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The Interaction of Psychostimulant Intake With Brain Aging: Effects On Behavioral Capacity, Oxidative Damage and Dopaminergic Markers

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ABSTRACT

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Frequent abuse of psychostimulants is known to induce changes in brain neurochemistry that are most profound in dopaminergic neurons. These changes could both impair dopamine neurotransmission and adversely affect psychomotor and cognitive functions. One hypothesized cause of these impairments is the adverse effects of psychostimulant-induced increases in oxidative stress. The current studies addressed the general hypothesis that chronic administration of cocaine and methamphetamine would create a change in neurochemistry in dopaminergic neurons and, as a consequence, increase oxidative damage. This would result in decreases in dopaminergic functions specifically in the nigrostriatal region and cause impairments in psychomotor functions.

To test this hypothesis we utilized an *in vivo* rodent model involving continuous chronic administration of cocaine or methamphetamine. Separate groups of mice were exposed to a 30-day treatment, involving continuous infusion of saline, 40 mg/kg of cocaine, or 2 mg/kg of methamphetamine. After discontinuation of the drug treatment, separate groups of the mice were tested for cognitive and psychomotor function at 11, 14, or 16 months of age i.e., 1 week, 3 months, or 5 months after treatment. The test used in this study included spatial learning and memory (swim maze), coordinated running

ability (accelerating rotorod), muscle and grip strength (wire suspension) and balance and coordination (bridge walking). Following completion of the behavioral tests brain regions were dissected. The regions we analyzed were the cortex, striatum, cerebellum, hippocampus, midbrain, and hindbrain. These regions were analyzed for carbonyl and thiobarbituric acid reactive substances concentrations to measure levels of protein and lipid oxidation, and Western blotting procedures to address dopaminergic protein expression.

Overall, both chronic administration of cocaine and methamphetamine resulted in significant impairments to psychomotor functions. These impairments were evident for both groups on wire hanging tests, bridge walking, and rotating rod tests, both initially following the treatment phase and throughout the age ranges that were analyzed. In addition, the cocaine treatment administered led to profound impairments on cognitive function in the 14-month-old age groups. This impairment was most evident on the reversal phase of the spatial swim maze tests.

The biochemical tests revealed that chronic cocaine and methamphetamine administration induced significant increases in protein oxidative damage in the striatum initially following the treatment phase. Psychostimulant-induced lipid oxidative damage was evident in the striatum in both the 14 and 16 month old age groups. Age related declines were evident in the midbrain, cortex and striatum. Overall neither treatment had any effect on the expression of the dopaminergic proteins that were analyzed.

The results from these studies warrant the conclusion that chronic cocaine and methamphetamine administration causes an increase in intracellular oxidative damage in the nigrostriatal neurons which decrease dopamine-mediated psychomotor functions. Overall there was not enough evidence to conclude that chronic abuse of these drugs induce impairments that would increase during senescence.

INTERACTION OF PSYCHOSTIMULANT INTAKE WITH BRAIN AGING: EFFECTS ON BEHAVIORAL CAPACITY, OXIDATIVE DAMAGE AND DOPAMINERGIC MARKERS

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CHAPTER I

INTRODUCTION

Psychostimulant addiction has been a major health problem over the last few decades. In the United States alone, there are approximately 43 million Americans who have tried cocaine or methamphetamine at least once in their lives. Furthermore, individuals who abuse these drugs suffer from several health problems, including arrhythmias, heart attacks, respiratory failure, strokes, seizures, and nausea (for review see Volkow, 2004). Despite the health dangers associated with the use of these drugs, many continue to abuse psychostimulants chronically, with several million people facing the risk of becoming addicted. The two psychostimulants that are most widely abused currently are cocaine and methamphetamine, both powerful stimulants that possess strong reinforcing properties and elicit euphoric and addictive effects. Another psychostimulant, methylphenidate is a widely prescribed medication in the treatment of attention-deficit hyperactivity disorder and is pharmacologically related to cocaine (Swason et al, 1998; Edwards et al., 2008). Estimated methylphenidate treatments in some studies have been reported to be up to 6-7% of all school-aged children (Kollins et al., 2001). While little is known about the abuse potential of methylphenidate the widespread use among young people have prompted some concerns about its long-term effects (Askenasy et al., 2007).

Routes of administration for psychostimulant abuse can vary greatly, ranging from smoking, snorting, injecting, or ingesting the drugs dissolved in some liquid. Individuals who chronically abuse psychostimulants frequently re-administer the drugs over a short

period of time in a binge pattern of use. However, the binge patterns associated with cocaine and methamphetamine are not identical. For example, cocaine addicts may binge on cocaine for 30 minutes at a time over the course of several days (Dackis and Gold, 1985; Gawin, 1989). In contrast, methamphetamine addicts tend to self-administer the drug every two hours across a three- to ten-day bout that is followed by a period of abstinence. One reason for the slightly different binge patterns may be due to the longer duration of action of methamphetamine when compared with cocaine.

The characteristic binge pattern of psychostimulant use results in a sustained increase in plasma and brain concentrations of the drug that is a hallmark of psychostimulant abuse (Gawin and Kleber, 1985). Binge usage of psychostimulants is thought to result in acute tolerance to the reinforcing effects, contributing to the observation that addicts "chase" the high by using greater quantities of the drugs (Gawin and Ellinwood, 1988). Drug use is discontinued typically because the supply is exhausted, or because of drug-induced psychosis or other adverse effects. Once drug use ceases, the individual may experience a withdrawal syndrome include dysphoria, psychosis, lack of mental and physical energy, and anhedonia. It is also during this period that addicts are most likely to relapse (Gawin and Ellinwood, 1988; Gold, 1992). It is worth noting that the withdrawal syndrome associated with psychostimulant abuse, while significant, is not as severe as the withdrawal syndrome associated with some other classes of drugs like opioids or alcohol. In addition there is still considerable debate over what role the withdrawal syndrome plays in the development of addictions to these different classes of drugs (for discussion, see Weiss et al., 2001). Psychostimulant addiction is a dynamic problem involving

several factor including changes in brain neurochemistry, as well as familial and social aspects (Volkow and Li, 2005). Currently there are no effective medications available to treat psychostimulant addiction that target all of it's complex components (O'Brien et al., 2005).

In summary, the evidence above indicates that psychostimulant abuse, characterized by a binge type pattern of use followed by the onset of withdrawal once drug administration is terminated, is currently a major societal problem. However, beyond its significance as an immediate health problem with societal impact, the exposure to psychostimulants during adult life, on either a short- or long-term basis, has significant potential to produce a more global deleterious effect on health and functional status in individuals during later life. Psychostimulant abuse is well-known to elicit long-lasting and sometimes permanent neuroadaptive changes, which have been widely studied for the purpose of defining their role in processes of addiction and relapse (for reviews (Koob, 1999; Nestler, 2004; Volkow and Li, 2005). Furthermore, psychostimulant exposure may directly elicit or promote neurotoxic effects (Sulzer et al., 1995). Notwithstanding, few studies have considered the potential for psychostimulant-induced neuroadaptive or toxic effects to interact with processes of brain aging. This possibility was addressed directly in the current studies using a mouse model of psychostimulant intake and behavioral analogues of psychomotor and cognitive decline. The potential anatomical targets, aging processes, and neural functions involved in psychostimulant/brain aging interactions are considered briefly in the sections that follow.

Anatomy and function of brain dopamine-containing neurons

Neurons that release the neurotransmitter, dopamine, mediate a variety of critical functions, though most notably they are a key component of neural circuitry underlying the neuropsychological constructs of motivation, reward and voluntary movement (Bernheimer et al. 1965; Berridge and Robinson 1998, Miller et al. 1990, Pessiglione et al. 2006). The two most widely studied dopaminergic pathways are the mesolimbic/mesocortical and the nigrostriatal. The mesolimbic dopamine pathway is critically important in motivation/reward processes and contains neurons that originate in the A10 region of the ventral tegmental area (VTA) of the midbrain and project to the nucleus accumbens and other structures of the basal forebrain, as well as the caudate putamen (Bjorklund and Lindvall, 1975). A well established body of evidence indicates that the ability of psychostimulants to produce euphoric effects in humans, and elicit locomotion and drug-seeking behavior in animals, is critically dependent upon the activity of dopamine neurons of the mesolimbic/mesocortical system. It has been theorized by Wise and Bozarth that psychostimulants like other addictive substances activate central dopaminergic neurons of the mesolimbic/mesocortical systems which results in increases in forward locomotion and simultaneous activation of the positive reinforcement circuitry of the brain (for review see (Wise, 1987).

The nigrostriatal pathway plays a primary role in movement initiation and control and contains dopamine neurons that originate in the substantia nigra *pars compacta* (A9) and project into the striatum. The nigrostriatal dopamine projections are viewed as a critical enabling circuit within the extrapyramidal motor system of the brain, a loop that

conditions motor output of the cortex and is necessary for both initiation and control of movement patterns. The functional significance of the nigrostriatal dopamine system is illustrated in Parkinson's disease (PD) a condition involving selective degeneration of nigrostriatal dopamine-containing neurons that results in rigidity and bradykinesia.

Functional aspects of dopamine neurotransmission

At the cellular level, dopamine neurotransmission is regulated by a number of specialized proteins and synthesized in a multistep process. First tyrosine is hydroxylated to L-DOPA by the enzyme tyrosine hydroxylase (TH). This is the rate limiting step in dopamine biosynthesis and is subject to regulation by a number of processes. Short term regulation of TH activity occurs through the phosphorylation of specific serine residues by protein kinases (Haycock, 1990; Kumer and Vrana, 1996; Lindgren et al., 2000). Increases in tyrosine hydroxylase phosphorylation have been shown to accelerate enzymatic activity both *in vivo* and *in vitro* (Dunkley et al., 2004). The second step in this process is the formation of dopamine from L-DOPA by aromatic-L-amino acid decarboxylase. Once synthesized, dopamine is packaged in storage vesicles by way of vesicular monoamine transporters (VMAT). These specialized proteins use energy from vesicular proton gradients to sequester dopamine and other monoamine neurotransmitters from the cytosol into storage vesicles. After packaging, dopamine vesicles are then transported to the synapse and can be released into the synaptic cleft in response to neuronal action potentials through a calcium-dependent process.

Once released, dopamine exerts its actions by binding to receptors of various subtypes located pre- or post-synaptically on neurons in the limbic, cortical, or striatal targets. At all sites of neurotransmission, the action of dopamine is terminated by its rapid reuptake through dopamine transporters (DAT). These proteins mediate the reuptake of dopamine (as well as of toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by an electrogenic Na^+ and Cl^- -transporter-coupled mechanism. Once back inside the cell, cytosolic dopamine can be processed through one of two pathways. The neurotransmitter can either be packaged once again into presynaptic storage vesicles, or it can be degraded to its metabolites homovanillic acid (HVA), or dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase B. Non-sequestered (free) dopamine can be considered redox-active, as its autoxidation or metabolism by monoamine oxidase both result in reactive oxygen species like hydrogen peroxide and superoxide radicals which are potentially harmful to the cells (Cubells et al., 1994; Hastings et al., 1996). Therefore alterations in the expression or function of these proteins can have profound consequences on neuronal health and vulnerability. For example VMAT over-expression has been reported to be protective against MPTP, L-DOPA and methamphetamine neurotoxicity in mice (Liu et al., 1992; Larsen K, 2002; Larsen 2002). In addition, mice with reduced levels of VMAT are more susceptible to the toxicities of MPTP and L-DOPA (Gainetdinov et al., 1998; Kariya et al., 2005). Altered expression of DAT can elicit profound changes as well. (Bezard et al., 1999) reported that mice lacking the DAT were protected against MPTP toxicity.

In summary, several specialized proteins play important roles in the biosynthesis and release of the neurotransmitter dopamine. Potentially any perturbation in the expression or function of these proteins could increase the susceptibility of the neurons to toxins or oxidative damage.

Neuroadaptive and toxic consequences of chronic psychostimulant use

Both cocaine and methamphetamine are indirect dopamine agonists that induce euphoric effects by binding to the dopamine transporter with high affinity and increasing levels of dopamine within the synaptic cleft. The mechanisms by which cocaine and methamphetamine influence dopaminergic neurotransmission have been extensively studied, and it has been well established that long-term abuse of either drug elicits neuroadaptive responses leading to relatively long-lasting shifts in dopaminergic homeostasis. For example, in both nigrostriatal and mesolimbic dopamine systems, chronic cocaine administration results in reduced vesicular monoamine transporter expression, increased expression and membrane trafficking of DAT, enhanced alpha synuclein expression, increased levels of tyrosine hydroxylase, amplified autoreceptor sensitivity, as well as numerous other macromolecular changes (Alburges and Wamsley, 1993; Masserano et al., 1996; Brown et al., 2001; King et al., 2002)

Recently, researchers have concluded that cocaine administration significantly increases oxidative stress in important dopaminergic pathways. For example, Dietrich et al. (2005) reported that an acute injection of cocaine in rats increased hydrogen

peroxide/lipid peroxide, and increased mitochondrial hydrogen peroxide generation in the striatum. In addition, they reported that cocaine down-regulated the expression of the mitochondrial genome, which is thought to be a precursor to mitochondrial dysfunction (Manczak et. al., 2005). Furthermore, these investigators found that cocaine amplified the expression of both superoxide dismutase and glutathione peroxidase in the frontal cortex and the striatum. In another study, chronic cocaine administration decreased catalase activity in the prefrontal cortex and striatum of mice (Macedo et al., 2005). Such alterations in antioxidant enzymes support the idea that cocaine increases oxidative stress, as these proteins are the brain's first line of defense against harmful reactive oxygen species.

Consistent with this hypothesis are recent findings in which the exposure of human neuronal progenitor cells to cocaine resulted in increases in oxidative stress and up-regulation of pro-inflammatory genes, followed by apoptotic cell death (Poon et al., 2007). Although the persistence and long term consequences of these cellular losses remain unexplored, it is conceivable that these alterations could account for long-lasting functional impairments that have been reported in association with psychostimulant addiction. For example (Gillen et al., 1998) reported cognitive and motor impairments in cocaine addicts following short periods of abstinence. Others have reported that chronic cocaine users exhibited persistent problems in cognitive function, psychomotor speed, and manual dexterity (Bolla et al., 1999). In a recent study investigating the long-term effects of cocaine in mice, Mendez and colleagues reported that 15 days of chronic cocaine administration resulted in cognitive impairments on spatial learning that were

evident as long as three months after the initial exposure (Mendez et al., 2007). It remains unclear whether these effects are the result of toxicity associated with cocaine or simply a manifestation of the withdrawal syndrome associated with abstinence from the drug.

Unlike cocaine, the toxicity of methamphetamine has been widely acknowledged. While the exact mechanism of toxicity is still being debated, increasing evidence suggests that reactive oxygen species are involved. Sulzer et al. (1995) proposed that methamphetamine, which is a weak base, acts via the VMAT to promote the collapse of the transporter's vesicular proton gradients. Such a collapse would result in a redistribution of dopamine from storage vesicles to the cytosol (Mundorf et al., 1999). In addition, methamphetamine rapidly decreases the amount of VMAT in striatal dopaminergic neurons (Eyerman and Yamamoto, 2007). Under these conditions, dopamine would be auto oxidized and produce reactive oxygen species which would damage the cell or impair its function (Cubells et al., 1994). Methamphetamine increases synaptic dopamine by entering into the DAT and reversing the carrier-mediated dopamine uptake system, which would cause the release of dopamine into the synapse (Raiteri et al., 1979). The excessive extracellular dopamine would then be oxidized enzymatically to form dopamine quinones and reactive oxygen species, which would also contribute to increases in oxidative damage.

Chronic methamphetamine exposure results in significant increases in reactive oxygen species and reactive nitrogen species, which can be harmful to dopaminergic neurons (Imam et al., 2001; Thomas et al., 2004; Miyazaki et al., 2006).. Methamphetamine has the ability to diffuse through mitochondrial cell membranes and

disrupt the electrochemical gradients, releasing reactive oxygen species into the cytosol. This increase in oxidative damage can trigger both apoptotic and necrotic cascades leading to neuronal death (for review see (Seiden et al., 2001). Post-mortem studies of brains from methamphetamine addicts are in accordance with a loss of structural and/or functional integrity in dopaminergic systems, revealing significant decreases in total levels of dopamine and its metabolites, DAT, VMAT, and TH (Larsen et al., 2002; Johnson-Davis et al., 2004; Johanson et al., 2006; Cheng et al., 2007)

In accordance with the apparent neurotoxicity, impairments in a number of cognitive and psychomotor tasks are associated with methamphetamine addiction (Simon et al., 2002). For example, methamphetamine abusers were found to display significant disruptions in both verbal memory and motor functions (Volkow et al., 2001b), while others performed significantly poorer on tests that measured memory recall (Simon et al., 2000). It is important to note that various researchers have reported similar findings on the short term effects of methamphetamine, but little is known about the long term effects of the drug on cognition or psychomotor functions.

In summary, there is ample evidence that demonstrates the ability of cocaine and methamphetamine to produce neuroadaptive changes, which may lead to increased oxidative stress and short term impairments in cognitive and psychomotor performance. Neuroadaptive changes impacting on oxidative stress could involve expression of alpha synuclein, TH, DAT, or VMAT.

Aging and the Dopamine System

Aging of the brain involves progressive alterations in various biological processes, including alterations in cellular metabolism, changes in hormonal and neurotransmitter levels, and loss of neuronal function for (reviews see Goldstein, 1993; Albers and Beal, 2000; Butterfield and Kanski, 2001; Yankner et al., 2008). These changes are correlated with mild to moderate degrees of cognitive impairment and losses of psychomotor function that can be distinguished from more severe impairments linked to neurodegenerative conditions such as AD and PD. Indeed in the absence of neurodegenerative disease, aging does not appear to involve any significant loss of neurons in most areas of the cortex and hippocampus (Morrison and Hof, 1997). The underlying causes of functional impairments linked to normal aging are not fully understood, although the oxidative stress theory of aging is the most prevalent. The oxidative stress theory of aging evolved from the free-radical theory proposed by Harman in the 1950's, which suggested that declines in physiological efficiency reflected accumulation of oxidative damage as a result of increasing free-radical attacks on macromolecules (Harman, 1956). Free radicals (molecules with an unpaired electron in an outer shell) could be shown to interact with important cellular components, including DNA, proteins, lipid membranes, and metabolites, thereby disrupting cellular functions and under some conditions producing cell death.

The most abundant and damaging free radicals are referred to as reactive oxygen species (ROS) because they are derived from oxygen metabolism (Gerschman et al., 1954). To regulate ROS generated during the course of energy production by cells, endogenous enzymatic and non-enzymatic antioxidants are present that can remove or

decrease concentrations of local ROS (Cadenas, 1997). However, despite the numerous endogenous antioxidative defenses, it is postulated that there is an inherent and progressively worsening cellular imbalance favoring ROS in most cell types. In accordance with this view, there is an increase in ROS generation with age that is not offset by endogenous antioxidant defenses, resulting in an increasingly pro-oxidizing state within cells. Furthermore, the molecular consequences of the pro-oxidizing state accumulate with age in different tissues, in the forms of oxidatively modified protein, lipid and DNA (for review see (Halliwell, 1984; Halliwell and Gutteridge, 1984). Increases in ROS production are thought to be the primary contributors to age-related increases in oxidative stress, although there is some evidence of age-related decline in selected antioxidant defenses that may also contribute. In any case, during senescence, the imbalance between the amount of ROS and level of antioxidants is hypothesized to damage or peturb cellular processes, which would then result in declines in psychomotor and cognitive performance (Forster et al., 1996; Sohal et al., 2002).

Dopaminergic neurons have several attributes that make them vulnerable to the damaging effects of reactive oxygen species, including high lipid and iron content, and low antioxidant concentration (Dexter et al., 1989; Dexter et al., 1994). Dopamine metabolism is thought to play a major role in the declines of dopaminergic neurons that occur during senescence. Dopamine is a highly reactive molecule and, when not sequestered in synaptic vesicles, can auto-oxidize or be metabolized to form toxic molecules like hydrogen peroxide, superoxide, hydroxyl radicals or dopaquinones (Grahm et al., 1979; Cohen, 1984; Graham, 1984; Slivka and Cohen, 1985). The

potential for “redox-active” dopamine to generate ROS via these mechanisms, coupled with the already high concentrations of iron in dopamine neurons, make them particularly sensitive to the deleterious effects of oxidative stress (Youdim, 1988; Naoi and Maruyama, 1999).

In accordance with the high vulnerability of dopamine neurons to oxidative stress, dopamine-rich areas of the brain, such as the midbrain and striatum, showed relatively greater age-related accumulation of protein oxidative damage in rodents, when compared with other regions such as the cortex, cerebellum and brainstem (Dubey et al., 1996). Furthermore, when compared with other brain regions, the striatum of aged mice exhibited the largest accumulation of iron (Sohal et al., 1999) and the largest pro-oxidizing shift in glutathione redox potential, a sensitive indicator of cellular oxidative stress (Sohal and Forster, 2007). Caloric restriction to lower oxidative stress and decrease oxidative damage accumulation in the striatum is associated with a preservation of motor functions (Dubey et al., 1996).

The integrity and functions of the two major components of the dopaminergic system, the nigrostriatal and the mesolimbic/mesocortical regions, are clearly diminished during aging. It has been widely reported that dopaminergic neurons in the nigrostriatal pathway decline in number 5-10% per decade of life, in accordance with the decline of psychomotor function associated with senescence (Hiral et al., 1968; Carlson and Winblad et al., 1976; Mann and Yates et al., 1983; Emborg et al., 1998; Ma et al., 1999). These apparent losses of integrity with aging are much less severe when compared with pathological degeneration of dopamine neurons in Parkinson’s disease, which may

involve over 90% loss of dopamine-containing cells (Hornykiewicz et al., 1963). Based on these observations, it has been speculated that it is the age-related loss of nigrostriatal dopamine neurons that is linked to mild loss of motor function and confers an increase in risk for more severe loss associated with Parkinson's disease (for review see Fahn, 2003).

Despite the number of earlier reports of dopamine cell loss, others have failed to identify changes in cell number and instead suggested that aging involves a progressive decline in function and integrity of dopamine neurons without cell loss (Cruz-Muros et al., 2007). Evidence for the functional decline of dopamine neurons is based on reports of age-related decreases in dopamine synthesis (Watanabe, 1987; Ota et al., 2006), its release and uptake (Friedemann and Gerhardt, 1992), decreased tyrosine hydroxylase (Haycock et al., 2003), and decreased dopa decarboxylase (Kish et al., 1995). Based on recent studies in which the mesolimbic and nigrostriatal dopamine systems were considered separately, (Cruz-Muros et al., 2007) concluded that in normal aging, the mesolimbic dopamine system was in fact more vulnerable to aging than the nigrostriatal pathway, the former but not the latter subject to an axonal degenerative process. In both the nigrostriatal and mesolimbic regions, aged rats exhibited reduced levels of dopamine, dopa decarboxylase, and phosphorylated tyrosine hydroxylase. Unphosphorylated tyrosine hydroxylase and L-dopa levels remained at levels comparable to younger rats when only the nigrostriatal region was considered. In contrast, greatly reduced levels of unphosphorylated tyrosine hydroxylase and L-dopa were measured in the mesolimbic region of the aged rats. The mesolimbic region of the aged rats also exhibited morphological signs of degeneration in nerve terminals, with accumulations of alpha

synuclein and phosphorylated forms of amyloid precursor protein. Although it is not yet clear whether or not such physiological changes during aging are correlated with behavioral changes, it can be speculated that loss of efficiency in mesolimbic dopaminergic function would contribute to age-related changes in processes of positive reinforcement or motivation.

Other age-related changes in the dopaminergic system have been demonstrated. For example, decreased DAT/VMAT function and expression were associated with the aged dopamine brain system (Bannon et al., 1992; Cohen, 2000). Based on the previous discussion, both of these effects should increase oxidative stress and vulnerability to toxins such as methamphetamine and, indeed, the effects of chronic methamphetamine on dopaminergic functioning were significantly larger on aged animals than on young animals (Miller et al., 2000; Imam and Ali, 2001). Specifically, the aged mice had significantly larger and more persistent depletions of striatal dopamine, DOPAC, and HVA concentrations following methamphetamine. In addition, these animals exhibited increased degeneration and elevations in glial fibrillary acidic protein, both of which are hallmarks of neuronal degeneration.

In summary, these reports demonstrate that the major dopaminergic pathways of the brain are especially vulnerable to oxidative stress, show marked accumulation of oxidative damage, and show significant declines in functional integrity during the aging process. Moreover, aging would appear to confer an enhanced sensitivity to the toxic effects of psychostimulants.

Role of Alpha-synuclein in Dopaminergic Neurotransmission.

Alpha synuclein is a 140-amino acid phospho protein which is abundant in the brain. In the late 1990s, scientists discovered that two forms of familial Parkinson's disease resulted from mutations in alpha synuclein, implicating the protein in the pathology of the disease (Polymeropoulos et al., 1997; Kruger et al., 1998). In addition, there is an increasing body of evidence which implicates impaired alpha synuclein regulation in a number of other neurodegenerative diseases, including Lewy body disease, Multiple Systems Atrophy (MSA), and Alzheimer's disease (for review, see (Dev et al., 2003)). One of the principal commonalities in these pathologies is the presence of insoluble aggregates of alpha synuclein located in degenerated neurons. Although the exact function of alpha synuclein remains a matter of debate, recent discoveries have suggested the protein plays a primary role in the trafficking of storage vesicles throughout the cell. Abnormal alpha synuclein in yeast resulted in disruption of ER-Golgi vesicular trafficking and enhanced toxicity (Cooper et al., 2006).

In addition to its possible role in trafficking, there is also evidence linking abnormal expression of the protein with disruption of dopamine neurotransmission. Several lines of evidence support this hypothesis: 1) Alpha synuclein can bind to, and alter the number and function of membrane DAT (Lee, 2001; Wersinger and Sidhu, 2003a); (2) Alpha synuclein has an inhibitory effect on tyrosine hydroxylase activity by increasing the amount of the less active form of enzyme i.e., the dephosphorylated form (Perez et al., 2002); (3) Mutations of alpha synuclein in MESC2.10 cells result in down-regulation of vesicular monoamine transporter (VMAT) and enhanced cytoplasmic dopamine,

indicating the protein may regulate vesicular dopamine storage (Lotharius et al., 2002); (4) Mice lacking alpha synuclein exhibit a reduction in striatal dopamine, altered dopamine release, and decreased locomotor responses to amphetamine (Abeliovich et al., 2000); (5) Overexpression of alpha synuclein in Drosophila melanogaster, results in degeneration of dopaminergic neurons and motor deficits (Feany and Bender, 2000). Age-related increases in alpha-synuclein are associated with nigrostriatal dopamine depletion in monkeys (Chu and Kordower, 2007)

Although it must be noted that the exact function of alpha synuclein, the long term effects of altering its expression, and the changes in alpha synuclein expression following chronic psychostimulant administration remain poorly understood, it is well established that alterations in alpha synuclein expression can have effects on proteins that are involved in dopaminergic neurotransmission. For example Mash et al., (2008) reported that alpha synuclein protein levels are increased in serum from recently abstinent cocaine abusers. It was reported by Walker and Grant, (2006) that peripheral blood alpha synuclein mRNA levels are elevated in cynomologus monkeys that chronically self-administer ethanol. Taken together these reports add to the idea that alpha synuclein plays a major role in the regulation of dopamine neurotransmission.

Rationale for the Current Studies

Previous research has suggested that long-term abuse of either cocaine or methamphetamine can elicit both neuroadaptive changes and toxic effects, which can both impair dopamine neurotransmission and adversely affect psychomotor and cognitive

functions. Based on the evidence presented in the previous sections, it was hypothesized that a major cause of these adverse effects is an increase in oxidative stress affecting mesolimbic and nigrostriatal dopamine neurons, linked to pro-oxidizing intracellular and extracellular concentrations of dopamine itself. Neuroadaptive shifts in activity and/or expression of TH, DAT, VMAT, and alpha synuclein, elicited by chronic psychostimulant use, were postulated as key elements in the initiation and maintenance of this process, based on their respective roles in regulating intracellular and extracellular concentrations of dopamine. The current studies addressed this general hypothesis using an *in vivo* rodent model involving continuous sub chronic administration of cocaine or methamphetamine. Following psychostimulant treatment and recovery, the mice were tested for adverse effects on cognitive and psychomotor performance using a behavioral test battery, and subsequently the brain concentrations of oxidized protein and lipid were measured as signatures of oxidative stress. Concurrently, expression of DAT, TH, and alpha synuclein were determined. If the behavioral consequences of psychostimulant use were linked to oxidative stress elicited by adaptive shifts in DAT, TH, or alpha synuclein, it was expected that these elements would be affected concurrently.

An important goal of the current study was to address the possibility that, because of their ability to elicit oxidative stress, psychostimulants could lead to an acceleration of age-related losses of function in the oxidation-sensitive nigrostriatal or mesolimbic dopamine systems that are linked to age-related declines in functional performance. This hypothesis was addressed by comparing separate groups of the mice for oxidative damage, DAT, TH and alpha synuclein, and for impairments of cognitive and

psychomotor function, at different times (i.e. at different ages) following the psychostimulant treatments. If psychostimulant treatment results in acceleration of age-related changes in mesolimbic or striatal dopamine systems, this would be detected as a difference in the rate of age-associated changes in dopamine functional markers, oxidative damage, and age-related functional declines.

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CHAPTER II

MATERIALS AND METHODS

One hundred and forty-four male C57BL/6JNia mice were obtained from the National Institute on Aging when 10 months of age. The mice were acclimated to the UNTHSC vivarium under a 12-hour light/dark cycle beginning at 0700 hrs for 1 week prior to treatment and were maintained in the same room for up to 7 months. They were housed two per cage in clear polycarbonate cages (12 x 17 x 12.5 cm) and had access to food and water *ad libitum*. The colony room was maintained at 23 ± 1°C. Serological testing on sentinel mice revealed no positive results during the period of this experiment. The mice were euthanized by cervical dislocation after the experiment and brain tissue was quickly dissected and frozen for later analysis. All procedures involving the mice were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center at Fort Worth.

Experimental design

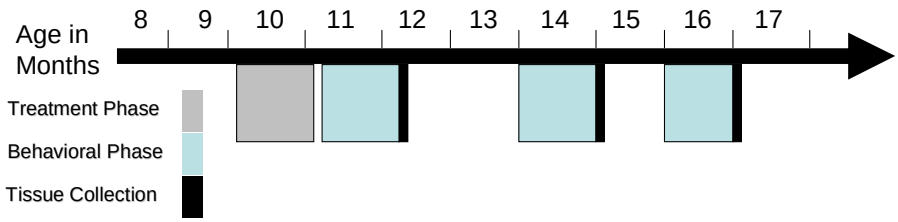
Figure 1 depicts a flow diagram of the experimental design, which included a treatment phase followed by behavioral testing of the mice at different ages. Following 2 weeks of acclimation, the mice were assigned to one of three treatment groups (n= 47-48) that received cocaine (40 mg/kg/day), methamphetamine (2 mg/kg/day), or an equivalent volume of the vehicle (0.9% saline) for a period of 4 weeks. Each treatment group was further subdivided and assigned to be tested when they were approximately 11, 14, or 16 months of age (i.e., 1 week, 3 months, or 5 months following the end of the treatment

phase). Based on previous studies of psychomotor function in C57BL/6 mice (e.g., de Fiebre et al., 2006), it was expected that performance of the control groups would undergo detectable but modest decline over the period from 11 to 16 months, an outcome that would maximize ability of the experimental design to detect an accelerated decline in the treatment groups. The sample size for each of the age x treatment groups, assigned *a priori*, was 16 based on a power analysis performed using variance estimates for data from 300 untreated mice previously tested in the Morris water maze. The effect size used in the analysis was based on one predicted outcome of the study, that orderly degrees of impaired performance would be observed at each age after the treatment exposure when compared to control groups, with a maximum impairment of 70 to 80% of the difference between young and old controls. A power greater than 90% was obtained for detection of this outcome in a 2-way analysis of variance with an alpha level set to 0.01. The sample sizes in the completed experiment ranged from 12-19 mice per group.

During the treatment phase, animals were implanted with Alzet mini-pumps for 4 weeks, after which the pumps were removed and the mice were tested for psychomotor and cognitive performance using an age-sensitive battery of behavioral tests at the three testing periods (see Figure 1; Table 1.) Both body weight and food intake were recorded over the course of the treatment phase and during the testing period. The mice were euthanized 3 to 7 days following the last behavioral test for removal of brain tissue. The brains were harvested and dissected into 6 regions: cerebellum, cortex, hippocampus, striatum, hindbrain, and midbrain. The tissue was then analyzed using Western blots and biochemical assays for oxidative damage.

Figure 1. Timelines of cocaine or methamphetamine treatment and behavioral testing. Following 2 weeks of acclimation, the mice were assigned to one of three treatment groups (n=47-48) that received continuous infusions of cocaine (40 mg/kg/day), methamphetamine (2 mg/kg/day), or an equivalent volume of the vehicle (0.9% saline) for a period of 4 weeks. Each treatment group was then subdivided and assigned to be tested at approximately 11, 14, or 16 months of age. After the testing phase, various brain regions were collected and analyzed for oxidative damage and protein expression as described in this section.

Figure 1.


Age in Months
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Treatment Phase
Behavioral Phase
Tissue Collection

Cocaine and methamphetamine infusion

On day one of the treatment schedule, each mouse was surgically implanted with an osmotic mini-pump continuously infusing 40 mg/kg/day of cocaine, or 2 mg/kg/day, of methamphetamine, or saline for 4 weeks. Alzet osmotic pumps (model 2004 from DURECT Corporation, PO Box 530 Cupertino, CA, 95015-0530) were filled with appropriate concentrations of methamphetamine, cocaine, or saline for continuous infusion of the intended doses. The pumps were primed by warming them in a beaker of saline in a water bath at 37°C for 4 hours prior to surgical implantation. The minipumps were modified by adding a microdialysis fiber to the output portal to disperse the drug over a wider surface area, thereby allowing for the continuous infusion of high doses of cocaine without the development of necrotic skin lesions. The mice were injected with (0.2cc) lidocaine (Abbott) at the dorsal midline incision site and then anesthetized by inhalation with Isoflorane (Vedco, Inc.). A 2 cm vertical incision was made with scissors and a large subcutaneous pocket was formed. The minipump was then inserted into this pocket with the delivery portal toward the head. The opening was closed with metal surgical autoclips. After 4 weeks the pumps were removed using the same procedure and the residual amount of liquid measured.

Analysis of dopamine markers

The samples were homogenized at 4°C in 20mM Tris, pH 7.4, 150nM NaCl, 5 mM EDTA, 5mM EGTA, and 2% Triton X-100, 0.1% SDS containing protease inhibitor cocktail *(Roche Diagnostics, Indianapolis, IN)*. The protein concentrations were determined using the BCA method. Equal concentrations of protein extract

(approximately 5-20 µg total protein) were dissolved in SDS-sample buffer (0.5ml dH20, 200 mM Tris-HCl pH 6.8, 40% glycerol, 0.07% Bromphenol blue, and 400 mM SDS), and separated on 15% gel using gel electrophoresis at a voltage of 100 V for 2 hours. The gels were then electroblotted onto nitrocellulose membrane at a voltage of 100V for 1 hour *(Bio-Rad, Richmond, CA)*. Membranes were blocked in 5% skim milk in TBS-Tween 20, overnight at 4°C. The membranes were then incubated with anti-tyrosine hydroxylase (1:1000), anti-DAT(1:500) *Chemicon, Inc., Temecula, CA*, anti-alpha synuclein (1:500) *Santa Cruz Biotechnology, Santa Cruz, CA* or anti-beta actin antibodies (1:10,000) *Chemicon, Inc., Temecula, CA*, over night at 4°C. Membranes were washed 3 X 10 min with TBS-Tween-20 and incubated with the secondary antibodies at room temp for 1 hr, following which membranes were washed 3 X 10 min with TBS-Tween-20 followed by washing 2 X 5 min with TBS. Bound antibodies were visualized using enhanced chemiluminescence (*ECL, Amersham Pharmacia Biotech*).

Quantification of oxidative damage

The amounts of macromolecular oxidative damage were analyzed using two techniques according to established protocols (Sumien et al., 2005).

Protein carbonyls. The method used for carbonyl analysis was modified from Levine et al. (1994). The samples were homogenized in buffer containing 5mM sodium phosphate, pH 7.4, 5mM EDTA, 2mM BHT and 0.1% Triton X-100, and protease inhibitors(1 tablet/10ml,Roche Diagnostics, Indianapolis, IN). The samples were then centrifuged at

1000 rpm for 5 min at 4°C. Supernatant was saved and BCA protein assay was employed to determine protein concentration of each sample. Brain homogenates were diluted in homogenizing buffer to a protein concentration of 1mg/ml. Homogenates were added to 2N HCL for blanks or 10 mM dinitophenylhydrazine (DNPH) in 2N HCl for samples in a 1:5 ratio. The samples were then incubated for 1 hour at room temperature in the dark, following which the proteins were precipitated by the addition of 10% trichloroacetic acid (TCA), and then centrifuged at 5000 rpm for 5 minutes. After discarding the supernatant, the pellets were washed at least 3 times with 1ml ethanol/ethyl acetate (1:1). The samples were then dissolved in 500 μl of denaturing buffer (100 μM of sodium phosphate buffer with 3% SDS at pH 6.8) overnight, and read at 360 nm. The protein concentration was determined using BCA protein assay and read at 278 nm on a spectrophotometer. Using the final protein concentration and the extinction coefficient of DNPH (22.0 mmol/cm), nmols of carbonyl/mg of protein was calculated.

Thiobarbituric acid reactive substances (TBARS). Measurement of TBARS was done according to the method of Ohkawa et. al. (1979). Fifty microliters of brain homogenate were added to 300 μl of 20% acetic acid (pH 3.5), 40 μl 8.1% of SDS, 110 μl of water and 300 μl of thiobarbituric acid (TBA). To get a standard curve, 0, 10, 20, 30, 40 or 50 μl of 1,1,3,3-tetramethyoxypropane (TMP, 6.07 μM) was adjusted to a final volume of 50 μl, with 40 % EtOH. The standards and samples were then capped and incubated at 95°C for 1hr. After incubation the samples were allowed to cool to room temperature and were then centrifuged at 3,000 rpm for 10 min. Following which the samples and standards were read on a fluorometer (excitation: 525 nm, Emission: 550 nm, k=5). Using the

standard curve the amount of TBARS was calculated as nmol TBARS/mg protein.

Behavioral Experiments

In order to assess the long- and short-term effects of the cocaine and methamphetamine treatments on nervous system function, mice were administered selected components (summarized in Table 1) of a test battery described previously (Sumien et al., 1996; de Fiebre et al., 2006) for detecting age-related declines in different psychomotor and cognitive functions. While control groups in these studies were expected to exhibit modest declines in psychomotor performance over the ages tested in these studies, only small declines (if any) of cognitive performance were expected (e.g., de Fiebre et al., 2006). While not a bioassay of dopaminergic function, per se, the psychomotor tests are sensitive to cortical, striatal and cerebellar dysfunction (Table 1). Ability of mice to learn the spatial swim test is thought to be dependent upon intact function of the hippocampus (Burke & Barnes, 2003), whereas learning of the reversal component additionally requires frontal cortical function.

Tests of reflexive and psychomotor function

Locomotor activity. Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously (Forster and Lal, 1991). Each mouse was placed in a clear acrylic test cage (40.5 x 40.5 x 30.5 cm) that was surrounded by a metal frame lined with photocells. The test cage was enclosed in a dimly-lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). During a 16-min period, movements in the horizontal plane as

well as a vertical plane 7.6 cm above the floor were detected by the photocells and processed by software to yield 4 different variables describing horizontal, vertical, stereotypic, and spatial components of spontaneous activity in the apparatus.

Wire suspension. Mice were administered the wire suspension test for four consecutive days (2 trials/day). For each trial (lasting a maximum of 60 s), the mouse was allowed to grip a horizontal wire with the front paws when suspended 27 cm above a padded surface. The latency to tread (a reflexive grasping of the wire with the hindlimbs) and the latency to fall were recorded and averaged over the four sessions. In C57BL/6 and heterogeneous (HET) mice, there is a marked age-related decline in wire suspension performance attributable to loss of hindlimb and grip strength (Sumien et al., 2004; 2006).

Bridge walking. Each mouse was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges, each mounted 50 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each bridge was presented three times, and the measure of performance was the average latency to fall (up to a maximum of 60 s) across all bridges. The reliability and age sensitivity of this measure has been described for groups of C57BL/6 mice (de Fiebre et al., 2006).

Coordinated running. Motor learning and maximum running performance were measured using an accelerating rotorod test described previously (Forster and Lal, 1999). The apparatus was a motor-driven treadmill (Accuscan Instruments, Model # AIO411RRT525M) that consisted of a 3-cm diameter nylon cylinder mounted

horizontally at a height of 35 cm above a padded surface. On each trial, the mouse was placed on the cylinder, which then began rotating with increasing speed until the animal fell to a well-padded surface. Ability of the mice to improve running performance was assessed in a series of training sessions (two per day), each consisting of four trials at 10-min intervals. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to show improvement over three consecutive sessions, which represented each mouse's maximum stable level of performance. In previous studies, the average latency to fall on the final session was consistently shorter in aged when compared to young mice (Sumien et al., 2004; 2006).

Tests of cognitive function

Spatial learning, memory, and cognitive inflexibility (reversal learning) were measured using a swim maze test as described previously (Forster et al., 1996, de Fiebre et al., 2006). On a given trial, the mouse was allowed to swim in a 120-cm diameter plastic tank filled to 34 cm from the top edge with colored water (non-toxic white paint) that was maintained at 24 ± 1º C. An escape was provided by means of a small 10 × 10-cm platform hidden from view 1.5 cm below the surface of the water. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (San Diego Instruments, San Diego CA, Model # SA-3).

During a *pretraining* phase, the tank was covered by a black curtain to prevent pre-exposure of the mice to visual cues present outside of the tank. In this way, mice learned the motor components of swimming and climbing onto the platform without learning its

location in the tank. On each trial, the mouse was placed at one end of a 10 × 65-cm (W × L) straight alley that had a platform at the other end, and allowed to swim until it reached the platform or a maximum latency of 60 s had elapsed. The mice were given four sessions of pretraining (two per day), each consisting of five trials spaced at 5-min intervals. The pretraining phase data were considered in the assessment of the effects of treatment on psychomotor function, as the swimming reflex differs qualitatively from ambulation in terms of the forelimb and hindlimb movements involved.

After pretraining, the black curtain was removed from above the tank, and the mice were tested for their ability to learn the location of the platform using spatial cues. Testing for spatial performance was divided into three phases: acquisition (eight sessions with the platform in a fixed location), retention (two additional sessions after a 66-h delay interval), and reversal (four sessions with the platform at a new, fixed location). Each session consisted of five trials, at 10-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Two sessions were conducted per day, separated by a period of at least 2 h during which the mice were returned to the home cages. After the fifth trial of session 8, a probe trial was given in which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it. On this trial, spatial bias for the platform location was evaluated in terms of the (i) percentage of time spent in the platform quadrant, (ii) percentage of time spent within 40- and 20-cm diameter annuli surrounding the platform location, and (iii) entries into the platform zone itself. A total learning index was defined as the average

path length that each mouse took to reach the platform on sessions 2-4 during acquisition (acquisition learning index) and sessions 12-14 during reversal (reversal learning index). Maximum spatial learning was defined as the minimum path length that each mouse took to reach the platform over the two final sessions of initial acquisition (sessions 7 and 8).

Statistical analysis of data

For analysis of dopamine markers, oxidative damage, and most behavioral tests, the results for each measure were subjected to a two-way analysis of variance and the effects of Age and Treatment (3 x 2) were considered as between-groups factors. Separate analyses were performed considering cocaine or methamphetamine as the treatment in each. Restricted sets of planned individual comparisons between age groups (14 or 16-month-old vs 11 month-old within each treatment) and treatment groups (methamphetamine or cocaine vs saline at each age), were performed using single degree-of-freedom F tests within the Age x Treatment interaction. For experiments involving repeated measurements (i.e., rotorod performance, body weight, spatial swim maze measures), three-way analyses were performed with Time or Sessions as a within-groups factor. The alpha level was set at 0.05 for all analyses of variance and individual comparisons. Pearson correlation analyses were performed to probe for the degree of association, across all individual and experimentally- induced variation, between measures of behavioral performance and oxidative damage. Based on reproducible associations reported in previous studies involving brain/behavior relationships, a criterion of r = 0.5 or higher, accounting for 25% of the variance, was determined, *a priori* as indicating a biologically significant relationship (Thangthaeng et al., 2008).

Table 1: Summary of Behavioral Test Battery

Behavioral Test	Capacity	Brain Region	No. of Sessions	No. of Days
Psychomotor				
Wire suspension	reflex, strength	cortex	1	1
Bridge walking	balance; coordination	cerebellum; striatum	4	4
Rotorod	coordination; balance	striatum; cerebellum	8-11	5-6
Alley swim	Coordination	cortex; striatum	4	2
Cognitive				
Swim Maze:				
Acquisition	spatial learning; memory	hippocampus	8	4
Retention	spatial memory	hippocampus	2	1
Reversal	cognitive flexibility	frontal cortex	4	2

CHAPTER III

RESULTS AND DISCUSSION

General assessment: food and water intake, weight, and locomotor activity

To provide an assessment of the effect of treatments on the general health and energy balance of the mice in these studies, food and water intake, body weight, and locomotor activity were measured during the treatment period (Table 2). In general, the saline control groups consumed both more food and more water than psychostimulant-treated groups, and the methamphetamine-treated mice, overall, consumed the least amount of both water and food when compared to the control and cocaine-treated groups. However, analysis of variance revealed that there was no effect of either treatment on food, weight, or water intake. Analysis also failed to indicate any significant effect of cocaine or methamphetamine on locomotor activity during the treatment period (all p values > 0.05).

Effect of Psychostimulants on Psychomotor Performance during Aging

A battery of tests of psychomotor function was administered to separate groups of the psychostimulant treated mice after they had reached 11, 14, or 16 months of age. These tests had different requirements for strength, balance, and sensorimotor coordination and based on previous studies were expected to reveal modest age differences in performance of the control mice.

[1] Values for food/water intake and body weight represent the average of 48 cocaine, 47 methamphetamine, and 47 saline- treated mice (mean ± SE). Food and water intake was measured during the second week of the pretreatment phase, while the body weights were taken during the first and fourth week.

[2] Arousal levels were assessed from locomotor activity counts in the horizontal plane of the test apparatus on week 4 of the treatment phase. Values for locomotor activity levels represent a subset (N=12) of the mice in each treatment group.

Table 2: Food/water Intake, Body Weight and Locomotor Activity of Mice during Cocaine or Methamphetamine Treatment

	Treatment Group		
Assessment	**Cocaine**	**Methamphetamine**	**Saline**
Intake [1]			
Food(g/day)(2w)	5.27±0.22	5.13±0.29	5.56±0.13
Water(ml)(2w)	5.12±0.21	4.96±0.34	5.39±0.31
Body Weight(g)[1]			
Week 1	31.35±0.59	31.17± 0.55	31.03± 0.28
Week 4	32.12±0.44	31.89±0.67	32.3±0.46
Locomotor Activity[2]	2579±65.4	2538±83.03	2446±73.70

Treading reflex and wire hanging test

The effects of age and treatment for methamphetamine and cocaine on wire hanging tests are shown in Figure 2 (upper panels). Significant differences ($p<0.05$) for planned comparisons between age and treatment groups are indicated in the figure. The control groups showed a significant delay in the treading reflex (raising and gripping the wire with the hindpaws) from 11 to 14 months of age, although there was no further increase in treading latency from 14 to 16 months. The 11-month-old control group performed the wire tread reflex an average of 12.1 seconds better than the 14-month-old group and 9.4 seconds better than the 16-month-old group.

In contrast to the control group, the methamphetamine group (left panel) showed a nearly linear increase in latency to tread. The 11-month-old methamphetamine group had a latency to tread that that was an average of 9.6 seconds shorter than the 14-month-old group and 14.5 seconds shorter than the 16-month-old group. Impairments were evident in the performance of the 16-month-old methamphetamine groups when compared to the age-matched controls, with the 16-month-old control group performing the wire tread reflex an average of 10.7 seconds faster than the 16-month-old methamphetamine group.

The cocaine groups showed age differences in treading latency that paralleled those in the control group, with significant increases occurring from 11 to 14 months, but no further increase by 16 months. However, in contrast to the effect of methamphetamine, the cocaine-treated mice performed more poorly (by 7.3 to 7.9 seconds) than controls at 11 and 14 months, but not at 16 months of age.

For the 2 x 3 analyses of variance performed separately for cocaine and methamphetamine, there were significant main effects of Age, Cocaine, and Methamphetamine on the treading reflex latency (all p values < 0.008), but no significant interaction of age with either treatment (all p values > 0.232).

On the latency to fall measure for the wire hanging test, the control groups showed a significant decrease in latency from 11 to 14 months, but no further decrease was evident after 14 months of age. The 11-month-old control group was able to remain on the wire an average of 18.7 seconds longer than the 14-month-old group and 16.9 seconds longer than the 16-month-old group. Overall, the methamphetamine-treated animals showed a significantly shorter latency to fall from the wire at all three ages, and showed a greater decrease in latency as a function of age when compared with the control groups. The 11-month-old methamphetamine group was able to remain on the wire an average of 16.4 seconds longer than the 14-month-old group and 22.1 seconds longer than the 16-month-old group. The 11- and 16-month-old methamphetamine groups were significantly impaired when compared to their age-matched control groups. On average, the 11-month-old control group was able to remain on the wire 7.9 seconds longer than the age-matched methamphetamine group, while the 16-month-old control group was able to remain on the wire 13.1 seconds longer than the age- matched methamphetamine group.

Significant decreases in latency from 11 to 14 months of age were evident in the cocaine group, but this group showed increases from 14 to 16 months, to a performance level that matched that of the 16-month-old controls. The 11-month-old cocaine group was able to remain on the wire an average of 13.6 seconds longer than the 14-month-old

group. Both the 11- and 14-month-old cocaine groups were significantly impaired when compared to their age-matched control groups. The 11-month-old control group was able to remain on the wire an average of 14.2 seconds longer when compared to the 11-month-old cocaine group, and the 14-month-old control group was able to remain on the wire an average of 9.1 seconds longer than the 14-month-old cocaine group.

The analyses of variance performed on cocaine and methamphetamine for latency to fall from the wire each revealed an overall effect of Age, Cocaine, and Methamphetamine, as well as an interaction between Methamphetamine and Age (all p values <0.008).

Bridge walking test

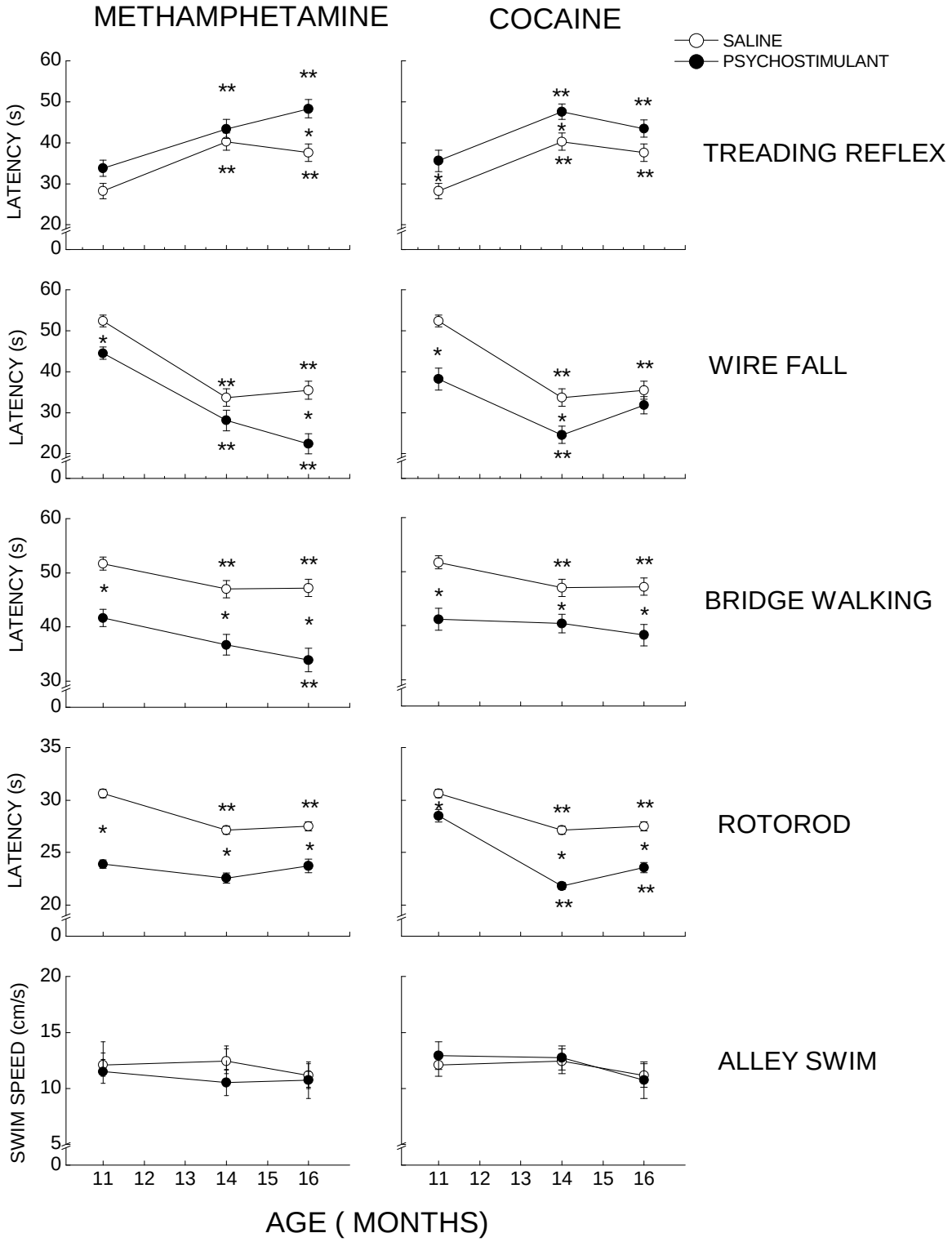
The effects of age and treatment on performance of the mice on the bridge test are shown in Figure 2 (middle panels). An age-related decrease in ability to remain on the bridges was evident in all three treatment groups, and this resulted in a significant main effect of Age ($p<0.003$) when data were analyzed for cocaine or methamphetamine. Planned comparisons confirmed that the control groups at 14 and 16 months showed a shorter latency to fall from the bridge when compared with the 11-month-old controls.. Both the methamphetamine- and cocaine-treated mice in all three age groups were markedly impaired in their bridge-walking performance when compared to their age-matched controls. The 11-month-old control groups were able to remain on the bridges an average of 10 seconds longer than the 11-month-old methamphetamine mice and 10.5 seconds longer than the 11-month-old cocaine group. At 14 months of age the control groups were able to remain on the bridge an average of 10.3 seconds longer than the

methamphetamine mice and 6.4 seconds longer than the age-matched cocaine treated mice. At 16 months of age the control groups were able to remain on the bridge an average of 13.3 seconds longer than the methamphetamine-treated mice and 8.9 seconds longer than the cocaine-treated mice. Analyses of variance conducted on the bridge-walking test results for both cocaine and methamphetamine revealed an overall effect of Age, Methamphetamine, and Cocaine (all p values<0.003) but there were no interactions between the any of the factors (all p values >0.493).

Straight alley swim test

As an additional indicator of psychomotor performance, the speed of swimming during the pretraining phase of the swim maze test was considered. The bottom panels of Figure 2 show the average speed over the four sessions (five trials each) in the 65-cm straight alley described in Materials and Methods. The 11-month-old cocaine group had the highest average swim speed at 12.9 cm/s while the 14-month-old methamphetamine group had the slowest speed at 10.5 cm/s. However, in contrast to the results for other measures of reflexive and psychomotor performance, analyses of variance and planned comparisons for both cocaine and methamphetamine failed to reveal significant effects of Age, Treatment, or the interaction of those factors (all p values >0.434)

Figure 2. Performance of psychostimulant-treated mice on behavioral tests of reflexive and psychomotor performance. Ten- month-old mice received, for a period of 30 days, continuous infusions of saline, methamphetamine (2 mg/kg/day, left panels), or cocaine (40 mg/kg/day, right panels). The treatments were then discontinued and after 1 week, 3 months, or 5 months (when the mice were 11, 14, or 16 months of age), the mice received the behavioral tests. Values represent the mean ± SE for the 11-month-old saline group (N=18), 11-month-old methamphetamine group (N=19), 11-month-old cocaine group (N=11), 14-month-old saline group (N=16), 14-month-old methamphetamine group (N=13), 14-month-old cocaine group(N=17), 16-month-old saline group (N=12), 16-month-old methamphetamine group (N=15), and 16-month-old cocaine group (N=15). * Denotes a significant difference between psychostimulant-treated and age-matched control groups ($p<0.05$). **Denotes a significant difference from the 11-month-old group of matching treatment ($p<0.05$). Note that broken ordinate scales are shown in each of the panels of this figure, and that data for the control group are plotted in both the left and right panels.


METHAMPHETAMINE
COCAINE
SALINE
PSYCHOSTIMULANT
TREADING REFLEX
WIRE FALL
BRIDGE WALKING
ROTOROD
ALLEY SWIM
LATENCY (s)
SWIM SPEED (cm/s)
AGE (MONTHS)

Rotorod test

The effect of age and psychostimulant treatment on the performance of mice on the rotating rod tests are shown in figures 2 and 3. The rotorod results are summarized in figure 2 (for comparison with the other psychomotor test results), as the average latency to fall from the rotating cylinder over testing sessions 1 through 7. The control groups had a decreased level of performance from 11 to 14 months of age, with no further decrease evident after 14 months. Overall, the mice in the11-month-old control group were able to remain on the rotating rod an average of 3.51 seconds longer than the 14-month-old control groups and 3.14 seconds longer than the 16-month-old control groups. Performance of the methamphetamine groups was poor relative to controls at all ages and did not show any significant change as a function of age. The 11-month-old methamphetamine mice on average remained on the rotating rod 6.7 seconds less than their age-matched controls. The 14- and 16-month-old methamphetamine groups on average remained on the rotating rod 4.6 seconds and 3.8 seconds less than their age-matched controls, respectively.

The cocaine-treated groups showed an apparent decrease in performance from 11 to 14 months of age, but no further decrease was evident after 14 months. Both the 14- and 16-month-old cocaine groups had significantly poorer performance when compared to the 11-month-old cocaine group. The 11-month-old cocaine groups were able to remain on the rotating rod an average of 6.7 seconds longer than the 14-month-old cocaine groups, and 4.9 seconds longer than the 16-month-old group. The cocaine-treated mice had significant impairments across all three age groups when compared to their age-matched

controls. Overall the 11-month-old cocaine groups on average remained on the rotating rod 2.1 seconds less than their age-matched controls. The 14-month-old cocaine animals remained on the rotating rod 5.3 seconds less than their age-matched controls and the 16-month-old cocaine groups remained on the rotating rod 3.9 seconds less than their age-matched controls.

Analyses of variance performed on the rotorod results shown in figure 2, revealed significant main effects of Age, and Cocaine in addition to the interaction of those factors (all ps <0.001). While a main effect of Methamphetamine was observed, there was no interaction between these factors.

Effect of psychostimulant treatment on motor learning

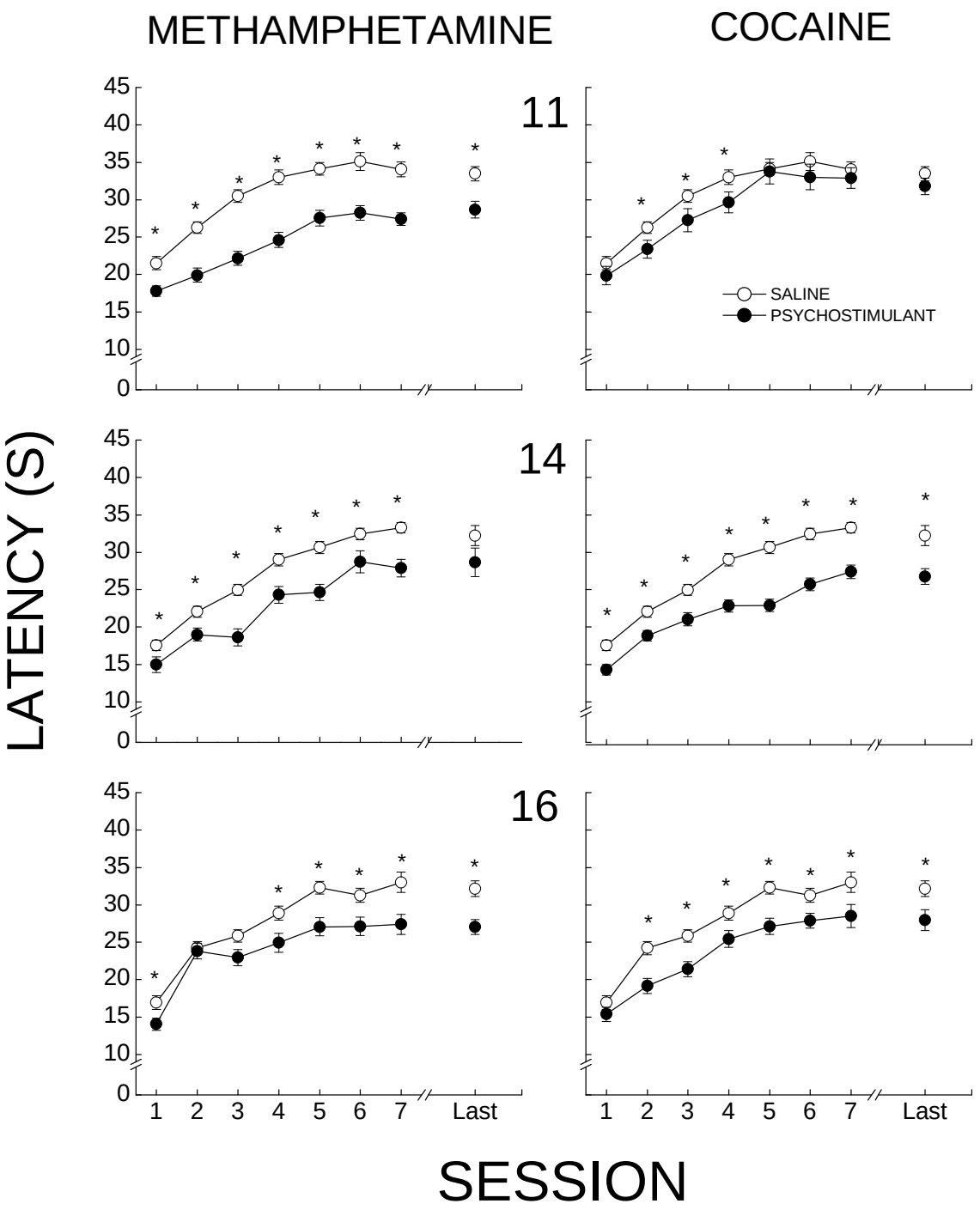
To determine whether or not psychostimulant treatments possibly influenced motor learning independently of motor performance capacity, the session by session data were considered in statistical analyses (figure 3). Control groups were able to improve their level of performance from session 1 to session 7 in all three age groups. In the 11-month-old control group the animals in general reached a peak level of performance at session 6, while both the 14- and 16-month-old groups reached a peak level of performance at session 7. The 11-month-old and 14-month-old methamphetamine-treated mice reached a level of peak performance during session 6. The peak level of performance was achieved at session 7 for the 16-month-old methamphetamine group.

Overall, impairments of running performance were evident in the 11-month-old and 14-month-old methamphetamine groups across all of the sessions tested. For the 16-

month-old methamphetamine group, significant impairments were evident on sessions 4 through 7 and on the initial session. After reaching their stable maximum level of performance, the control groups remained on the rotating rod an average of 4.8 seconds longer than the 11-month-old methamphetamine group. The 14-month-old control group, upon reaching a stable maximum level of performance, was able to remain on the rotating rod an average of 3.6 seconds longer than the 14-month-old methamphetamine group and 5.5 seconds longer than the 14-month-old cocaine groups. The 16-month-old control group, upon reaching a stable maximum level of performance, was able to remain on the rotating rod an average of 5.1 seconds longer than the 16-month-old methamphetamine groups, and 4.2 seconds longer than the 16-month-old cocaine animals.

Coordinated running impairment was evident in the 11-month-old cocaine group on sessions 2 through 4, although this group was able to reach a performance level achieved by the 11-month-old control mice on their final session. Impairments were evident in the 14-month-old cocaine-treated mice on all sessions across the test, and this was also the case for the 16-month-old cocaine animals except for the first testing session. Analysis of data for both cocaine and methamphetamine revealed that there was an overall effect of Age and Treatment in addition to an Age x Treatment x Session interaction (all p's <0.019).

Figure 3. Coordinated running performance measured by latency to fall from an accelerating rotorod as a function of treatment, age, and testing session. Each value represents the mean ± SE. * Denotes a significant difference between treatment group and age-matched control for a particular session ($p<0.05$). The number of mice in each group is the same as indicated in the legend to figure 2. Note that broken ordinate scales are shown in each of the panels of this figure, and that data for control groups are plotted in both the left and right panels.


METHAMPHETAMINE
COCAINE
11
14
16
LATENCY (S)
SESSION
SALINE
PSYCHOSTIMULANT
0
10
15
20
25
30
35
40
45
1
2
3
4
5
6
7
Last

Effect of Psychostimulants on Cognitive Performance During Aging

Cognitive performance in the psychostimulant-treated mice of different ages was assessed by considering their session by session performance during the acquisition, retention, and reversal phases of the swim maze test described in the Materials and Methods section and Table 1 (see figure 4).

Swim maze learning, retention, and reversal

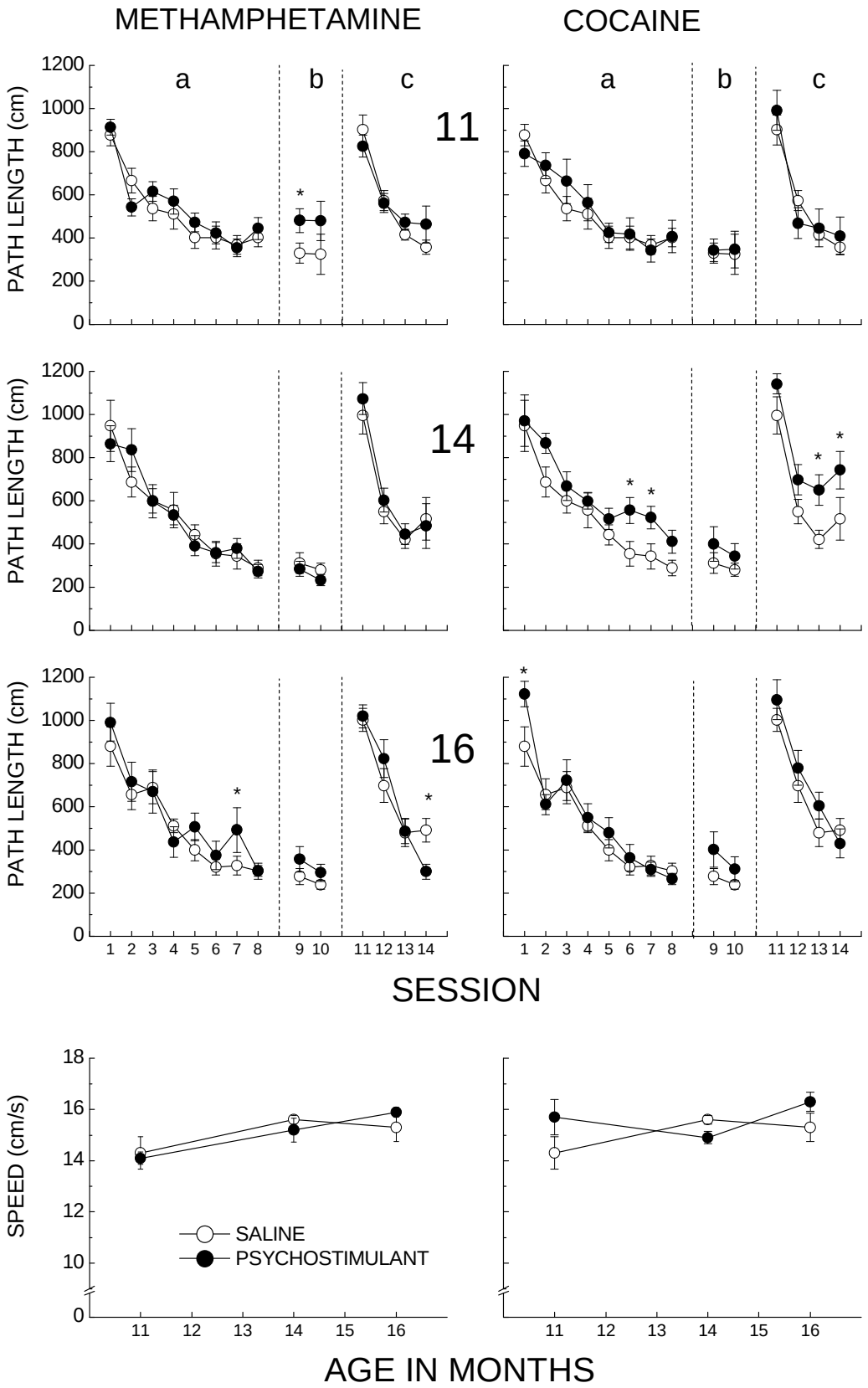
In accordance with data from the pretraining phase (see figure 2), there was no overall effect of either age or treatment on the swim speed over the 14 sessions in which the mice were trained for spatial learning, retention, and reversal (figure 4, bottom panels). The control mice in all three age groups were able to learn the location of the platform as evidenced by the decreasing path length across most of the sessions during the acquisition phase (figure 4, upper panels). Control groups showed their longest distance to find the platform on the first session of tests in all three age groups, and were able to locate the platform traversing the shortest distance by the eighth session of the acquisition phase. During the retention phase, control mice in all three age groups were able to remember the location of the platform as evidenced by path lengths that were nearly equivalent or better than the most efficient acquisition sessions. During the reversal phase, the control mice were able to learn the new location of the platform, which was evidenced by a decrease in path length over sessions 11 through 13. There was no further decrease in path length on session 14 for the 16- and 14-month-old control groups.

During the acquisition phase, both the methamphetamine- and cocaine-treated mice

exhibited peak distance in path length on session 1 which was evident in all three age groups. Both the 14- and 16-month-old psychostimulant groups had peak performance on session 8 of the acquisition phase. The 11-month-old cocaine and methamphetamine groups exhibited peak performance on session 7. The psychostimulant-treated animals were able to learn the location of the platform as evidenced by the decreasing path length exhibited across most of the tests sessions in the acquisition phase. This effect was evident across all three age groups tested. During the retention phase, which began 66 h following session 8, all of the psychostimulant-treated groups had path lengths that were nearly equivalent or shorter than their path lengths on session 8.

During the reversal phase, the methamphetamine-treated mice learned the new location of the platform as evidenced by the decrease in path length for the 11- and 16-month-old groups over sessions 11 through 14. The 14-month-old methamphetamine groups had decreasing length to platform on sessions 11 through 13. Similarly, the 11 and 16-month-old cocaine-treated groups were able to learn the new location as evidenced by decreasing path length over sessions 11 through 14. However, it was apparent that the 14-month-old cocaine groups had difficulty learning the new location, as there was a decrease in path length on sessions 11 and 12, but no further decrease thereafter. The only impairments associated with methamphetamine treatment occurred during the retention phase in the 11-month-old group, where methamphetamine-treated mice performed more poorly than controls on session 9. In contrast, the 14-month-old cocaine treatment resulted in significantly poorer performance relative to controls on sessions 6 through 8 and sessions 13 and 14.

Figure 4. Top panels: The effect of age (top to bottom) and treatment (left to right panels) on spatial learning in different phases of a swim maze test. Dashed lines indicate breaks between the acquisition (a), retention (b), and reversal (c) phases of the swim maze test as described in Materials and Methods. Bottom panel: Effect of age on swimming speed averaged over all phases of the swim maze test. All values represent the mean ± SE. * Denotes a significant difference between the cocaine or methamphetamine treatment group and age-matched controls ($p<0.05$). The number of mice in each group is the same as that indicated in the legend to figure 2. Note that data for control groups are plotted in both the left and right panels.


METHAMPHETAMINE
COCAINE
a
b
c
11
14
16
PATH LENGTH (cm)
SESSION
SPEED (cm/s)
SALINE
PSYCHOSTIMULANT
AGE IN MONTHS

Analyses of variance performed separately for the acquisition, retention, and reveral phases supported the observations above and in figure 1. For analyses of methamphetamine data during the acquisition and reversal phases, only a main effect of Sessions was obtained ($p<0.001$), whereas there was no main effect or interaction involving the Treatment factor (all $p>0.099$). On the other hand, similar analyses for cocaine each revealed a significant Age x Session interaction on all three phases, and a main effect of Treatment and Age on acquisition and reversal phases in addition to a significant Age x Session interaction (all p values <0.04). Analyses for the retention phase data revealed a main effect of Age in addition to an Age x Session interaction (all p values <0.044).

The effect of age and treatment on different swim maze performance indices

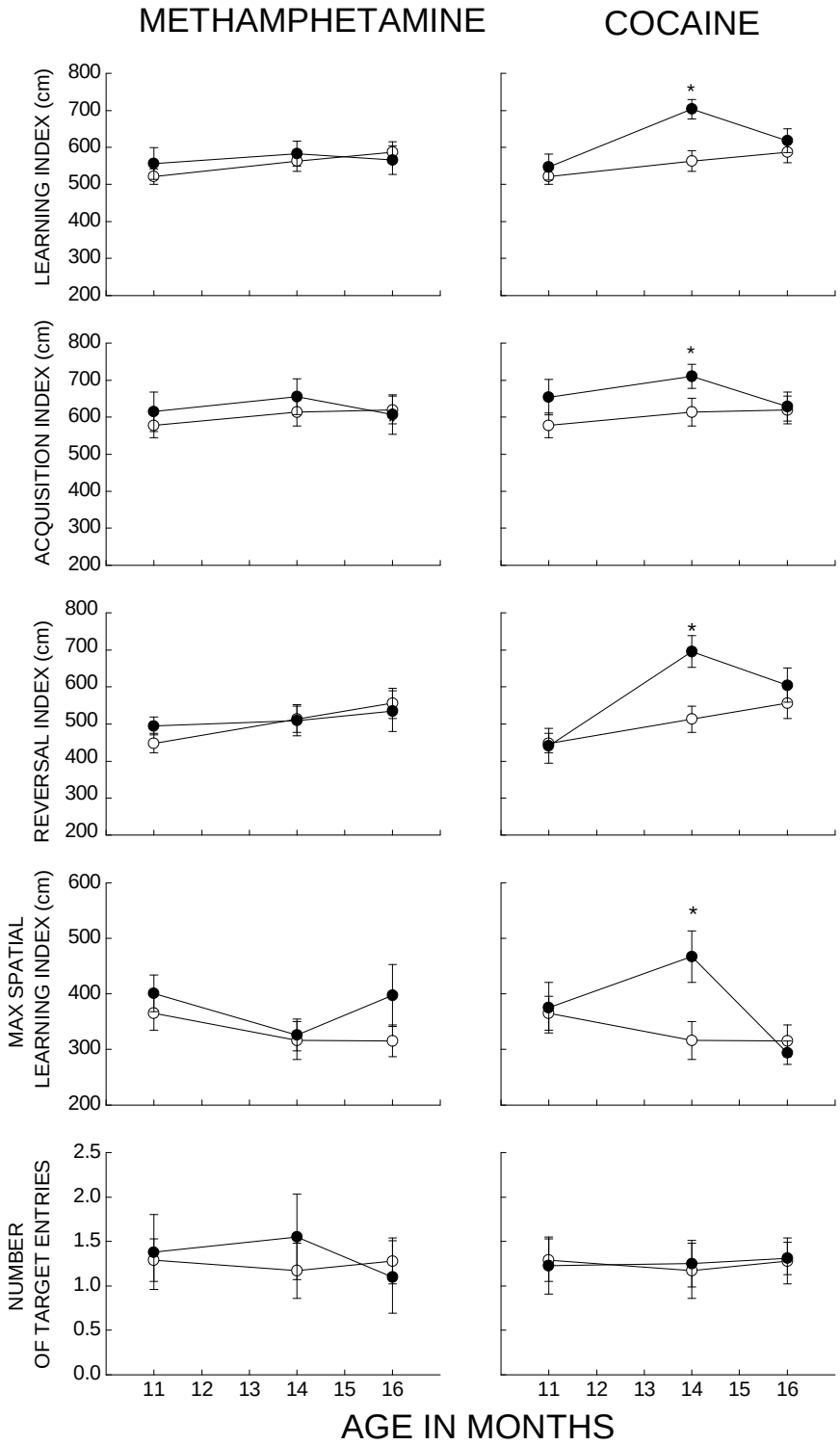
The session by session swim maze data were considered as a function of age by calculating performance indices that addressed age-sensitive components of spatial performance. For the total learning index, which reflected ability to learn both during initial acquisition and reversal, the control mice had decreasing performance (reflected in increased path length) as a function of age. When acquisition and reversal were considered separately, it was apparent that difficulty learning the reversal was largely responsible for this trend. There was little change as a function of age for the acquisition index, max spatial learning index, or the target entries during the probe trial conducted

after session 8. The cocaine-treated mice exhibited a worsening of their performance from 11 to 14 months, with no further decreases evident after 14 months of age, on the total learning index, acquisition index, reversal index, and maximum spatial learning index. On the other hand, age differences in performance of the methamphetamine-treated mice generally paralleled that occurring for the control groups.

There was no significant difference between control and methamphetamine-treated mice for any of the performance indices analyzed, at any of the ages tested. However, when compared to the age-matched control groups, the 14-month-old cocaine-treated mice had significant impairments for the total learning, acquisition, reversal, and max spatial learning indices. When compared with their age-matched controls, the 14-month-old cocaine group took 137 cm longer to locate the platform on the sessions comprising the total learning index, 185 cm longer on the sessions comprising the reversal index, and 151 cm longer on the sessions comprising the max spatial learning index.

Results for the 2-way analyses of variance for these data supported the above observations, revealing for cocaine a significant effect of Treatment, Age, and their interaction for the total learning index, max spatial learning and reversal learning index (all p values <0.047). In contrast, the analysis for methamphetamine failed to indicate a significant treatment effect or interaction (all p values >0.209)

Figure 5. The effect of age and treatment on various calculated indices of performance on the swim maze test. Each value represents the mean± SE. * Denotes a significant difference between the cocaine- or methamphetamine-treated group and the age-matched controls ($p<0.05$). The number of mice in each group is the same as that indicated in the legend to figure 2.


METHAMPHETAMINE
COCAINE
LEARNING INDEX (cm)
ACQUISITION INDEX (cm)
REVERSAL INDEX (cm)
MAX SPATIAL LEARNING INDEX (cm)
NUMBER OF TARGET ENTRIES
AGE IN MONTHS
*

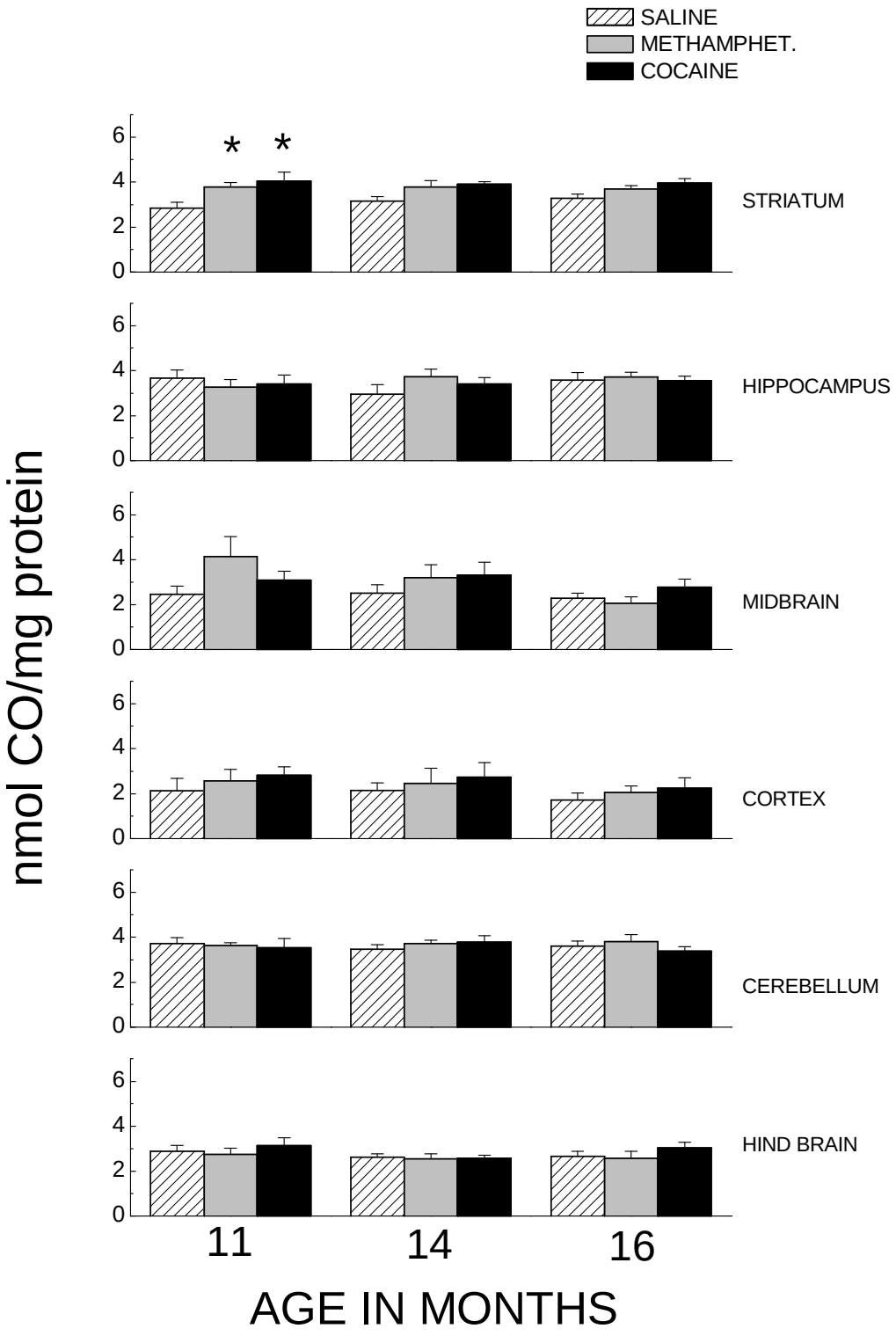
Effect of Psychostimulant Treatment on Markers of Oxidative Damage

The concentrations of protein carbonyl groups and thiobarbituric acid reactive substances (TBARS), markers of protein and lipid oxidative damage, were considered as a function of age and treatment in the brains from subsets of the mice tested for psychomotor and cognitive performance. These measurements were made in different regions to evaluate the hypothesis that behavioral impairments related to methamphetamine or cocaine treatments were the result of increases in oxidative stress targeting dopamine-rich areas such as the midbrain and striatum.

Protein Carbonyls

For the control groups, carbonyl content of all brain regions was relatively constant across each of the age groups tested, with the exception of the cerebellum for which an age-related decrease was evident (figure 6). However, carbonyl concentration varied markedly as a function of brain region. In the 11- month-old control group the hippocampus had the largest concentration of carbonyls (3.7 nmol/mg protein), followed by the cerebellum, hindbrain, striatum, midbrain, and cortex which had the smallest carbonyl concentration at 2.1 nmol/mg protein. The regional differences were more or less stable as a function of age; by 16 months, the hippocampus still exhibited the highest carbonyl concentration, with cortex containing the lowest.

Figure 6. The effect of age and treatment on the protein oxidative damage in various brain regions. The regions shown are the striatum (N=6), the hippocampus (N=5), the mid brain (N=5), the cortex (N=5), the cerebellum (N=5), and the hind brain (N=5). Each value represents the mean ± SE. * Denotes a significant difference between the treatment group and age-matched control ($p<0.05$).


SALINE
METHAMPHET.
COCAINE
nmol CO/mg protein
STRIATUM
HIPPOCAMPUS
MIDBRAIN
CORTEX
CEREBELLUM
HIND BRAIN
0
2
4
6
*
*
11
14
16
AGE IN MONTHS

The carbonyl concentration tended to increase as a function of age in the striatum, though this effect was not statistically significant. There was a significant increase in the protein carbonyl concentration in the striatum of 11-month-olds in response to both cocaine and methamphetamine treatment. The same trend was evident in the 14- and 16-month-old groups, although planned comparisons did not suggest that the differences at these ages were significant. However, analyses of variance conducted on the carbonyl data from the striatum did indeed suggest significant main effects of Cocaine and Methamphetamine (ps <0.002), and did not indicate significant Treatment x Age interactions (all p values >0.313). None of the other analyses of any particular brain region suggested main effects or interactions involving cocaine or methamphetamine treatments (all p values > 0.398)

TBARS content

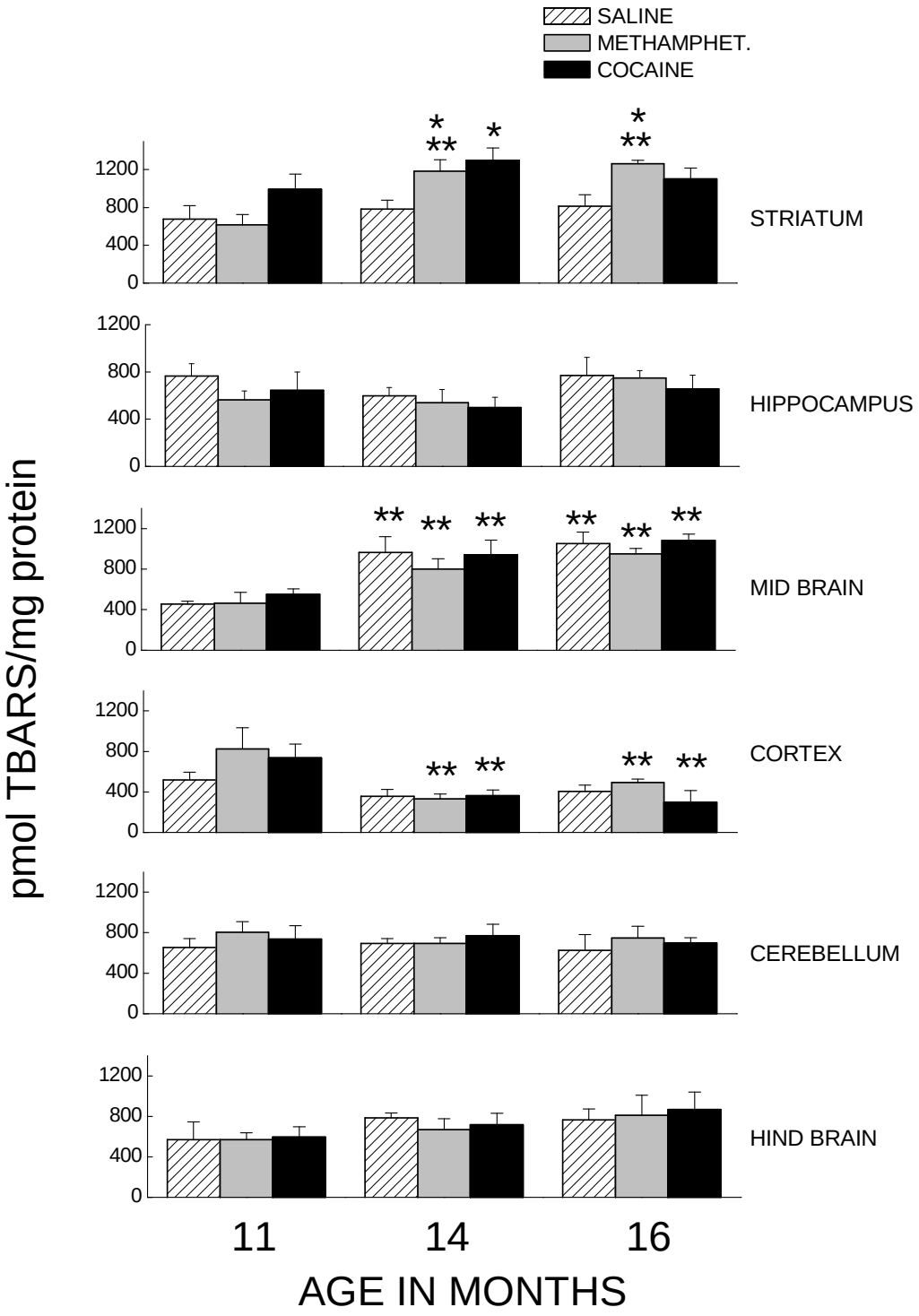
The effects of age and treatment on TBARS concentration are shown in Figure 7. Like the carbonyl content, there was there were no significant increases in TBARS concentration associated with age for the control mice, with the exception of the midbrain, in which the TBARS concentration was increased by 14 months of age. There was little or no further increase in TBARS by 16 months of age. In contrast, the TBARS concentration in the cortex tended to decrease by 14 months of age, although this effect was not statistically significant.

The regional differences in the TBARS concentrations of the 11-month-old saline-

treated groups were similar in pattern to that observed for carbonyls (hippocampus > cerebellum > striatum > hindbrain > cortex > midbrain), with the smallest concentration of TBARS in the midbrain at roughly 455 pmol/mg protein, and the highest concentration of TBARS in the hippocampus at 765 pmol/mg protein. This pattern was shifted by 16 months of age, primarily as a result of age-related increases in TBARS concentration in the midbrain and hindbrain, together with significant decreases in the cerebral cortex. By 16 months of age, the midbrain had the highest TBARS concentration (1054 pmol/mg protein) whereas the cortex had the lowest (407 pmol/mg).

At 11 months of age, there was a variable trend in the direction of an increase in TBARS in response to either cocaine or methamphetamine treatment, depending on the brain region examined. However, none of these effects were significant. Relative to the respective control groups at 14 and 16 months of age, however, the TBARS concentration was higher in the striatum of the cocaine- and methamphetamine-treated groups. For the methamphetamine-treated groups, these differences reflected an age-related increase in TBARS that was not paralleled by similar increases with age in the control group. While the cocaine group at 14 months differed significantly from the age-matched control group, it was not significantly different from the 11-month-old cocaine group. When the TBARS concentration for the striatum was considered in analyses of variance for cocaine or methamphetamine, each analysis revealed a significant main effect of Age and Treatment (ps <0.005), but failed to suggest a significant interaction of treatment and age (all p values >0.06).

Figure 7. The effect of age and treatment on lipid oxidative damage in various brain regions. The regions shown are the striatum (N=6), the hippocampus (N=5), the mid brain (N=5), the cortex (N=5), the cerebellum (N=5), and the hind brain (N=5). Each value represents the mean ± SE. * Denotes a significant difference between the treatment group and age-matched control ($p<0.05$).


SALINE
METHAMPHET.
COCAINE
pmol TBARS/mg protein
STRIATUM
HIPPOCAMPUS
MID BRAIN
CORTEX
CEREBELLUM
HIND BRAIN
0
400
800
1200
*
**
11
14
16
AGE IN MONTHS

When brain regions other than the striatum were considered, there was no indication that cocaine or methamphetamine had affected TBARS concentration. Analyses of variance for the midbrain and cortex indicated significant main effects of Age (ps <0.0001), but none of the non-striatal regional analyses suggested a significant effect of Cocaine, Methamphetamine, or an interaction of these treatments with Age (all p values >0.06).

Associations between Oxidative Damage and Cognitive or Psychomotor Function

Based on the finding that both methamphetamine and cocaine treatments resulted in significant increases in markers of both protein and lipid oxidative damage, a correlation analysis was performed to determine whether or not performance of individual mice on the psychomotor or cognitive tests was quantitatively related to amounts of oxidative damage. These analyses focused on behavioral performance indices that were significantly affected by the methamphetamine or cocaine treatments, and included behavioral performance and oxidative damage data for all three treatment and age groups. Pearson correlation coefficients for these analyses are shown in Tables 3 and 4, for samples ranging from N=45 to N=53.

Overall, there were no “strong” correlations (r > 0.5 as defined in previous studies) between oxidative damage and behavioral performance, although some of the correlations reached statistical significance (generally, those above r=0.246 for 1-tailed p<0.05). The strongest relationships were between the striatal TBARS concentration and

impaired performance on a number of the tests of psychomotor function, including the treading reflex and wire grip test, and the bridge-walking test (r=0.227 to -0.344). It is worthy of note that for the treading reflex and wire grip test, a correlation was detected in the opposite direction for TBARS concentration in the cerebral cortex, a region in which the TBARS concentration was observed to decline as a function of age in the methamphetamine and cocaine-treated mice. The strongest relationships for carbonyl concentration also involved the striatum, with impairment correlations involving the wire grip, bridge walking, and rotorod tests (r=-0.265 to -0.306).

All of the learning indices for the swim maze (Table 4) were not strongly correlated with hippocampal lipid or protein damage. The strongest relationship was evident between the hippocampal carbonyl content and max spatial learning index which had a r value of 0.113. The strongest association for swim maze performance involved the cortex, for which the TBARS concentration was weakly correlated with the total learning index (r=0.226) and the acquisition learning index (r= 0.362).

Analysis of Protein Expression for DAT, TH, and Alpha synuclein.

Protein expression for markers of the integrity and function of brain dopamine-containing neurons was evaluated in the midbrain and striatum of the brain for all of the treatment and age groups. These determinations were made in subsets of four mice that were different from those used to determine TBARS and carbonyls. The results for these determinations are shown in figures 8 through 10.

Table 3. Pearson correlations among psychomotor tests and oxidative damage

TBARS

	Brain Region[1]					
Psychomotor/Reflex	**ST**	**HP**	**MB**	**CX**	**CB**	**BRS**
Wire tread (s)	0.227	-0.199	-0.210	-0.331	-0.181	0.047
Wire fall (s)	-0.344	0.272	0.251	0.382	0.138	0.023
Bridge fall (s)	-0.327	0.209	-0.220	-0.032	0.024	0.068
Rotorod fall (s)	-0.078	-0.162	0.123	-0.006	0.035	0.045

Carbonyls

	Brain Region[1]					
Psychomotor/Reflex	**ST**	**HP**	**MB**	**CX**	**CB**	**BRS**
Wire tread (s)	0.167	0.001	-0.041	-0.172	0.135	0.046
Wire fall (s)	-0.296	-0.088	0.167	0.165	0.048	0.014
Bridge fall (s)	-0.306	-0.259	-0.221	0.004	0.226	0.021
Rotorod fall (s)	-0.265	-0.037	-0.012	0.154	-0.077	0.023

[1] Abbreviations: CX, cerebral cortex; CB, cerebellum; MB, midbrain; BRS, brainstem; HP, hippocampus; ST, striatum

Table 4. Pearson correlations among swim maze learning indices and oxidative damage

TBARS

	Brain Region[1]					
Learning/Memory	**ST**	**HP**	**MB**	**CX**	**CB**	**BRS**
Swim Maze						
Total learning index (cm)	0.210	0.056	0.109	0.226	0.078	-0.053
Acquisition learning index (cm)	0.119	-0.072	0.123	0.362	0.103	-0.035
Reversal learning index (cm)	0.223	0.163	0.034	0.006	0.034	0.026
Maximum spatial learning (cm)	-0.167	-0.037	0.051	0.008	0.206	0.035

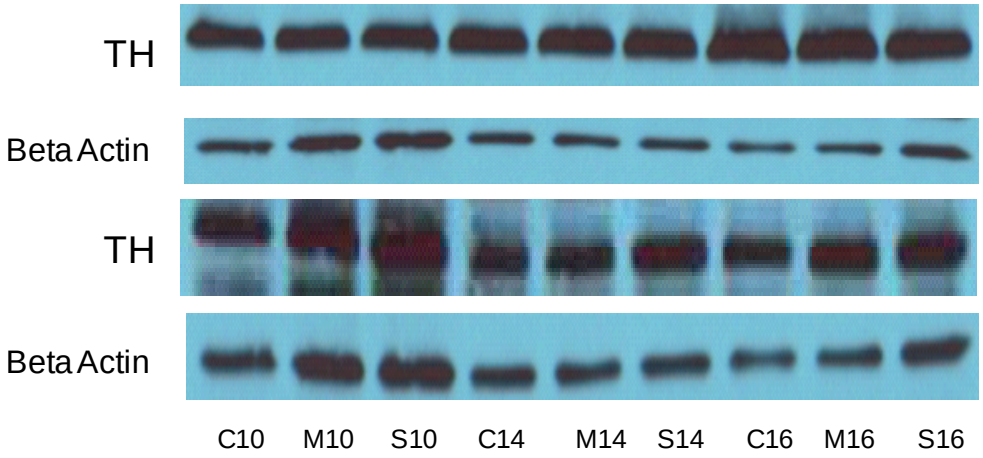
Carbonyls

	Brain Region[1]					
Learning/Memory	**ST**	**HP**	**MB**	**CX**	**CB**	**BRS**
Swim Maze						
Total learning index (cm)	-0.076	0.048	0.233	-0.079	0.056	0.024
Acquisition learning index (cm)	-0.149	-0.05	0.356	0.068	0.137	0.037
Reversal learning index (cm)	0.051	0.14	-0.02	-0.186	0.042	0.212
Maximum spatial learning (cm)	0.114	0.113	-0.068	-0.024	0.226	0.133

In general, there were no main effects of Cocaine, Age or Methamphetamine on the expression of DAT, TH, or alpha synuclein in any of the dopamine-rich regions that were tested. Of all of the dopamine markers, only the results for DAT and alpha synuclein suggested any variability in response to treatments, however, none of the effects was significant. Tyrosine hydroxylase failed to show any change in expression for either the midbrain or the striatum. In addition, no change in expression was associated with age for any of the proteins that were probed. Because a relatively small sample was evaluated, power analyses were performed to estimate the size of any effect that would not have been detected in the studies of the dopamine markers. In each of these analyses P was set at less than 0.05 (1-tailed), power was set at 80%, and a pooled variance was estimated for each marker based on data for all experimental groups. Under these conditions, the current studies would have detected a decrease in each marker of 50 to 60%.

Figure 8. The effect of age and treatment on tyrosine hydroxylase expression in the midbrain and striatum. (A) Representative immunoblots from the midbrain and striatum. Beta-actin was used as loading control. (B) Densitometric analysis of tyrosine hydroxylase immunoblots, normalized with beta-actin. Each value represents the mean ± SE of 4 mice for each test group.

A


TH
Beta Actin
TH
Beta Actin
C10
M10
S10
C14
M14
S14
C16
M16
S16

B

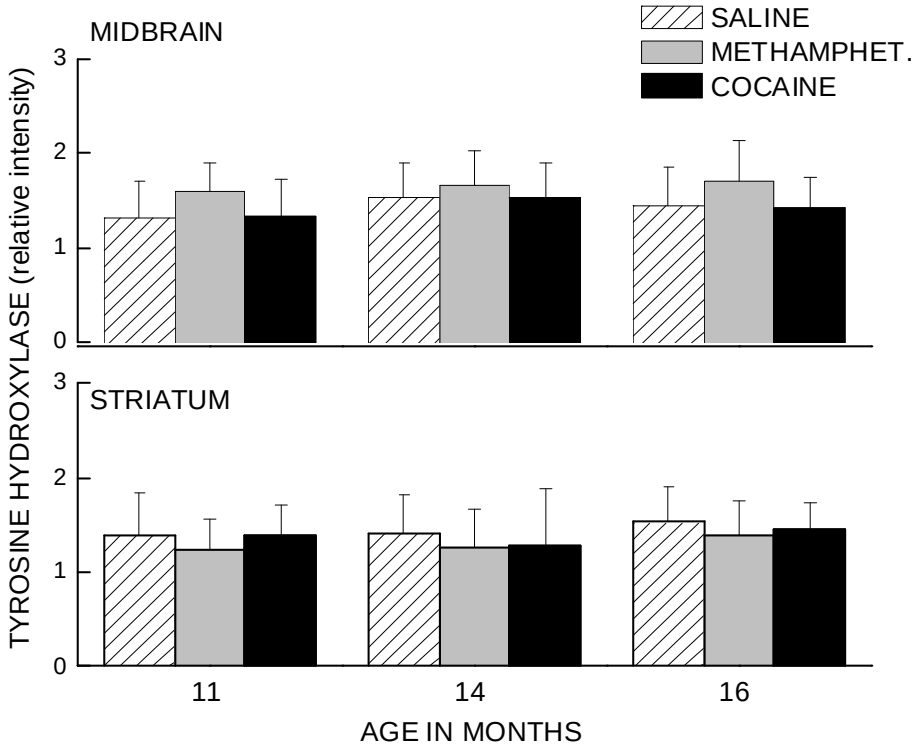
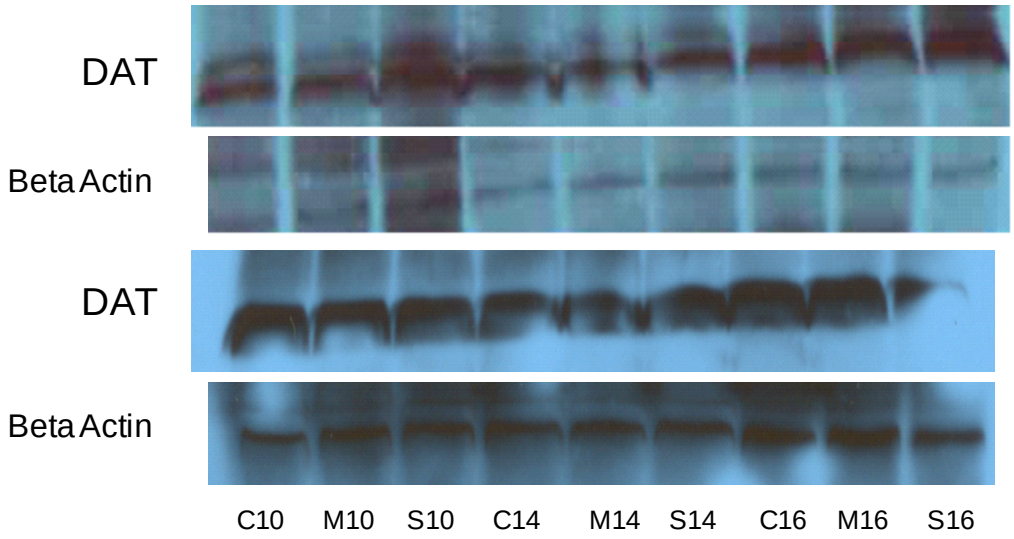

MIDBRAIN
SALINE
METHAMPHET.
COCAINE
STRIATUM
TYROSINE HYDROXYLASE (relative intensity)
0
1
2
3
11
14
16
AGE IN MONTHS

Figure 9. The effect of age and treatment on dopamine transporter expression. (A) Representative immunoblots from the midbrain and striatum. Beta-actin was used as the loading control. (B) Densitometric analysis of dopamine transporter immunoblots, normalized with beta-actin. Each value represents the mean ± SE of 4 mice for each test group.

A


DAT
Beta Actin
DAT
Beta Actin
C10
M10
S10
C14
M14
S14
C16
M16
S16

B

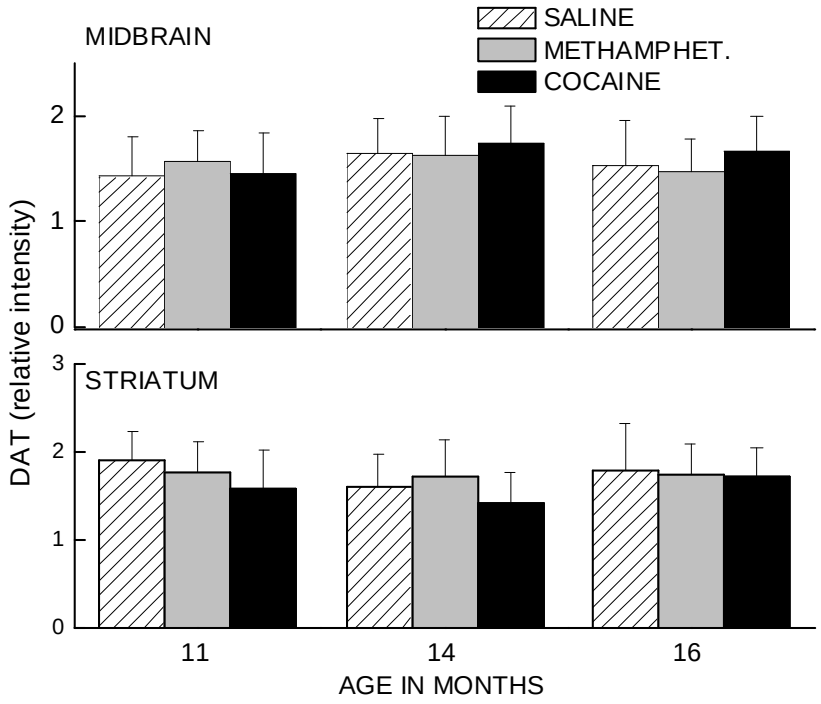
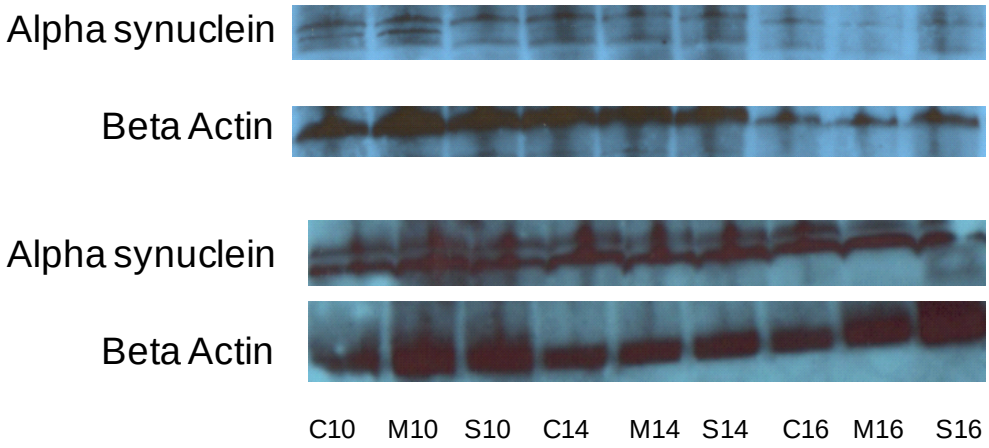
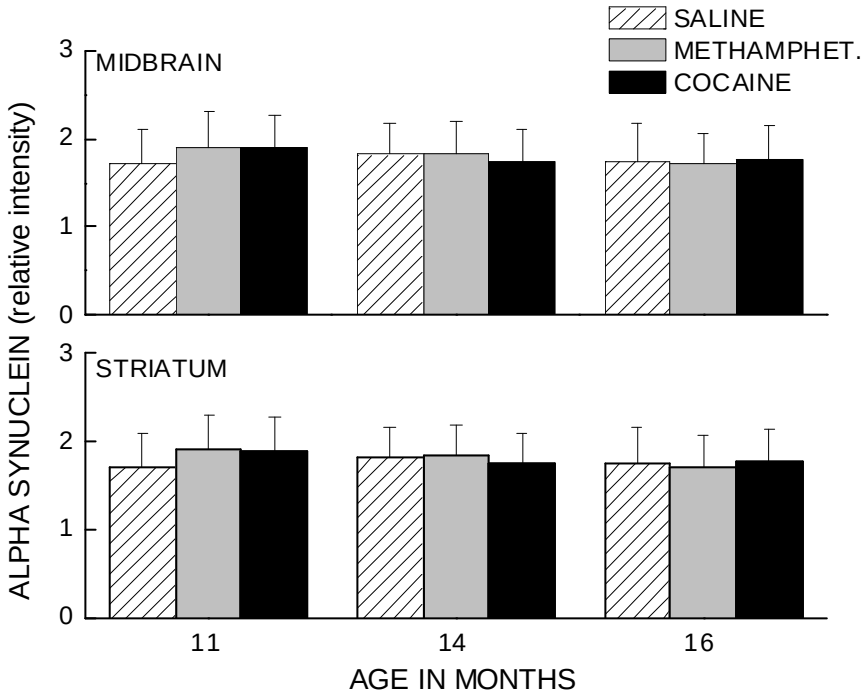

SALINE
METHAMPHET.
COCAINE
MIDBRAIN
STRIATUM
DAT (relative intensity)
0
1
2
3
11
14
16
AGE IN MONTHS

Figure 10. The effect of age and treatment on alpha synuclein expression. (A) Representative immunoblots from the midbrain and striatum. Beta-actin was used as loading control. (B) Densitometry analysis of alpha synuclein immunoblots, normalized with beta-actin. Each value represents the mean ± SE of 4 mice for each test group.

A


Alpha synuclein
Beta Actin
Alpha synuclein
Beta Actin
C10 M10 S10 C14 M14 S14 C16 M16 S16

B


SALINE
METHAMPHET.
COCAINE
MIDBRAIN
STRIATUM
ALPHA SYNUCLEIN (relative intensity)
0
1
2
3
11
14
16
AGE IN MONTHS

CHAPTER IV

GENERAL DISCUSSION

Dopaminergic neurotransmission and synthesis is a tightly regulated process in the brain that is altered by regular intake of abused psychostimulants such as cocaine or methamphetamine. Dopamine-containing neurons are vulnerable to the effects of reactive oxygen species, and the function of these neurons is compromised during the aging process. In addition, chronic administration of psychostimulants has been shown to have profound neuroadaptive and toxic effects on dopaminergic function, in both the nigrostriatal and mesolimbic dopamine neural pathways. In this study we hypothesized (i) that the use of psychostimulants could produce long-lasting behavioral impairments caused by increases in oxidative stress (ii) that psychostimulant-induced increases in oxidative stress may be linked to neuroadaptive changes in dopaminergic function and (iii) that oxidative stress and neuroadaptive changes associated with chronic psychostimulant administration could interact with normal aging processes leading to accelerated declines in psychomotor and possibly cognitive functions. Accordingly, the present study employed a mouse model of chronic psychostimulant administration to address the long-term effects on oxidative stress, behavioral capacities, and indices of the integrity and functional status of dopamine-containing neurons. To our knowledge this was the first study to fully address the possibilities described above.

A major finding in this study was that reflexive and psychomotor performance, as measured on the rotating rod, wire hanging, and bridge walking tests, was significantly impaired for up to 5 months following the administration and discontinuation of the psychostimulant cocaine or methamphetamine. While methamphetamine- induced impairments of psychomotor function were the most robust, impairments from both drugs were observed over all of the age ranges tested. The current studies also provided evidence, though not unambiguous, that psychostimulant treatment could accelerate the normal process of age-related decline in psychomotor function.

Another significant finding in the current studies was that the psychomotor impairments associated with both cocaine and methamphetamine were accompanied by increases in protein and lipid oxidation that were restricted to the striatum, a dopamine-rich area of the brain containing neural circuits critical to normal expression of motor behavior. However, the increase in oxidative stress was not associated, as hypothesized, with long-lasting shifts in expression of alpha synuclein, DAT, or tyrosine hydroxylase.

An additional noteworthy finding in the current study was that cocaine, but not methamphetamine, produced impairment of cognitive function as demonstrated by results from the test of spatial performance using the swim maze. The cocaine-associated impairment was not detected immediately following treatment, was present three months later, and could then not be detected thereafter. These main findings are considered in light of current and previous literature, as well as several caveats, in the sections that follow.

Animal Model and Target Age Groups

These studies employed a mouse model of chronic psychostimulant abuse in which 10-month-old C57BL/6 mice were exposed to a 28-day continuous administration of 2.0 mg/kg/day of methamphetamine, 40 mg/kg/day of cocaine, or saline as a control group. Evaluating the status of the mice from these groups either 1 week, 3 months, or 5 months later provided information on dopaminergic cell neurochemistry, oxidative stress levels and the consequences on behavior functions following chronic psychostimulant abuse. Additionally, these testing intervals represented a sufficient period of life to evaluate potential interactions with aging processes. It had been established that at approximately 10 months of age, declines in psychomotor functions are already in progress (Shukitt-Hale et al., 1998; Forster and Lal, 1999). Therefore, the relatively short age points were chosen so that senescence would not impair function to such an extent that further observations of increased impairments (due to treatments) would not be observable by the behavioral or biochemistry tests we employed. All of the tests included in the study have been established as effective in detecting age associated impairments of the C57BL/6 mice and, more specifically, the psychomotor tests employed are sensitive to brain aging and oxidative damage associated with the striatum, cerebral cortex, and cerebellum (Forster et al., 1996). By analyzing three distinctive time points these studies were expected to detect accelerated age-related declines, or alternatively, any possible recovery of function from the psychostimulant administration. The pattern of results tended to confirm both types of outcome depending on the particular behavioral test and psychostimulant treatment.

Among psychostimulant addicts, the route of administration can vary greatly, but one common attribute is the frequent re-administration of the drugs over a short period of time in a binge type pattern of use. Therefore we chose to employ osmotic minipumps as a route of administration. They provide substained plasma levels of drug with little fluctuation. In addition, doses were chosen as to give a reasonable comparison of the effects of the two psychostimulants tested during this study. Because of the difference in plasma half-life for cocaine and methamphetamine, as well as the differences in potency at different sites within the nervous system, it was difficult to equate amounts of each compound to be administered on that basis. However, the doses of the two compounds administered on a daily basis were equivalent to the extent that each represented exposure to an amount that would have been sufficient to produce the maximum locomotor stimulant effect if given as a single dose via the intraperitoneal route. Equivalence on this dimension was established through an analysis of dose response curves for locomotor stimulant effects of methamphetamine and cocaine in psychostimulant-naive mice. Also as for methamphetamine, 2 mg/kg had previously been shown not to be neurotoxic (Ricaurte et al., 1984). In conclusion, the animal model utilized during these studies, was an effective way to answer the questions posed by the working hypothesis.

The Effects of Psychostimulants on Behavioral Functions

These studies showed that chronic administration of cocaine and methamphetamine in middle aged animals resulted in decreases in behavioral capacities thought to be mediated by dopaminergic functions. These impairments manifested themselves in several of the

psychomotor behavioral tests employed. For example in rotorod, wire hanging, and bridge walking tests, the results suggested psychomotor impairments associated with both of the psychostimulants. With only one exception, these impairments were evident one week following the treatment phase, for both cocaine and methamphetamine. For bridge-walking and rotorod performance, the deficits persisted or became more pronounced by 16 months of age in the psychostimulant-treated mice. On the other hand, data for the treading reflex and wire fall tests suggested somewhat different patterns for cocaine and methamphetamine. Specifically, data for cocaine-treated mice indicated recovery of function, to nearly the level of the age-matched controls, whereas for methamphetamine-treated mice, the degree of impairment became more pronounced as a function of age. It was noteworthy that while the psychostimulant treatments led to very robust impairments in motor tasks requiring ambulation with fore- and hindlimbs, such as the rotorod and bridge-walking tests, there was no apparent effect of the treatments on swimming performance, which involves inhibition of the forelimbs together with paddling by the hindlimbs.

Analysis of the rotorod performance data afforded the opportunity to assess whether or not the psychostimulant treatments had any effect on motor learning in any of the age groups. The relatively poor performance in the psychostimulant-treated mice during the learning phase of this test may indeed suggest a motor learning deficit. However, this finding was somewhat ambiguous in light of the fact that the psychostimulant-treated mice generally did not achieve a maximal level of stable performance that was equivalent to controls. Thus, the impaired rotorod performance of cocaine- and methamphetamine-

treated mice is most likely attributable to a limitation in capacity for coordinated running as opposed to impairment in their ability to improve performance with practice.

A significant age-related decline in psychomotor performance of the control groups was detected in this study, for the treading reflex, wire grip, bridge-walking, and rotorod tests. These findings were in accordance with previous studies and confirmed that a detectable amount of “aging” had occurred over the period of 5 months surveyed. Unfortunately, the existence of impaired performance in the psychostimulant-treated groups at 11 months of age made it difficult to evaluate the hypothesis that these treatments may accelerate normal age-related declines in psychomotor performance. Notwithstanding, results for the tests of the treading reflex, wire grip, and bridge-walking suggested a more pronounced effect of age in methamphetamine-treated mice when compared with the control groups. On the other hand, none of the results for cocaine-treated mice suggested an acceleration of age-related decline, and in some cases (e.g., for wire grip) the results suggested a recovery rather than an acceleration.

While the psychomotor impairments associated with psychostimulant treatment were substantial and long-lasting, there were few effects on cognition as measured in the spatial swim maze test. Only the 14 month old cocaine groups had any significant impairment in performance, and these effects were not global, but specifically involved impaired ability to learn a new hidden platform location after a previous location had been established.

A significant finding related to the behavioral outcomes in the psychostimulant studies was the observation of increases in protein and lipid oxidative damage restricted

to the striatum, a brain region clearly linked to both cognitive and psychomotor function. The effect on protein oxidation, measured as protein carbonyls, was most evident one week following the psychostimulant treatment period but appeared to persist up to 16 months of age. Lipid peroxidation, inferred by increases in TBARS, was increased at 14 and 16 months in the striatum. Thus, there was at least a temporal association between persistence of psychomotor impairments in the mice and measures of oxidative stress. The western blotting analysis revealed that the expression of the important dopaminergic proteins mentioned in the previous chapters was not changed in response to either of the psychostimulants utilized in the study. Therefore the long-term effects associated with these drugs appear to be independent of any change in dopaminergic protein expression.

Possible Explanation of Outcomes and Previous Studies

Our present findings suggest that chronic psychostimulant administration leads to enduring deficits in psychomotor function. The impairments associated with the treatment were evident in several of the psychomotor tests initially following the psychostimulant administration (i.e. the 11 month old group) and endured through the 14 and 16 month testing groups. It can be argued that longer age ranges were needed to truly justify this explanation. For example if the results were attributable to an initial insult from the psychostimulant treatment or withdrawal, subsequent impairments in function would be expected to be short term as the nigrostriatal dopaminergic neurons recover in function. As the neurons recover it is not unreasonable to expect some if not complete recovery of psychomotor function and possibly cognitive functions. Therefore one could

conclude that psychostimulant administration could result in initial impairments to dopaminergic function, and subsequent recovery. However because of the increase in oxidative damage, neuronal pathways could be set in motion which would lead to apoptosis, neurodegeneration and more declines in psychomotor function as the animals moved into advanced age. Another possibility is that the persistant increases in oxidative damage evident in the study would lead to progressive declines in psychomotor function and prove our initial hypothesis correct. Therefore longer age ranges would have to be employed, to fully test these possibilities.

Cocaine and methamphetamine administration have been shown to significantly increase oxidative stress in nigrostriatal and mesolimbic dopaminergic neurons (Dietrich et al., 2005; Macedo et al., 2005; Poon et al., 2007). In addition to this, methamphetamine is a known neurotoxin. In 2001,Volkow et al., (2001a), found that methamphetamine abuse results in impaired psychomotor functions, and decreased the number of DATs in the striatum. This study, like the current one, involved both behavioral and biochemical measures which gave an overall picture of the consequences of chronic methamphetamine abuse. Volkow et al. (2001a) subjected methamphetamine addicts to PET scans for DAT number and a neuropsychological battery of tests to evaluate deficits in the striatum and frontal lobe. These researchers found that the methamphetamine abusers showed significant reductions in DAT number in the striatum which was evident for at least 11 months after abstinence from the drug. In addition they found that these individuals had persistent motor and memory impairments. Other studies from the same lab found that the damage from methamphetamine abuse was partially

recoverable after protracted abstinence. They found that both DAT and brain glucose metabolism was partially recoverable after 12-17 months of abstinence (Volkow et al., 2001b; Wang et al., 2004). This was different from the results observed in the current studies, for which after 3-5 months of withdrawal, there was no recovery of performance on most of the psychomotor tests. Also, as demonstrated by the spatial swim maze data, there were no impairments attributable to methamphetamine on cognitive functioning for any of the age groups tested. As noted in the previous chapters, animal models of methamphetamine abuse have yielded similar biochemical results. In vivo studies have revealed that chronic methamphetamine administration produces significant increases in reactive oxygen species and reactive nitrogen species, in addition to impairments in mitochondrial functions, and glucose metabolism (Imam and Ali, 2001; Thomas et al., 2004; Miyazaki et al., 2006).

The animal models used in previous studies were different from those used in the current studies in several important ways. First, most studies which examined the effects of methamphetamine utilized a high dose (> 10 mg/kg/day), and injections as the route of administration. Also, often the treatment phase was much smaller, lasting anywhere from a day to a week. One key attribute of the current model was the choice to utilize middle aged mice as subjects. This difference in age could have a profound influence on the possible neurotoxic effects of the drugs. For example Miller et al., (2000) found that age increased methamphetamine induced neurotoxicity in the striatum. They compared methamphetamine induced striatal dopamine depletion in young animals (1 month old) to the damage occurring in aged (12 month old) animals following chronic

methamphetamine administration. Like previous studies, a toxic dose of methamphetamine was utilized. The results showed that the 12- month- old animals displayed significant depletions of DA and its metabolites, an effect that was not observed in the one- month-old group.

Like methamphetamine, there are several studies examining the short- term effects of cocaine on cognition and psychomotor functions. Bauer, (1996) found that cocaine addicts exhibited increases in resting hand tremors, decreased reaction time, and delayed visual tracking, effects that were evident after 3 months of abstinence. As mentioned in a previous chapter Bolla et al., (1999), measured cognitive and motor impairments in cocaine addicts which was dose-related. These impairments were also evident after 28 days of abstinence from the drug. As far as we know our study is the first examining the long term effects of chronic cocaine on psychomotor functions in a rodent model. Our results, like the human studies, suggest significant psychomotor deficits are associated with chronic cocaine administration. However our studies suggest that these deficits persist through a significant period of time (roughly 20% of the mouse life-span), and they may be additive or interactive with normal aging processes affecting behavioral performance.

The only cognitive impairments observed in the current studies were deficits in the reversal index of the 14- month- old cocaine group, which were significantly different when compared to their aged matched control groups. One possible explanation for this effect is the ability of cocaine to cause functional changes in the mesocortical pathway which could result in decreases in incentive motivation, and cognitive and executive

functions (Robbins and Everitt, 1999; Rogers et al., 1999; Jentsch et al., 2002; Olausson et al., 2007). For example, imaging studies have revealed that drug-addicts have lowered cerebral blood flow and glucose metabolism in the frontal cortex both before and after the onset of drug-induced cravings (Volkow et al., 2000; Volkow et al., 2002). It has been suggested by Volkow et al., (2002) that these abnormalities result in the disruption of some of the higher cognitive and monitoring frontal cortical functions that play a distinctive role in the development of addiction. Specifically, the decrease in frontal cortex activity leads to deficits in tasks that require cognitive flexibility and higher executive functions (Jenstsch et al., 2002). For example, Mendez et al., (2007), found that rats exposed to a long-term regimen of cocaine performed significantly poorer on the water maze when compared to the control groups. These effects were long- lasting and endured up to 3 months following the treatment phase. One study performed by Jentsch et al., (2002) provides more germane evidence to this argument. Using Vervet monkeys, this laboratory examined the administration of cocaine on both acquisition and reversal of object discrimination. They reported that both an acute and chronic dose of cocaine administration caused impairments of reversal learning but not acquisition learning, which were evident initially following treatment and endured for up to 30 days following the withdrawal. Therefore it is reasonable to postulate that the effects observed in the 14-month-old cocaine treated group on the swim maze testing could be attributed to some impairment in frontal cortical functions. It is worth noting that we did not observe any treatment differences in oxidative damage in the cerebral cortex of the mice. Therefore as reported by Volkow et al., (2002) the impairments could be the result of decreased blood

flow or possibly glucose metabolism.

The effects of Age and Oxidative Stress on Dopaminergic neurons.

As discussed in previous chapters, dopaminergic neurons are vulnerable to the damaging effects of reactive oxygen species, and decline steadily during senescence (Dexter et al., 1989). These declines in nigrostriatal neurons specifically are associated with impairments in motor functions (Carlsson, 1978; Mann and Yates, 1983; Emborg et al., 1998; Ma et al., 1999). Longitudinal studies of age sensitive behavioral studies have yielded nearly linear declines of performance in psychomotor functions in various strains of mice including C57BL/6 mice from ages 4 to 24 months of age. Utilizing the same behavioral tests seen here, de Fiebre et al., (2006), reported a nearly linear decline in psychomotor performance which resulted in roughly a 23% decrease in performance on the elevated bridge tests, and roughly a 10% decline in learning index, in mice ranging from 6 months to 16 months of age. Sumien et al., (2004) reported roughly a 10% decline in maximum rotating rod performance when comparing young (5 months) to old (20 months) C57BL/6 mice. The aged animals exhibited a 24% decrease in wire hanging performance, and a 35% decrease in bridge tests. Overall the results reported here a similar to the declines associated during senescence reported in the literature.

These impairments in motor functions can also be induced by employing toxins such as 6-hydroxydopamine, (MPTP), or rotenone. These compounds are used extensively as models for the nigrostriatal neuronal toxicity and associated with the pathology of parkinson's disease. Several important findings have emerged from the study of these

compounds, and the etiology of Parkinson's disease which are relevant to the current studies. First each of these toxins involves the action of reactive oxygen species and cause declines in psychomotor function (for review see Bankiewicz et al., 2001). For example one of the earliest changes associated with the administration of MPTP and patients with Parkinson's disease is the decreasing of glutathione which is thought to occur from a reaction to the increase in reactive oxygen species in dopamine neurons (Sian et al., 1994; Owen et al., 1996). Postmortem tissue from patients with Parkinson's disease contain elevated levels of carbonyls and 4-hydroxynonenal, a lipid peroxidation product (Yoritaka et al., 1996; Alam et al., 1997a; Alam et al., 1997b; Seth et al., 2002). The administration of antioxidants, such as vitamin E can attenuate the toxicity associated MPTP (Lan and Jiang, 1997). In addition, these dopaminergic toxins produce superoxide, hydrogen peroxide, and hydroxyl radicals in vivo which are very harmful to neurons (for review see Drechsel and Patel, 2008). In our study we observed increases in oxidative stress both initially and several months following both the cocaine and methamphetamine administration. It is reasonable to assume that the increased oxidative damage from the psychostimulant administration, along with the decreased antioxidant compounds associated with aging, possibly resulted in impairments of the nigrostriatal neurons. The end result would be the declines in motor functions we reported.

Another important theme which has emerged from the studying of these compounds is the important roles, DAT, VMAT, and alpha synuclein play in the induction of toxicity and protection from these compounds. As mention in previous chapters, these proteins are important modulators of dopaminergic neurotransmission and homeostasis. The DAT

for example is critical in these processes. The protein moves between the plasma membrane and intracellular compartments possibly through an alpha synuclein mediated process (Wersinger et al., 2003). Once recruited to the plasma membrane the transporter protein then allows the entry of MPTP, methamphetamine, and 6-hydroxydopamine into the dopaminergic cells (Bezard et al., 1999; Cappelletti et al., 2005). For example (Qian et al., 2008) reported that the up-regulation of alpha-synuclein in PC12 cells resulted in an increase in DAT surface expression which made the cells more vulnerable to MPP+ induced neurotoxicity. Furthermore, mice lacking DAT are resistant to the neuro-toxicity associated with some of these compounds (Gainetdinov et al., 1997).

Taking into account the literature, we sought to evaluate what effects chronic psychostimulant administration would have on the long-term expression of the DAT. Ultimately; we did not detect any long-term changes in DAT expression. However, it is worth noting that previous experiments have yielded different results. In Hilburn et al., (2004) it was reported that a continuous infusion of cocaine lasting as little as three days was sufficient to induce a significant increase in DAT number evidenced by radioligand binding assays. This effect endured for 7 days following the psychostimulant administration, but it is not clear how long this effect might persist beyond that period. Therefore it is possible that any increases in DAT associated with the chronic psychostimulant administration could have been missed in the current studies due to our behavioral model and the testing periods that were employed.

Like the DAT, the functioning of the VMAT can have profound effects on dopaminergic neurons. It is thought that VMAT's play a protective role by sequestering

intracellular dopamine, and toxins into cytoplasmic storage vesicles (Staal and Sonsalla, 2000). If these substances are not packaged and remain in the cytosol they can induce harmful consequences to dopamnergic neurons in the nigrostriatal region. For example VMAT2 heterozygous knockout mice exhibit increased dopaminergic neuronal vulnerability to L-Dopa and MPTP toxicity (Gainetdinov et al., 1997; Kariya et al., 2005). Furthermore, overexpression of the VMAT gene rescues dopaminergic neurons from insoluble alpha synuclein- mediated neuronal degeneration, and methamphetamine induced toxicity (Larsen et al., 2002). These reports provide compelling support for the neuroprotective role of VMAT, and the need to sequester dopamine and other toxins into storage vesicles. It is worth noting that the VTA region has higher levels of VMAT than the neurons of the substantia nigra, which provides this area with a level of resistance to DA cell death (Takahashi et al., 1997). For example, several researchers have reported that nigrostriatal neurons have a greater vulnerability to the effects of hydrogen peroxide than their mesolimbic counterparts (Grant and Clarke, 2002). Also Maingay et al., (2006) reported that VTA neurons are resistant to human mutant alpha synuclein overexpression, which is the toxic form of the protein. This was not the case for neurons of the substantia nigra which exhibited profound cell loss. In addition to these differences in protein expression Werkman et al., (2001) reported that nigral neurons fire approximately 50% more than the neurons of the VTA. This higher activity would increase dopamine turnover in addition to increasing the metabolic demand placed on the neurons. These reports support what is observed in the pathology of Parkinson's disease where the neurons of the nigrostriatal region are affected more severely than other dopaminergic

regions (Uhl et al., 1985; Hirsch et al., 1988a; Hirsch et al., 1988b; Kish et al., 1988). Therefore it is reasonable to postulate that the extra protection exhibited by the mesolimbic neurons could possibly explain the regional differences we observed in oxidative damage. In our study we observed increases in oxidative damage in the striatum of our subjects. However no evident damage was observed in the midbrain or cortex containing the targets of the VTA. Another possible explanation for the results of this study is the observation that methamphetamine toxicity produces enduring decreases in the striatum, while only mildly affecting other dopaminergic regions like the prefrontal cortex and mesolimbic pathway (Seiden et al., 1976; Wagner et al., 1980; Eisch and Marshall, 1998).

While the exact function of alpha synuclein is the subject of current debate, recent data suggests that the protein helps regulate dopamine homeostasis. Alpha synuclein binds to and interacts with a number of cytoskeleton proteins suggesting that it has a role in intracellular trafficking (Payton et al., 2001). For example, this protein has been implicated in the regulation and targeting of DAT from the cytosol to the plasma membrane (Wersinger and Sidhu, 2003a). Other reports have suggested that alpha synuclein plays a role in the modulation of synaptic vesicle synthesis and recycling (Lotharius and Brundin, 2002; Sidhu et al., 2004). The one clear idea is that abnormal alpha synuclein expression or function is harmful to nigrostriatal dopaminergic neurons. For example, either over expression, knocking out, or mutations in the protein induce neurotoxicity or increases the lethality associated with known neurotoxins (Feany and Bender, 2000; Tanaka et al., 2001; Wersinger and Sidhu, 2003b; Drolet et al., 2004). In

the current study we did not observe any alterations in the expression of alpha synuclein, although t is worth noting that previous studies have yielded different results. Again in Hilburn et al. (2004), it was found that alpha synuclein expression was abolished in response to chronic cocaine administration in rats. Mash et al., (2003) reported that chronic cocaine abusers had increased expression of alpha synuclein in nigrostriatal neurons. It is reasonable to suspect that any alterations which occurred in these proteins could have arisen before the time points tested in the current studies. There were no evident changes in tyrosine hydroxylase expression associated with the drug treatments. It is worth mentioning that the psychostimulant treatment regimen we utilized may have induced changes in enzymatic activity. We chose to measure this protein to determine if there were any loss of dopaminergic neurons primarily, and therefore we did not analyze the levels of phosphorylated tyrosine hydroxylase. The psychostimulants seem to have the ability to alter the activity of the enzyme without changing the expression (Vrana et al., 1993).

Possible Caveats

While cocaine and methamphetamine are in the same class of drugs, they do differ in both half life, and potency. Methamphetamine has a much longer half-life in addition to a much higher potency which could account for a longer withdrawal syndrome that could persist for several months (Ellinwood et al., 1998). Cocaine withdrawal is thought to last for several weeks in humans and up to 30 days in rodents following discontinuation of drug use (Gawin and Ellinwood, 1988). Both of these withdrawal syndromes are

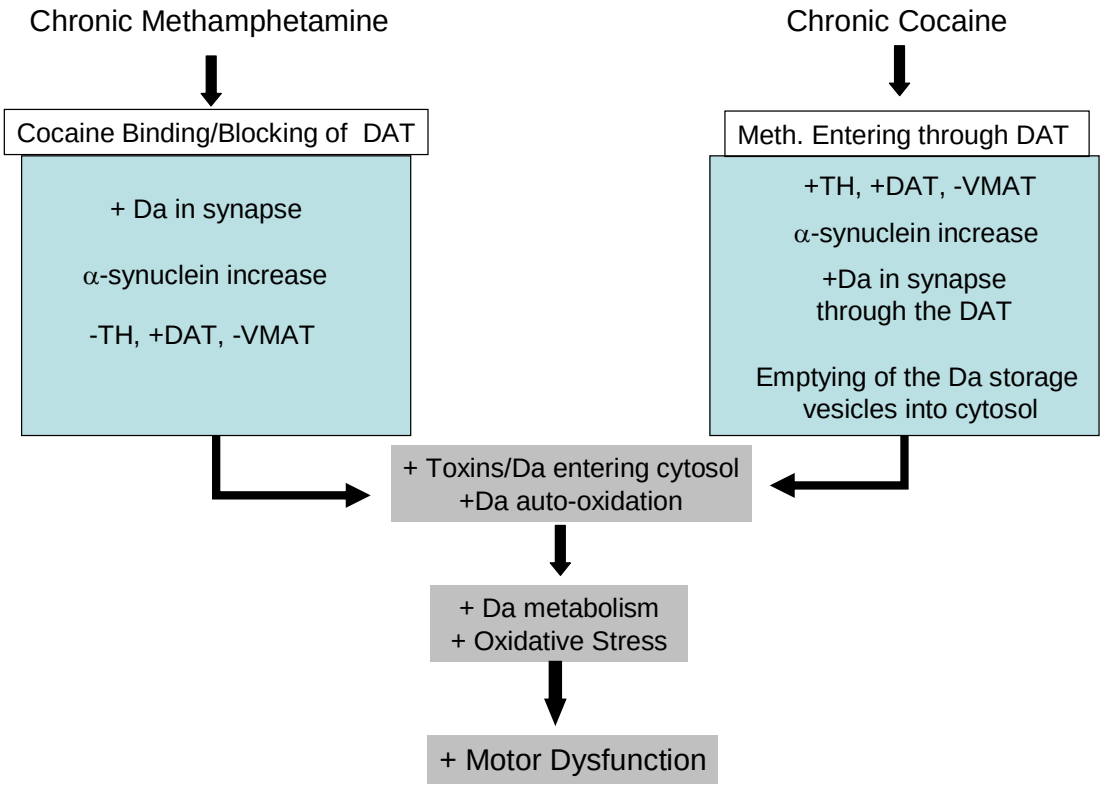
commonly associated with psychosis, paranoia, and anhedonia. Researchers have speculated that these result from the long- term dopamine deficiencies, which occur following binge use of the drugs. It is possible that the impairments in psychomotor and cognitive functions observed during this study were influenced by the withdrawal syndrome of the drugs. Indeed both withdrawal syndromes may have been present during the behavioral testing for the 11- month- old groups, though this would seem unlikely in the 14 and 16-month-old groups. It is unclear exactly how long the withdrawal syndrome for methamphetamine lasts in rodents and just how much of an effect it produced in our results without doing more analysis. Also, the age groups chosen for this study may not have been sufficient enough to portray the true long term effects of the drugs as discussed in previous chapters. Indeed longer age ranges could have produced evidence that the impairments associated with drugs rebounded back to those of basal levels. Dopaminergic neurons are very adaptable to insult (Volkow et. al., 2002). For example in Parkinson's disease patients' symptoms don't become evident until the dopaminergic neurons have declined by 85%. Another possibility is that the control groups would have become impaired while the performance of the psychostimulant groups remained relatively stable until the onset of senescence.

Conclusions and Possible Mechanisms

This study evaluated the long-term effects of the chronic administration of both methamphetamine and cocaine on the dopaminergic system in addition to psychomotor and cognitive functions. Overall, the psychomotor functions were impaired as a

consequence of the psychostimulants. Only the 14- month- old cocaine treated groups exhibited impairments on the cognitive measures we employed. Biochemical tests showed that these impairments were associated with increases in protein oxidative damage, while there was no evident change in the expression of DAT, TH, or alpha synuclein. While impairments were observed there was not enough evidence to prove the hypothesis that chronic psychostimulant administration results in an interaction in aging. Below we have outlined a possible mechanism for what might be occurring in the striatal dopaminergic neurons which would explain the declines in psychomotor functions (Figure 11). Chronic psychostimulant administration results in momentary alterations in the nigrostriatal regions. The drugs bind to and elicit their effects through the DAT, following which the cells want to decrease the amount of synaptic dopamine. To accomplish this alpha synuclein expression is increased which helps traffic the DATs from the cytosol to the plasma membrane. Increased DAT on the cytosol would allow more toxins, and redox dopamine into the cells. Dopamine modified alpha synuclein blocks chaperone mediated autophagy which would promote increases in protein aggregation. The decrease in VMATs would then decrease the storage vesicles in the neurons. The increase in intracellular dopamine would in turn increase dopamine turnover and result in the increase in oxidative damage.

Figure 11. Schematic representation showing possible mechanisms of psychostimulant-induced impairment of psychomotor function. Once administered, the psychostimulants would bind to and elicit their effects through the DAT, causing an increase in extracellular dopamine. As an adaptive response the neurons would activate regulatory mechanisms through DAT that would tend to decrease extracellular dopamine. This would cause up-regulation of alpha synuclein expression and increased trafficking of the DATs from the cytosol to the plasma membrane and decrease VMAT expression. Increased DATs would in turn allow intracellular accumulation of non-sequestered, redox-active dopamine, as well as toxins, because of the decline in VMAT. Also dopamine- modified alpha synuclein blocks chaperone mediated autophagy which would promote increases in protein aggregation. Together these events would cause damage to the dopaminergic neurons and lead to declines in psychomotor function.


Chronic Methamphetamine
Chronic Cocaine
Cocaine Binding/Blocking of DAT
+ Da in synapse
α-synuclein increase
-TH, +DAT, -VMAT
Meth. Entering through DAT
+TH, +DAT, -VMAT
α-synuclein increase
+Da in synapse through the DAT
Emptying of the Da storage vesicles into cytosol
+ Toxins/Da entering cytosol
+Da auto-oxidation
+ Da metabolism
+ Oxidative Stress
+ Motor Dysfunction

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