussion

Methamphetamine displaces monoamines like dopamine from their vesicles. Once released, the dopamine can be oxidized to reactive oxygen species. The combination of oxidative stress and mitochondrial dysfunction creates toxicity of striatal nerve terminals (Stokes et al., 1999; Zhu et al., 2006).These reactive species can cause an inhibition of mitochondrial ATP production which can lead to excitotoxicity, as a reduction in ATP would result in a loss of the ATP-dependent Mg-blockade of NMDA receptors, causing physiological concentrations of glutamate to mediate a calcium influx into the cell (Beal et al., 1993). Overexposure of the neurons to glutamate is cytotoxic, and a major determinant of lethal cell injury (Reiter, 1998).

Glutamate is the most abundant excitatory neurotransmitter; however, it becomes cytotoxic if released in excess, causing a calcium influx into the cell (Schwarcz and Meldrum, 1985). Methamphetamine causes a release of excess glutamate into the striatum via a direct pathway. Also, because methamphetamine displaces the dopamine in the vesicles, large amounts of it are released into the synaptic cleft and cytosol. There is an established relationship between the oxidative process of dopamine itself and nigrostriatal degeneration. During the auto-oxidation, synthesis and metabolism of dopamine, hydrogen peroxide (H_2O_2) is produced as a byproduct by tyrosine hydroxylase (TSH) and monoamine oxidase (MAO) (Lotharius and O'Malley, 2000). The hydrogen peroxide, which can react nonenzymatically to form highly reactive hydroxyl radicals, can then take part in a positive feedback loop causing further progression of oxidative damage, reduced ATP production and excitotoxicity (Jenner, 1998).

The central dopaminergic system that plays such an important role in motor activity is comprised of a surprisingly small number of neurons. Because of this, it is especially vulnerable and even minor insults may lead to irreparable functional deficits (Fariello et al., 1988). Dopaminergic neurons in the substantia nigra are rich in glutamate receptors, and thus highly susceptible to excitotoxicity. This emphasizes the importance of excitotoxicity as a target for possible neuroprotection in Parkinson's disease. Our earlier studies show that administration of methamphetamine to mice produces a loss of dopaminergic neurons and a syndrome that behaviorally, biochemically, and neurochemically resembles Parkinson's disease. In this study, we use this model to study whether amantadine exhibits neuroprotection against the toxicity caused by

methamphetamine. If amantadine proved to be a neuroprotectant substance, preventing the neuronal cell damage from excitotoxicity (Gualtieri et al., 1989), it could slow the progression of Parkinson's disease.

In this study, administration of methamphetamine caused a significant amount of dopamine depletion and a striatal environment ripe for oxidative damage, mitochondrial dysfunction and excitotoxicity. The neurochemical data obtained using HPLC-ECD showed that amantadine (1mg/kg) showed significant neuroprotection against methamphetamine induced dopamine depletion (n=6, p=<0.05). The *in vivo* biochemical results showed that methamphetamine caused significant generation of reactive oxygen species and that amantadine (1mg/kg) showed significant neuroprotection against methamphetamine induced generation of ROS (n=6, p=<0.05). The end product of excitotoxicity is the formation of reactive oxygen species (Murphy et al., 1989). Therefore, the reduction in ROS generated might indicate a reduction in excitotoxicity. The failure of the high dose to provide neuroprotection is likely due to the dopamine releasing effect of amantadine. Because dopamine itself can be oxidized into ROS, the higher dose of amantadine could be exacerbating the oxidative stress by releasing too much dopamine from the vesicles.

Overall, these results indicate that amantadine is partially neuroprotective against neurotoxicity and dopamine depletion caused by methamphetamine administration. Despite advances in pharmacotherapy that have improved quality of life, the mortality rate among Parkinson's disease sufferers remains largely unchanged. There is need for a proactive treatment strategy that could provide neuroprotection from the disease.

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6.7 Figures

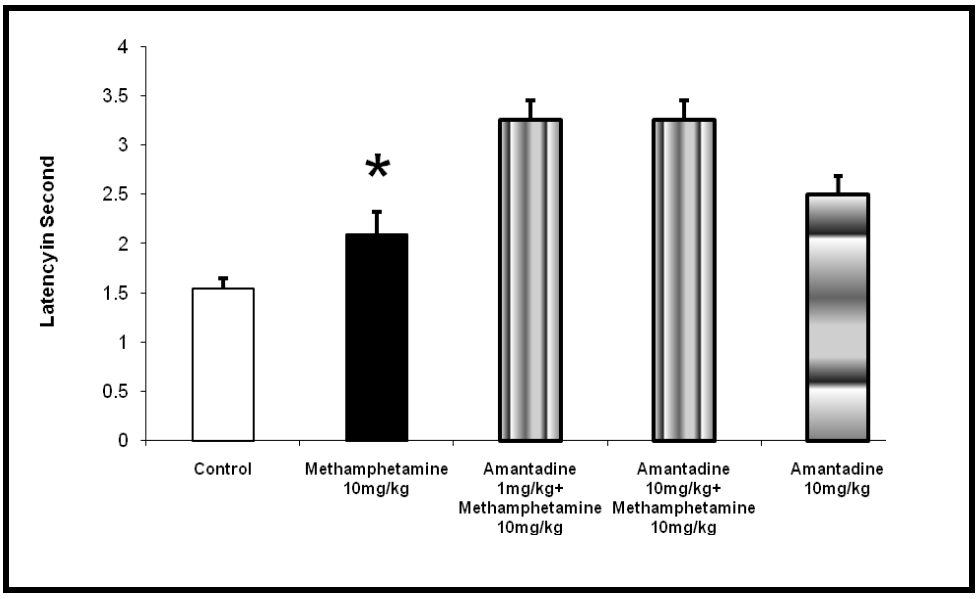

Figure 6.1. Effect of Amantadine on catalepsy: Amantadine (1 & 10mg/kg) did not block methamphetamine induced catalepsy (n=6). *significant (n=6, p*<.05) as compared to control animals.

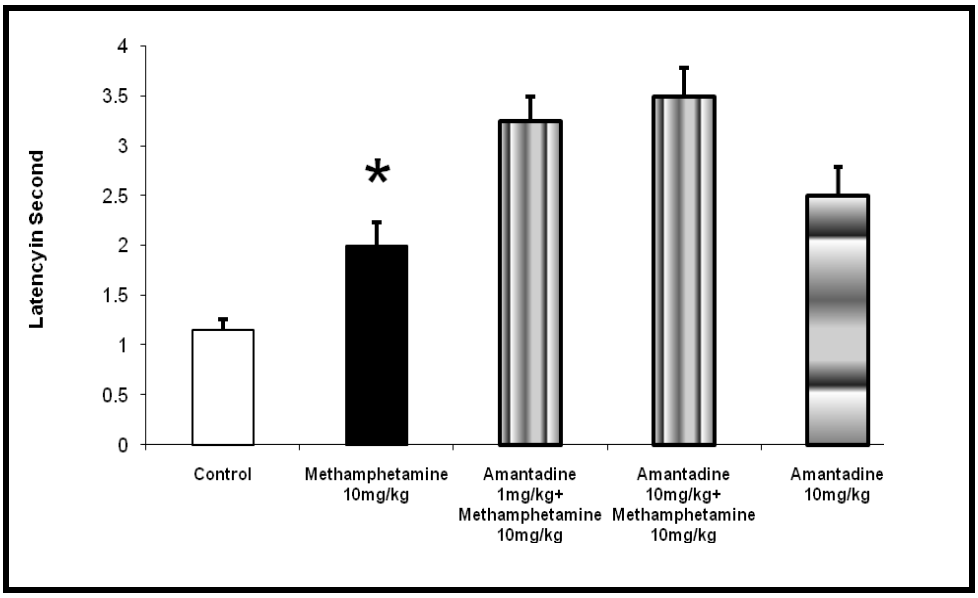

Figure 6.2. Effect of Amantadine on akinesia: Amantadine (1 & 10mg/kg) did not block methamphetamine induced akinesia (n=6). *significant (n=6, p*<.05) as compared to control animals.

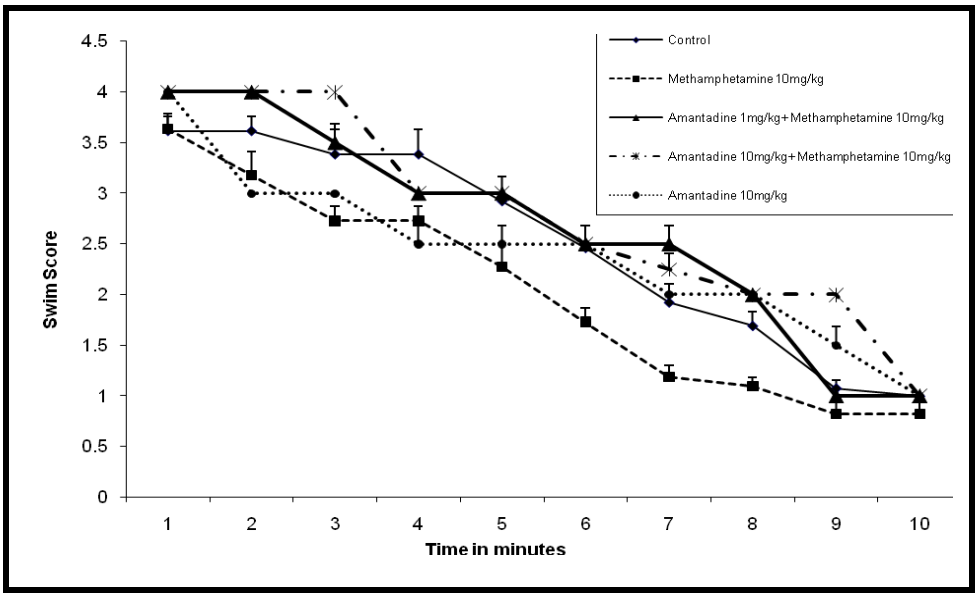

Figure 6.3. Effect of Amantadine on swim score: Amantadine (1 & 10mg/kg) did not block methamphetamine induced decrease in swim score (n=6). Scored by immobility time.

	average weight	straub tail	tremor
Control	24.3±0.6	0	0
Methamphetamine 10mg/kg	24.2±0.5	n=6	4.0±0.1
Amantadine 1mg/kg + Methamphetamine 10mg/kg	23±0.16	n=6	4.0±0.1
Amantadine 10mg/kg + Methamphetamine 10mg/kg	24.5±0.2	n=6	4.0±0.1
Amantadine 10mg/kg	22.2±0.5	0	0

Table 6.1. Effect of Amantadine on straub tail and tremor: Amantadine alone did not induce tremor or straub tail. Amantadine (1 & 10mg/kg) did not block/reduce methamphetamine induced straub tail and tremor (n=6).

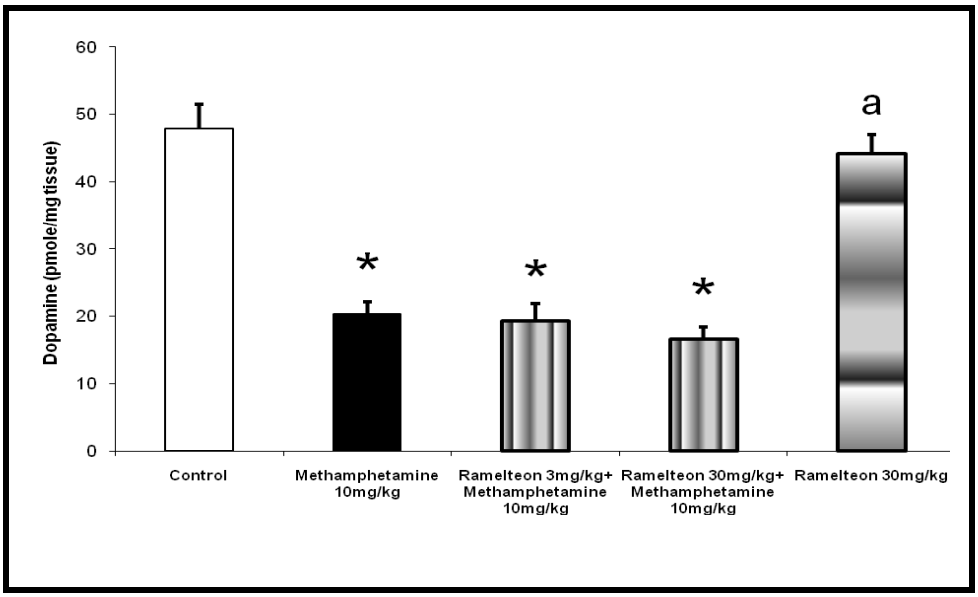

Figure 6.4. Effect of Amantadine on striatal dopamine content: Amantadine (1mg/kg) significantly blocked methamphetamine mediated dopamine depletion and amantadine (10mg/kg) did not significantly block methamphetamine mediated dopamine depletion (n=6). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^{a}<.05) as compared to methamphetamine animals.

	Control	Methamphetamine 10mg/kg	Amantadine 1mg/kg+ Methamphetamine 10mg/kg	Amantadine 10mg/kg+ Methamphetamine 10mg/kg	Amantadine 10mg/kg
DOPAC	49.12±11.6	19.45±3.8	27.45±3.5 * [a]	22.01±3.4 *	61.23±8.6 * [a]
HVA	24.73±1.7	18.10±1.7	16.35±0.7 *	16.16±0.7 *	20.67±1.3 *
5-HT	2.10±1.6	2.69±0.2	2.92±0.4	2.99±0.3	3.97±0.3 *
5-HIAA	11.37±1.7	9.86±0.5	6.60±0.5 *	7.39±0.4 *	25.11±0.6 *
NE	15.21±0.9	14.49±0.9	16.57±0.6	15.30±0.8	10.59±0.4 *

Table 6.2. Effect of Amantadine on striatal neurotransmitter and metabolite levels (expresed as average pmole/mg tissue ± SEM). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^{a}<.05) as compared to methamphetamine animals.

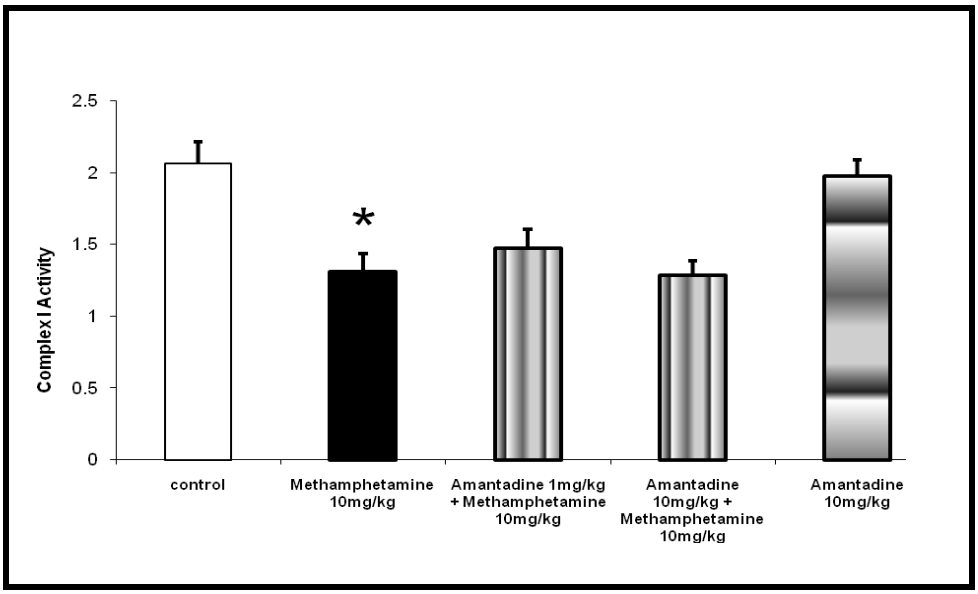

Figure 6.5. Effect of Amantadine on mitochondrial Complex I activity (*in vivo*): Amantadine (1 & 10mg/kg) did not block methamphetamine mediated decrease in Complex I activity (n = 6). Activity expressed as µmol/min/mg protein. *significant (n=6, p*<.05) as compared to control animals.

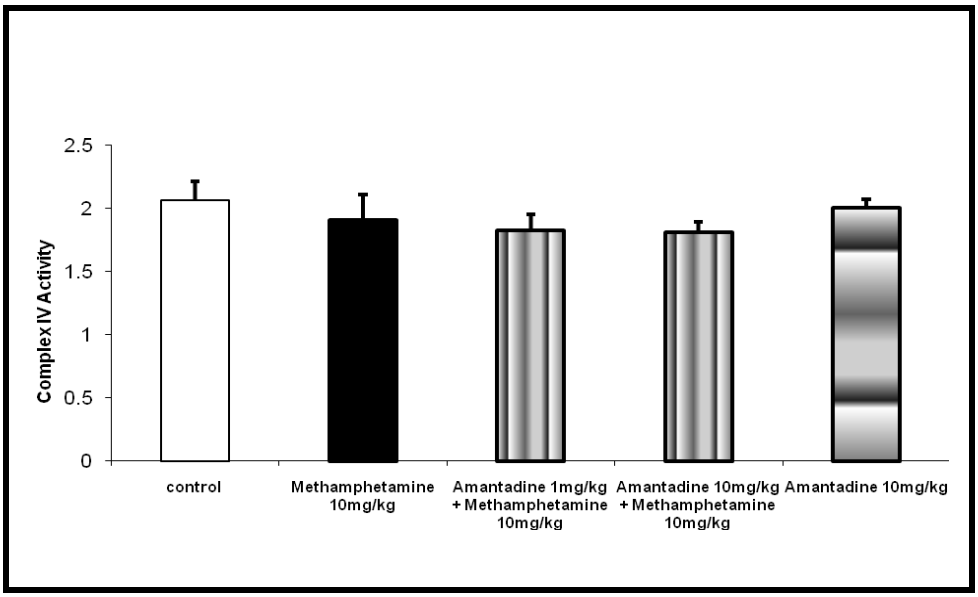

Figure 6.6. Effect of Amantadine on mitochondrial Complex IV activity (*in vivo*): Amantadine (1 & 10mg/kg) caused no significant change in complex IV activity (n=6). Activity expressed as µmol/min/mg protein.

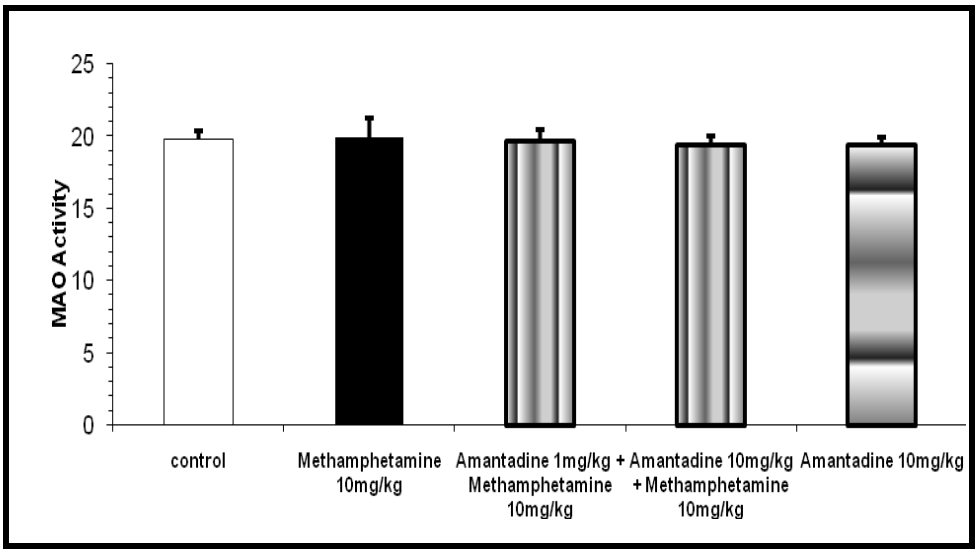

Figure 6.7. Effect of Amantadine on mitochondrial MAO activity (*in vivo*): Amantadine (1 & 10mg/kg) caused no significant change in MAO activity (n=6). Activity expressed as 4-OHQ/mg protein.

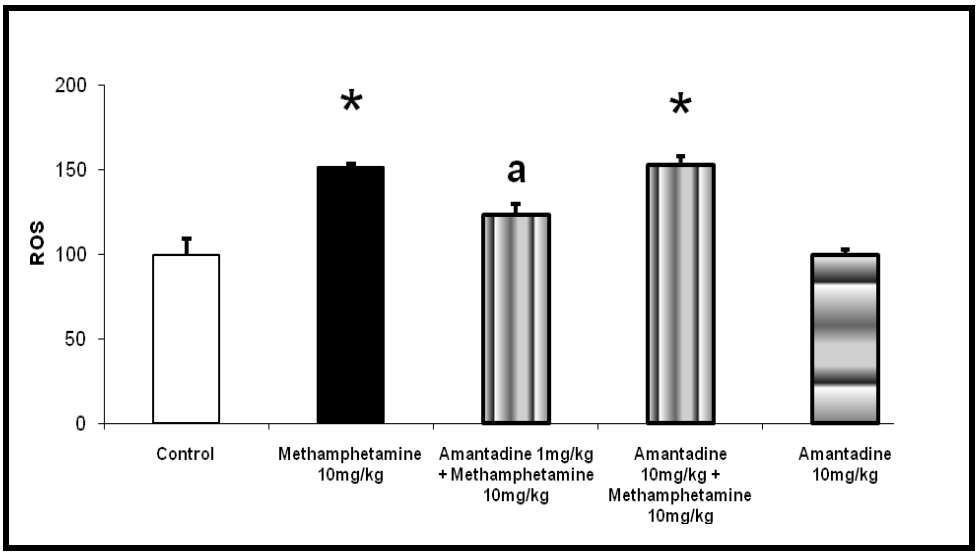

Figure 6.8. Effect of Amantadine on mitochondrial ROS (*in vivo*): Amantadine (1mg/kg) blocked methamphetamine mediated generation of ROS; amantadine (10mg/kg) did not block methamphetamine mediated generation of ROS (n = 6). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

7. CHAPTER 4: NEUROPROTECTION OF SALICYLIC ACID AGAINST NEUROTOXIC EFFECTS OF METHAMPHETAMINE

7.1 Overview

One of the leading causative theories in Parkinson's disease involves damage to neuronal cells induced by oxidative stress, apoptosis, mitochondrial dysfunction and inflammation. Salicylic acid is a known free radical scavenger. Thus, it could provide much needed neuroprotection against oxidative stress leading to neurodegeneration. We examined the effect of sodium salicylate (a sodium salt of salicylic acid) and methamphetamine on reactive oxygen species (ROS) generation, monoamine oxidase (MAO) activity and complex I & IV activities. Behavioral analysis evaluated the effect on movement. Neurotransmitter levels were evaluated using HPLC-ECD. Results showed that methamphetamine caused significant dopamine depletion in the striatum and also caused significant generation of reactive oxygen species and decreased complex I activity in the mitochondria. Behavioral analysis showed that methamphetamine had a significant effect on movement. Sodium salicylate (50 & 100mg/kg) significantly blocked methamphetamine mediated dopamine depletion in the striatum. Sodium salicylate significantly blocked methamphetamine mediated generation of reactive oxygen species and decreased Complex I activity. In addition, sodium salicylate significantly blocked methamphetamine induced movement abnormalities.

7.2 Introduction

Salicylic acid is a metabolite produced in the body following ingestion of aspirin, acetylsalicylic acid. It belongs to a class of drugs known as analgesics or antipyretics (Hardman, 1996). Aspirin was the first-discovered member of the class of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs), which all have similar effects and most have inhibition of the enzyme cyclooxygenase as their mechanism of action. Today, aspirin is one of the most widely used medications in the world, with an estimated 40,000 metric tons of it being consumed each year. In countries where Aspirin is a registered trademark owned by Bayer, the generic term is acetylsalicylic acid (Warner and Mitchell, 2002).

The etiology of Parkinson's disease remains unclear. However, one of the leading theories involves damage to neuronal cells induced by oxidative stress, or an imbalance between the generation of free radicals and the body's antioxidant defense systems (Cohen, 1998; Halliwell, 1989; Olanow, 1993). The high level of oxidative stress observed in Parkinson's disease sets off a chain of events leading to deficits in mitochondrial function, disruption of the electron transport chain, neuroinflammation, excitotoxicity and eventual neuronal cell death (Doble 1999).

In Parkinson's disease, nigrostriatal dopaminergic neurons undergo selective degeneration, leading to a shortage of dopamine in the striatum (Hornykiewicz, 1973; Caine and Langston 1983). A possible contributing factor to this is the brain and nervous system's particularly vulnerablity to free radical damage. It consumes a disproportionally large volume of oxygen for its small weight as compared to the rest of the body,

contributing to the formation of the highly reactive superoxide radical (Halliwell, 1989). Another possible contributing factor to the neurotoxicity and selective neurodegeneration seen in the substantia nigra is the presence of iron in this area, which can also contribute to the production of reactive oxygen species. In the presence of transition metals, hydrogen peroxide can be converted by the Fenton reaction, an iron mediated reaction that requires iron in the free ferrous form, to the highly reactive hydroxyl radical (Bharath et al., 2002). Oxidative stress causes an inhibition of mitochondrial ATP production, a major determinant of lethal cell injury. The damage to the striatal terminals and the nigral cell bodies observed in methamphetamine users is also indicative of oxidative stress, the same mechanisms indicated in Parkinson's disease (Imberti et al., 1993; Jones and Vale, 2000).

Salicylic Acid is a known free radical scavenger (Kataoka et al., 1997). It has been shown to provide neuroprotection against MPTP induced neurotoxicity (Mohanakumar et al., 2000). Thus, it could provide much needed neuroprotection against oxidative stress leading to neurodegeneration. With the ability of salicylic acid for free radical scavenging, it could play a much needed neuroprotective role in not only Parkinson's disease, but also in numerous other neurodegenerative disorders.

Another possibly neuroprotective factor is salicylic acid's anti-inflammatory mechanism. Researchers have examined neuroinflammation as a risk factor for Parkinson's disease for a long time (Landrigan et al., 2005). There is increasing evidence that one contributor to the later development of Parkinson's disease is inflammation of neuronal tissue. This inflammation involves the activation of brain immune cells,

microglia. Activation of these cells can be beneficial in the protection of the cells, but they can also release neurotoxic cytokines which could have a deleterious effect and produce neurodegeneration (Liu et al., 2003). The substantia nigra has the highest density of microglia in the brain, and studies have shown that Parkinson's disease patients have an even higher number of reactive glial cells than do patients without the disease (McGreer et al, 1988). Evidence of the involvement of inflammation came from studies showing that animals exposed to rotenone showed enhanced neurodegeneration in the presence of glia. Further studies showed synergistic effects when rotenone was combined with lipopolysacchide (Thiruchelvam et al., 2000). This would support the use of anti-inflammatory therapeutic agents in Parkinson's disease.

Our earlier studies show that administration of methamphetamine to mice produces a loss of dopamine in the striatum and a syndrome that behaviorally, biochemically, and neurochemically resembles Parkinson's disease. In this study, we use this model to study whether salicylic acid exhibits neuroprotection against the toxicity caused by methamphetamine. If salicylic acid proved to be a neuroprotectant substance, preventing neuronal degeneration, it could slow the progression of Parkinson's disease.

7.3 Materials and Methods

7.3.1 Animals

Male C57/Bl6 mice (4-6 weeks old) weighing 20-30 g were purchased from Charles Rivers. They were housed in a temperature controlled room with a 12h day and

night cycle with free access to food and water. They were housed for 2-4 days prior to experiments. All the experimental procedures were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Auburn University.

7.3.2 Drug administration

C57/Bl6 mice were separated into 5 groups (control, methamphetamine only, high dose sodium salicylate only, sodium salicylate high dose + methamphetamine and sodium salicylate low dose + methamphetamine). The groups were given i.p. injections once daily for one week with sterile water (control & methamphetamine groups), 100mg/kg sodium salicylate, dissolved in sterile water (sodium salicylate high dose), 100mg/kg sodium salicylate, dissolved in sterile water (sodium salicylate high dose + methamphetamine group), and 50mg/kg sodium salicylate, dissolved in sterile water (sodium salicylate low dose + methamphetamine group). On day 7 the methamphetamine, sodium salicylate high dose + methamphetamine and sodium salicylate low dose + methamphetamine groups were injected with methamphetamine (10mg/kg i.p., twice, 2hr apart). The animals were sacrificed 5 days after the last injection.

7.3.3 Behavioral Studies

Following methamphetamine administration, two examiners who were trained in evaluating different animal behaviors continuously observed mice. Tremor, akinesia, catalepsy, and straub tails were monitored during this period following standard scoring

procedures as described below. Animals were subjected to swim test on the 4th day after methamphetamine.

Straub tail: Straub tail was defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as Straub tail phenomenon was detected or not detected (Mohanakumar and Sood, 1986).

Akinesia: Akinesia usually refers to the impaired ability to initiate movements. It was measured by noting the latency in second(s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180s (Weihmuller et al., 1988; Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term implies the inability of an animal to correct an externally imposed posture. Catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Weihmuller et al., 1988; Murlikrishnan and Mohanakumar, 1998).

Swim test: Swim test was carried out in water tubs (40 l x 25 w x 12 h, in cm). The depth of water was kept at 8 cm and the temperature was maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were: 0 = hind part sinks with head floating, 1 = occasional swimming using hind limbs while floating on one side, 2 = occasional floating/swimming only, 3 = continuous swimming (Donnan et al., 1987).

7.3.4 Measurement of Neurotransmitters

To determine dopamine depletion in the striatum, methamphetamine injected animals were sacrificed on the 5th day and the striata was dissected out and analyzed for DA content employing HPLC-electrochemistry. Dopamine and its metabolites were separated in high pressure liquid chromatography. An electrochemical detector was used to quantify dopamine and its metabolites. The C18 octadecyl silica column (8 cm X 4.6 mm i.d.) was used for the separation of the monoamines. Brain tissue was sonicated in 0.4 N HClO4. These samples were then centrifuged and the 20 µl of supernatant used was injected in HPLC. The results were expressed as picomoles per milligram of tissue (Uthayathas et al., 2007).

7.3.5 Tissue Preparation for *In vivo* biochemical assays

Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi 2001).

7.3.6 Protein estimation

Protein was assayed using the coomassie plus protein assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

7.3.7 Mitochondrial Complex I Activity

Mitochondrial complex-I activity (NADH dehydrogenase activity) is based on the NADH oxidation. Oxidation of NADH by the NADH-dehydrogenase enzyme present in the tissue homogenate was measured spectrophotometrically at 340 nm. The mitochondrial P_2 suspension was added to the reaction mixture containing NADH, coenzyme Q_0 and phosphate buffered saline, to analyze its effect on NADH oxidation by monitoring the decrease in absorbance at 340 nm. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The change in absorbance indicates the amount of NADH oxidized. The mitochondrial complex-I activity is expressed as the amount of NADH oxidized/min/mg protein (Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

7.3.8 Mitochondrial Complex IV Activity

Complex IV activity was based on the cytochrome-C oxidation. The mitochondrial P_2 suspension was added to the reaction mixture containing cytochrome-C and phosphate buffered saline. The oxidation was measured spectrophotometrically. The absorbance was measured at 550nm for 2 min and the enzyme activity was expressed as cytochrome-C oxidized/mg protein(Ramsay et al., 1987; Wharton and Tzagotoff, 1967).

7.3.9 Assay of MAO activity

Total MAO activity was based on the amount of 4-hydroxyquinoline formed by the oxidation of kynuramine (Morinan and Garratt, 1985). 4-hydroxyquinoline was

measured fluorimetrically and the enzyme activity was expressed as 4-hyroxyquinoline formed/hour/mg protein (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan and Ebadi, 2001).

7.3.11 Assay of ROS production

Conversion of nonfluorescent chloromethyl-DCF-DA (2′,7′- dichlorofluorescin diacetate) to fluorescent DCF was used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. The generation of ROS was measured and reported as relative fluorescence intensity (Dhanasekaran et al., 2008).

7.3.12 Statistical Analysis

Results are expressed as the means±SEM. The statistical significance was evaluated by the one-way analysis of variance (ANOVA) using SigmaStat version 3. Values of *p* less than or equal to 0.05 were considered significant.

7.4 Results

7.4.1 Behavioral Analysis

Methamphetamine (10mg/kg) caused a significant motor impairment in the C57/BL6 mice. Intraperitoneal administration of sodium salicylate (50 & 100mg/kg) significantly blocked methamphetamine induced behavioral abnormalities including

catalepsy (figure 7.1), akinesia (figure 7.2), reduced swim time (figure 7.3), straub tails or tremor (table 7.1) (n=6, $p<0.05$).

7.4.2 Effect of salicylic acid and methamphetamine on neurotransmitters

Administration of methamphetamine (10mg/kg) caused a significant reduction in the levels of dopamine in the striatum. Sodium salicylate (50 &100mg/kg) significantly blocked methamphetamine induced dopamine depletion (n=6, $p=<0.05$), as compared to control animals (figure 7.4). Methamphetamine and sodium salicylate also affected levels of other striatal neurotransmitters (NE, 5HT, 5HIAA) and dopamine metabolites (DOPAC, HVA). The effect of sodium salicylate and methamphetamine on these neurotransmitters is shown in table 7.2.

7.4.3 *In vivo* biochemical analysis

Intraperitoneal administration of methamphetamine (10mg/kg) in mice significantly decreased complex I activity. Sodium salicylate (50 & 100mg/kg) did not block methamphetamine induced decrease in complex I activity (n = 6; figure 7.5). Neither sodium salicylate nor methamphetamine had a significant effect on complex IV activity (figure 7.6). Sodium salicylate and methamphetamine also had no significant effect on the total mitochondrial monoamine oxidase (MAO) activity (figure 7.7). Methamphetamine caused significant generation of reactive oxygen species and sodium salicylate (50 & 100mg/kg) did significantly block this effect (n = 6, $p=<0.05$) (figure 7.8).

7.5 Discussion

Salicylic acid irreversibly inactivates of the cyclooxygenase (COX) enzyme. This irreversible inactivation makes it different from other NSAIDs, such as ibuprofen, which are reversible inhibitors. The inhibition of the COX enzyme is responsible for its anti-inflammatory properties (Vane, 1971; Tohgi et al., 1992; Vane and Botting, 2003). However, the neuroprotective properties of salicylic acid are thought to be more because of its role as a scavenger of hydroxyl radicals than its COX-inhibitory action (Ferger et al., 1999; Mohanakumar et al., 2000).

Because methamphetamine displaces dopamine in the vesicles, large amounts of it are released into the synaptic cleft and cytosol. Once released, the dopamine can be oxidized to reactive oxygen species. These reactive species can cause an inhibition of mitochondrial ATP production. The combination of oxidative stress and mitochondrial dysfunction creates toxicity of striatal nerve terminals (Stokes et al., 1999; Zhu et al., 2006). The central dopaminergic system that plays such an important role in motor activity is comprised of a surprisingly small number of neurons. Because of this, it is especially vulnerable and even minor insults may lead to irreparable functional deficits (Fariello et al., 1988).

Salicylic acid has been shown to prevent the MPTP induced reduction in dopamine levels. It inhibits the action of this neurotoxin on the mitochondrial electron transport chain, a common source of free radicals in the cell. In addition, salicylic acid has been shown to block the neurotoxic effects of MPTP on the enzymatic defence

system of the brain such as superoxide dismutase, glutathione peroxidase and catalase (Ferger et al., 1999).

The reduction in glutathione levels induced by MPTP is significantly inhibited by salicylic acid. These results imply that salicylic acid is not only capable of scavenging free radicals, but also of enhancing the cell's own mechanisms against toxicity. In a MPTP mouse model of Parkinson's disease, salicylic acid blocked toxin-induced glutathione and dopamine depletion. This indicates its role as a hydroxyl radical scavenger and its neuroprotective properties. In addition, this study showed, for the first time, that salicylic acid pre-treatment significantly improved motor impairments (akinesia and catalepsy) caused by MPTP administration (Mohanakumar et al., 2000).

Our earlier studies show that administration of methamphetamine to mice produces a loss of dopamine in the striatum and a syndrome that behaviorally, biochemically, and neurochemically resembles Parkinson's disease. In this study, we use this model to study whether salicylic acid exhibits neuroprotection against the toxicity caused by methamphetamine. Our results showed that administration of methamphetamine caused a significant amount of dopamine depletion and a striatal environment ripe for oxidative stress, mitochondrial dysfunction and neuronal degeneration. The neurochemical data obtained using HPLC-ECD showed that sodium salicylate (50 & 100mg/kg) provided significant neuroprotection against methamphetamine induced dopamine depletion (n=6, p=<0.05). The *in vivo* biochemical results showed that methamphetamine caused significant generation of reactive oxygen species and that sodium salicylate (50 & 100mg/kg) showed significant neuroprotection

against methamphetamine induced generation of ROS (n=6, p=<0.05). Overall, these results indicate that salicylic acid is neuroprotective against neurotoxicity and dopamine depletion caused by methamphetamine administration. Despite advances in pharmacotherapy that have improved quality of life, the mortality rate among Parkinson's disease sufferers remains largely unchanged. If salicylic acid proved to be a neuroprotectant substance, preventing neuronal cell damage it could slow the progression of Parkinson's disease.

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7.7 Figures

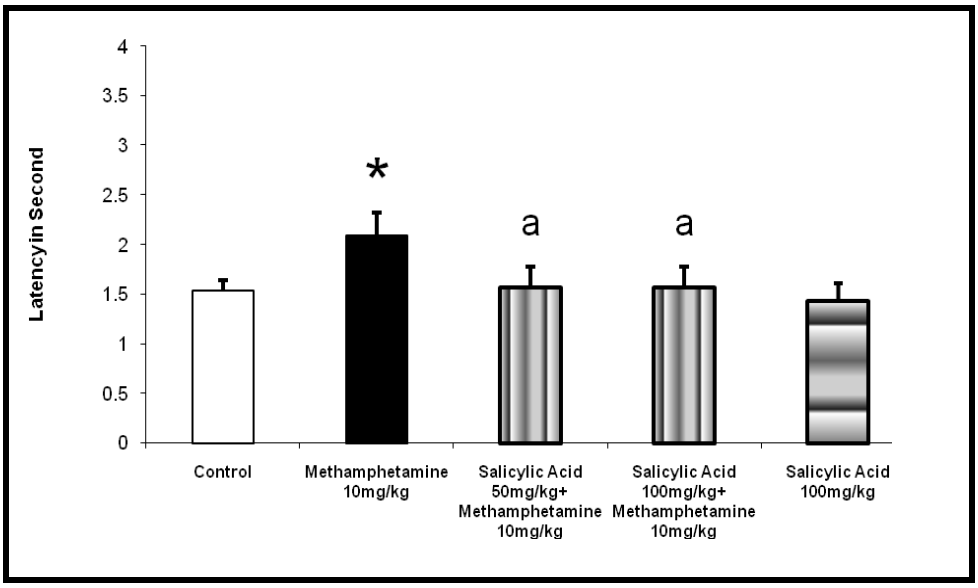

Figure 7.1. Effect of Salicylic acid on catalepsy: Sodium salicylate (50 & 100mg/kg) blocked methamphetamine induced catalepsy. *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

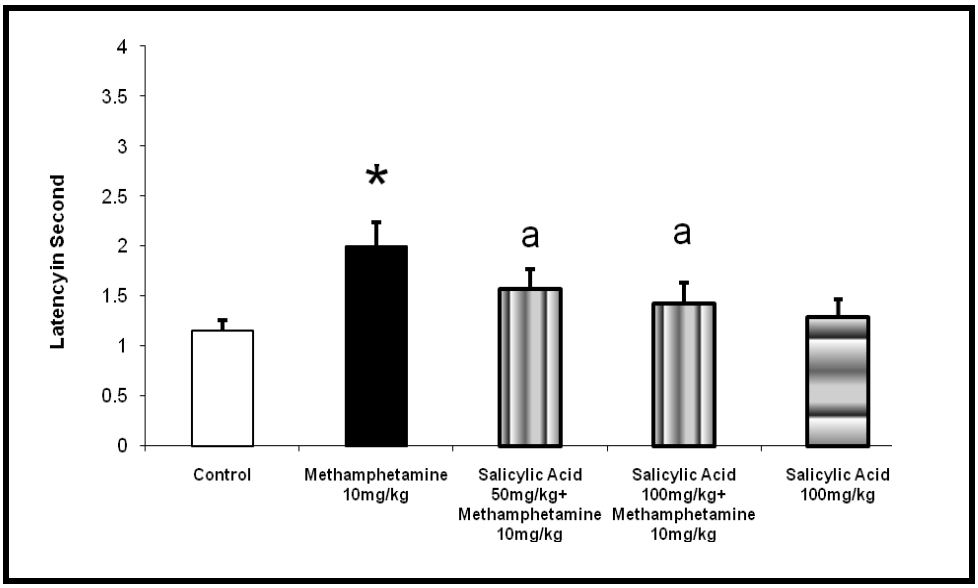

Figure 7.2. Effect of Salicylic acid on akinesia: Sodium salicylate (50 & 100mg/kg) blocked methamphetamine induced akinesia. *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

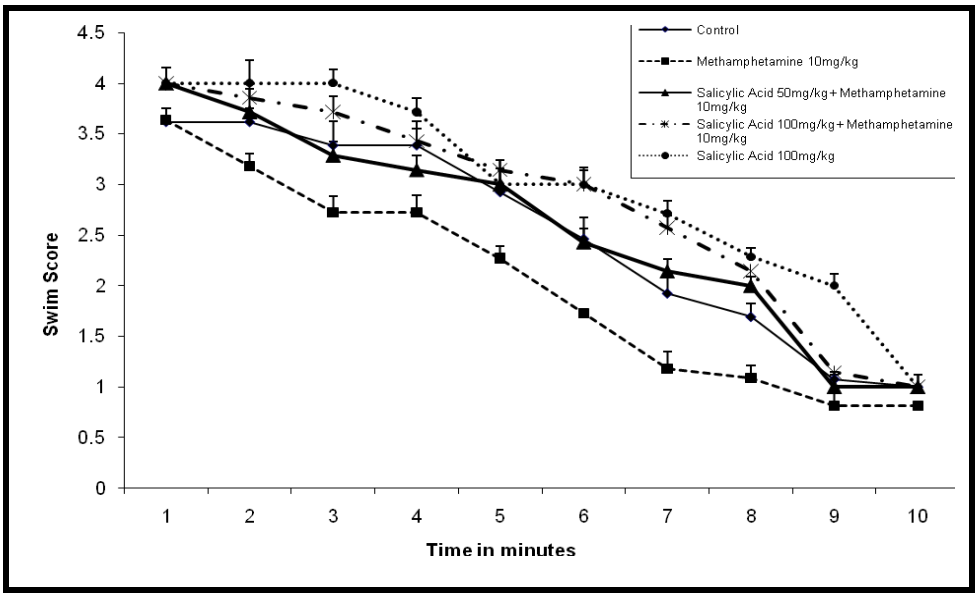

Figure 7.3. Effect of Salicylic acid on swim score: Sodium salicylate (50 & 100mg/kg) blocked methamphetamine induced decrease in swim score (n=6). Scored by immobility time.

	average weight	straub tail	tremor
Control	24.3±0.6	0	0
Methamphetamine 10mg/kg	24.2±0.5	n=6	4.0±0.1
Sodium salicylate 50mg/kg + Methamphetamine 10mg/kg	22.4±0.3	n=4	2.0±0.1
Sodium salicylate 100mg/kg + Methamphetamine 10mg/kg	23.9±0.3	n=1	1.5±0.1
Sodium salicylate 100mg/kg	23.6±0.4	0	0

Table 7.1. Effect of Salicylic acid on straub tail and tremor: Sodium salicylate alone did not induce tremor or straub tail. Sodium salicylate (50 & 100mg/kg) blocked/reduced methamphetamine induced straub tail and tremor (n=6).

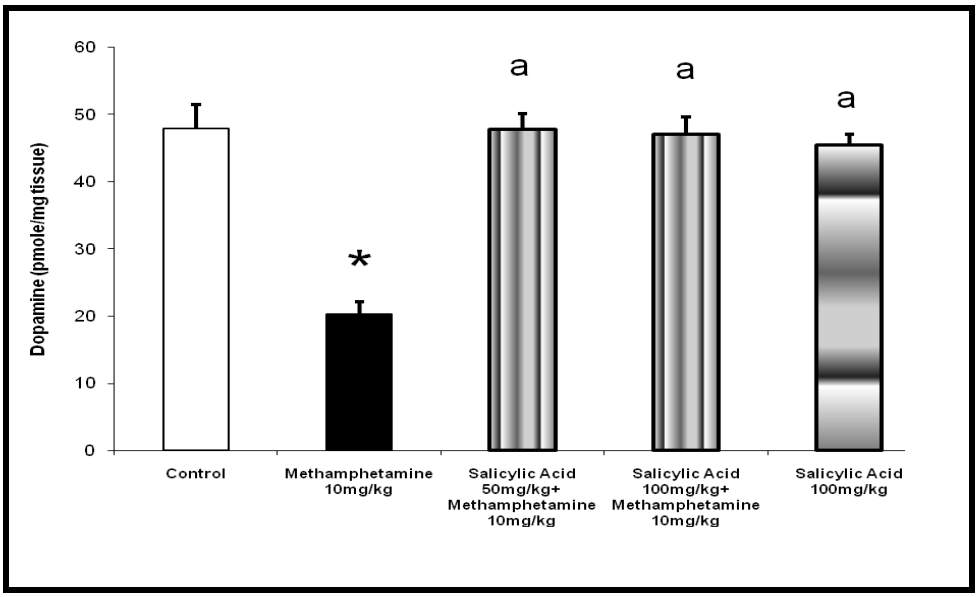

Figure 7.4. Effect of Salicylic acid on striatal dopamine content: Sodium salicylate (50 & 100mg/kg) blocked methamphetamine mediated dopamine depletion. *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

	Control	Methamphetamine 10mg/kg	Sodium salicylate 50mg/kg+ Methamphetamine 10mg/kg	Sodium salicylate 100mg/kg+ Methamphetamine 10mg/kg	Sodium salicylate 100mg/kg
DOPAC	49.12±11.6	19.45±3.8	14.51±0.1 *	12.84±0.7 *	14.85±1.5 *
HVA	24.73±1.7	18.10±1.7	25.82±1.9 [a]	22.87±1.6 [a]	19.23±2.0 [a]
5-HT	2.99±1.6	2.69±0.2	2.31±0.3	1.90±0.2 * [a]	1.75±0.4 * [a]
5-HIAA	11.37±1.7	9.86±0.5	9.82±0.8	9.22±0.6	7.59±0.7* [a]
NE	15.21±0.9	14.49±0.9	16.93±1.6	24.09±0.6	21.30±1.2 *

Table 7.2. Effect of Salicylic acid on striatal neurotransmitter and metabolite levels (expresed as average average pmole/mg tissue ± SEM). *significant (n=6, p*<.05) as compared to control animals. [a]significant (n=6, p^a<.05) as compared to methamphetamine animals.

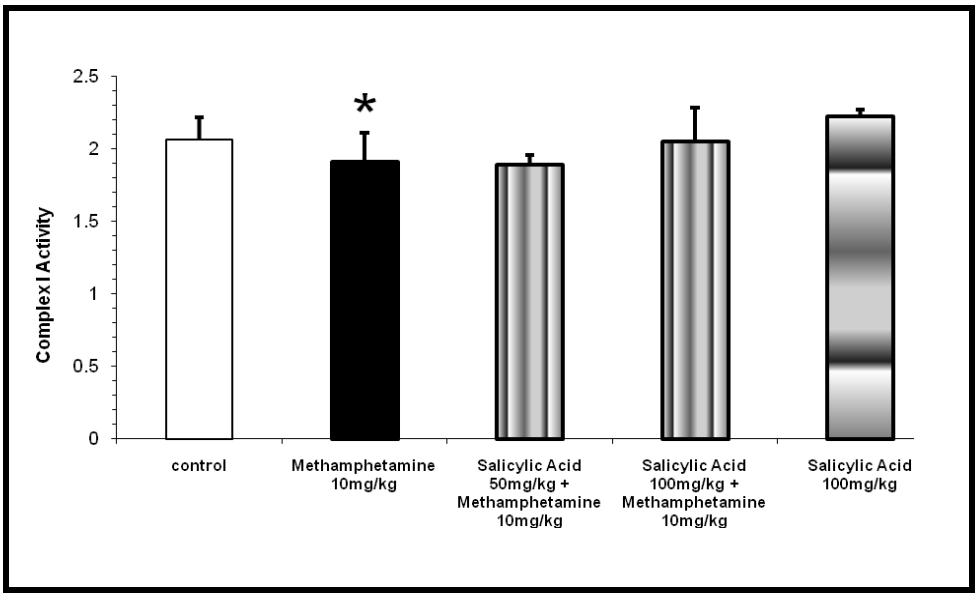

Figure 7.6. Effect of Salicylic acid on mitochondrial Complex I activity (*in vivo*): Sodium salicylate (50 & 100mg/kg) did not block methamphetamine mediated decrease in complex I activity (n = 6). Activity expressed as µmol/min/mg protein. *significant (n=6, p*<.05) as compared to control animals.

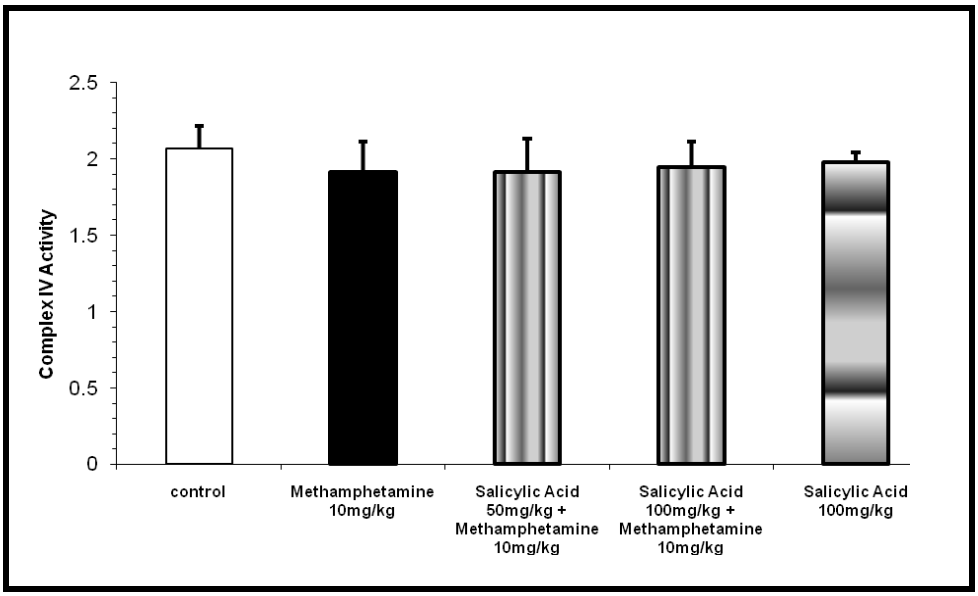

Figure 7.7. Effect of Salicylic acid on mitochondrial Complex IV activity (*in vivo*): Sodium salicylate (50 & 100mg/kg) caused no significant change in complex IV activity (n=6). Activity expressed as µmol/min/mg protein.

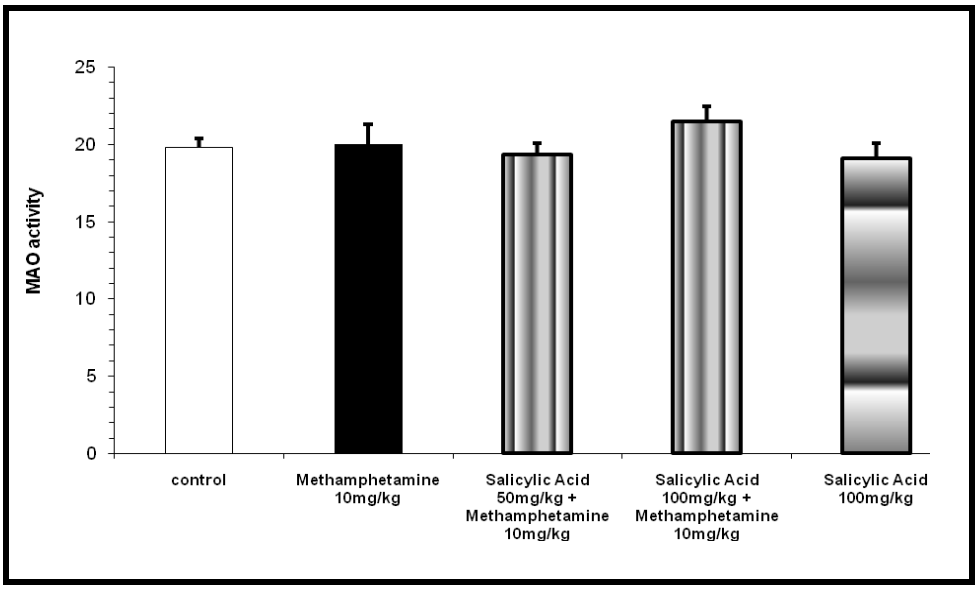

Figure 7.8. Effect of Salicylic acid on mitochondrial MAO activity (*in vivo*): Sodium salicylate (50 & 100mg/kg) caused no significant change in MAO activity (n=6). Activity expressed as 4-OHQ/mg protein.

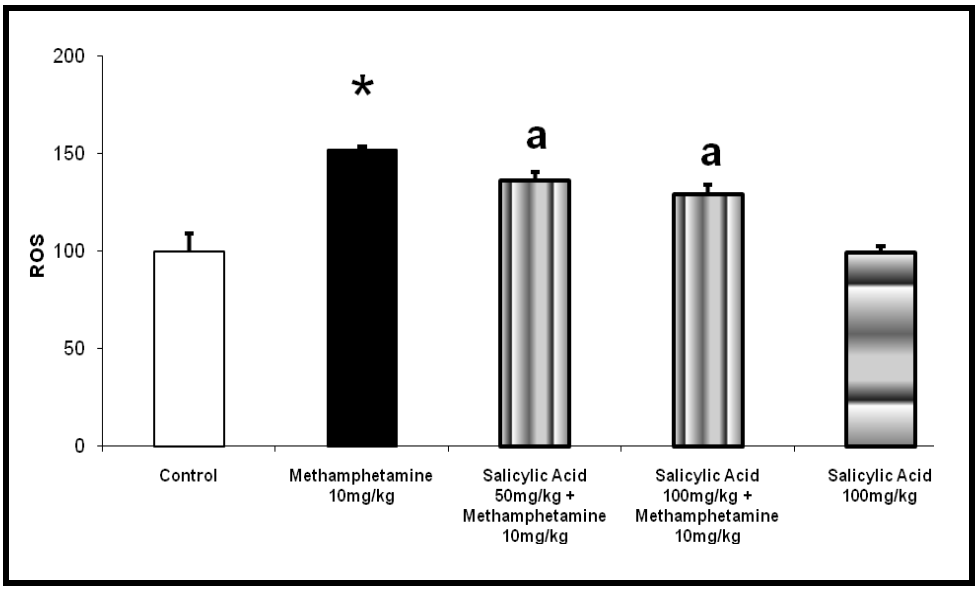

Figure 7.10. Effect of Salicylic acid on mitochondrial ROS (*in vivo*): Sodium salicylate (50 & 100mg/kg) blocked methamphetamine mediated generation of ROS. *significant (n=6, $p^*<.05$) as compared to control animals. [a]significant (n=6, $p^a<.05$) as compared to methamphetamine animals.

8. SUMMARY AND CONCLUSIONS

Previous studies have implicated exposure to toxins as contributors to the development of Parkinson's disease. Our research provided evidence that methamphetamine is significantly toxic to dopaminergic neurons.This study shows the significant loss of dopaminergic function induced by methamphetamine as evidenced by both behavioral and biochemical experiments and with neurochemical data. It also offers evidence of oxidative stress and mitochondrial dysfunction. This indicates that administration of methamphetamine to mice produces a dopamine depletion in the striatum and a syndrome that behaviorally, biochemically, and neurochemically resembles Parkinson's disease. These results indicate the usefulness of methamphetamine in making an animal model to study Parkinson's disease.

We used this model to ascertain if ramelteon, amantadine and salicylic acid exhibit neuroprotection against the toxicity caused by methamphetamine. In both methamphetamine toxicity and in Parkinson's disease, the high level of oxidative stress leads to deficits in mitochondrial function, disruption of the electron transport chain, initiation of excitotoxicity and neuroinflammation. All of these mechanisms are sufficient to cause apoptosis and/or necrosis of neuronal cells. Therefore, each mechanism could be considered a target for therapeutic intervention. Discovery of a neuroprotective

pharmacological agent could lead to proactive strategies to counteract the neurological damage caused by Parkinson's disease.

Overall, these results indicate that amantadine is partially neuroprotective but salicylic acid fully protected against methamphetamine-induced dopamine depletion. As previously stated, our long-term goal in this study is to facilitate and improve the care of patients with Parkinson's disease by developing cost cost-effective anti-Parkinson's drugs with the added benefit of a neuroprotective effect. There is a great need for neuroprotective and neurorestorative treatments for this disease.

Neuroprotection was observed in mice that were pretreated with sodium salicilate or amantadine before the administration of methamphetamine. Both drugs augmented dopaminergic neuronal function in the brain of methamphetamine treated mice and reduced striatal dopamine depletion. This provides strong supporting evidence for the use or continued use of these drugs as therapeutic agents to provide relief and protective benefits for Parkinson's disease.