DESCRIPTION: See instructions. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project (i.e., relevance to the **mission of the agency**). Describe concisely the research design and methods for achieving these goals. Describe the rationale and techniques you will use to pursue these goals.

In addition, in two or three sentences, describe in plain, lay language the relevance of this research to **public** health. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

High doses of METH produce long-term consequences indicative of neurotoxicity as revealed by cognitive deficits in humans and long-term decreases in markers of dopamine (DA) and 5HT neurotransmission in humans and animals. Our studies during the previous funding period and findings by others revealed that high levels of striatal glutamate (GLU) play an important role in METH toxicity. Nevertheless, there is no evidence of how striatal GLU transmission is increased by METH and if this produces excitotoxic damage. Moreoever, despite the neurochemical similarities between METH, environmental stress, and drug abuse, it is unknown if and how stress might enhance the excitotoxic effects of METH.

The hippocampus is also vulnerable to the toxic effects of METH and is particularly sensitive to stress and excitotoxic insult due the dense composition of GLU neurons and glucocorticoid receptors in this region. Despite numerous studies demonstrating that the hippocampus is involved in cognition and human METH abusers exhibit cognitive deficits, it is surprising that little is known about how METH damages the hippocampus or how stress affects the excitotoxic effects of METH.

The proposed project is a novel extension of our prior studies and will elucidate the neurochemical determinants and consequences of GLU-mediated excitotoxicity to the striatum and hippocampus and how they are affected by prior exposure to chronic unpredictable stress.

The overarching hypothesis that will be tested by the proposed specific aims is that excitotoxicity in the striatum is produced by METH, augmented by prior exposure to chronic stress, and mediated differentially by D1 and D2 receptors. Excitotoxicity will be paralleled by increased presynaptic storage and extracellular concentrations of striatal GLU resulting in oxidative stress to the vesicular monoamine transporter (VMAT2) and the mitochondrial electron transport chain to culminate in proteasomal inhibition and spectrin proteolysis. In addition, a stress-induced enhancement of GLU transmission in the hippocampus will be similarly evidenced by increased synaptic and extracellular GLU and consequently, decreased cellular bioenergetics, decreased proteasomal activation, and spectrin proteolysis.

PERFORMANCE SITE(S) (organization, city, state)

Methamphetamine (METH) abuse has increased across the U.S. at an alarming rate since the late 1980's. High doses of METH produce long-term consequences indicative of neurotoxicity as revealed by long-term decreases in markers of dopamine (DA) and 5HT neurotransmission in humans and animals, and cognitive deficits in humans. However, the mechanisms that mediate METH neurotoxicity are unknown.

Our studies during the previous funding period of the grant and findings by others revealed that high levels of striatal GLU (GLU) play an important role in METH toxicity. Nevertheless, there is no evidence of how striatal GLU transmission is increased by METH and if this produces *excitotoxic* damage. Moreover, despite the neurochemical similarities between METH, environmental stress, and drug abuse, it is unknown if and how stress affects METH toxicity.

The hippocampus is also vulnerable to the toxic effects of METH and is particularly sensitive to stress and excitotoxic insult due to its dense composition of GLU neurons and glucocorticoid receptors. Despite numerous studies demonstrating that the hippocampus is intimately involved in cognition and human METH abusers exhibit cognitive deficits, it is surprising that little is known about how METH damages the hippocampus or how stress interacts with the excitotoxic effects of METH.

The proposed project is a novel extension of our prior studies and will elucidate the neurochemical determinants and consequences of GLU-mediated excitotoxicity to the striatum and hippocampus and how they interact with prior exposure to chronic unpredictable stress.

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SPECIFIC AIMS

1. The determinants and time-dependency of the METH-induced increases in GLU transmission will be evaluated. The specific hypotheses are:
   A. METH increases GLU release in the striatum through the *initial activation* of substantia nigra D1 receptors and the increased expression of the vesicular GLU transporter-1 (VGLUT1) and increased vesicular GLU uptake.
   B. The initial activation of GLU transmission will be *followed by a more protracted disinhibition* of corticostriatal GLU transmission through diminished D2-mediated inhibitory tone.

2. The excitotoxic and biochemical consequences of METH-induced increases in GLU transmission will be studied. It is posited that:
   A. METH will produce evidence of excitotoxicity in the striatum and substantia nigra pars reticulata via diminished D2-mediated inhibitory tone.
   B. METH will decrease bioenergetic function as measured by decreases in mitochondrial Complex II activity and protein through GLUergic and pro-oxidative processes.

3. The effect of chronic stress on the excitotoxic effects of METH to the striatum and hippocampus will be examined. It is hypothesized that:
   A. Chronic unpredictable stress will enhance the excitotoxicity in the striatum produced by METH as evidenced by augmented decreases in DAergic markers, oxidation/nitration of vesicular monoamine transporter (VMAT2) protein, increased spectrin proteolysis, proteasomal inhibition, and the presence of Fluorojade-C staining. The enhanced markers of excitotoxicity will be paralleled by increased D1 receptor protein in substantia nigra. These changes will be blocked by NMDA, AMPA and/or D1 receptor antagonism, and chemical adrenalectomy (metyrapone).
   B. Chronic unpredictable stress will enhance the excitotoxicity in the hippocampus as evidenced by augmented GLU overflow, VGLUT1 expression, and spectrin proteolysis. Fluorojade-C staining, enhanced depletions of 5HT and SERT protein and decreased activity of the electron transport chain activity will also occur in a temperature and/or corticosterone-dependent manner.

BACKGROUND AND SIGNIFICANCE

Methamphetamine (METH) is a highly abused sympathomimetic amine with potent effects on the CNS resulting in psychomotor activation, mood elevation, anorexia, increased mental alertness, physical endurance, and hyperthermia. The abuse of METH by humans induces behaviors resembling paranoid schizophrenia characterized by symptoms such as paranoid delusions, aggressive behavior, hallucinations and disordered thought processes (Ellinwood, 1967). Thus, the side effects of METH and its abuse potential have prompted a concerted effort to determine its long-lasting effects on the brain.

High doses of METH are neurotoxic to rodents and nonhuman primates as evidenced by a loss of DA terminals and DA uptake sites (Wagner et al., 1980; Ricaurte et al., 1980; 1982, Stephans and Yamamoto, 1994; 1996a), an increase in fluorescent swollen axons (Ellison, et al, 1978), and an increase in Fink-Heimer silver staining indicative of degenerating cells (Ricaurte et al., 1982; 1984). These morphological changes are closely correlated with decreases in tyrosine hydroxylase-immunoreactive fibers (Ryan et al., 1988; Pu et al., 1994) and tyrosine hydroxylase (TH) and tryptophan hydroxylase activities (Hotchkiss and Gibb, 1980). Many of these changes endure for months (Bittner et al., 1981; Seiden et al., 1975/76) and collectively, are well accepted evidence of neurotoxicity (for review, see Seiden and Ricaurte, 1987).

The neurotoxic effects of METH exhibit brain region specificity. DA terminals in the striatum are affected most severely while those in nucleus accumbens, olfactory area, and frontal cortex are affected only minimally (Ricaurte et al., 1980). The DA cell bodies in the substantia nigra and ventral tegmental area that project to these forebrain regions are relatively spared from METH-induced damage although there is evidence of a severe loss of TH immunoreactivity in the substantia nigra of human METH abusers (Wilson et al., 1996) and of mice exposed to high doses of METH (Trulson et al, 1985; Sonsalla et al., 1996). The brain regions most prone to METH-induced serotonergic toxicity are the hippocampus, amygdala, frontal cortex, and striatum. In contrast, the hypothalamus is one of the few brain regions relatively resistant to depletions of both tissue DA and serotonin content (Ricaurte et al., 1982). In contrast, METH does not damage norepinephrine (Wagner et al., 1980) or acetylcholine (Hotchkiss et al., 1979) systems.

More recent evidence in mice suggests that the neurotoxic effects of METH may not be limited to DA and 5HT terminals. METH produces intraneuronal ubiquitin inclusions associated with striatal GABA cells in mice (Fornai et al., 2003; Fornai et al., 2004), increased TUNEL staining in the mouse striatum (Deng et al., 2001) and apoptosis of GABA neurons in the rat striatum (Jayanthi et al., 2005). Despite these observations, the mechanisms mediating the damage to these non-monoaminergic cells are unknown. The possibility exists that these changes could be mediated by METH-induced increases in extracellular GLU (Nash and Yamamoto, 1992; Stephans and Yamamoto, 1994). Since striatal GABA neurons express NMDA, AMPA, and metabotropic GLU receptors (Albin et al., 1992; Bernard and Bolam, 1998), it is possible that GLU mediated excitotoxicity plays a role in the damage to striatal soma and terminals after METH. **Specific Aim 2A will investigate this possibility.**

Glutamate, Methamphetamine, and Proteolysis

Glutamate and other excitatory amino acids (EAAs) have been linked to a number of neurodegenerative disorders (reviewed by Lipton and Rosenberg, 1994; Olney, 1980; Calabresi et al., 2000; Przedborski, 2005). Glutamate also appears to mediate the toxicity produced by METH. Sonsalla et al (1989) were the first to implicate GLU by demonstrating that an N-methyl-d-aspartate (NMDA) antagonist, MK801 blocks the decreases in TH activity and DA tissue content after METH. Their findings have since been extended by others using noncompetitive and competitive NMDA antagonists (Fuller et al., 1992; Baldwin et al, 1993; Weihmuller et al., 1992). Our laboratory was the first to demonstrate that METH itself or d-amphetamine administered to iprindole-treated rats increases the extracellular concentration of striatal GLU measure*d in vivo* (Nash and Yamamoto, 1992; 1993). This was subsequently confirmed (Abekawa et al., 1994; Bowyer, et al., 1993; Mora and Porras, 1993; Zhang et al., 2001; Bustamante et al., 2002) and extended to show that the GLU-derived neuronal nitric oxide (Itzhak and Ali, 1996) and the mGlu5 metabotropic receptor are involved (Battaglia et al., 2002; Golembiowska et al., 2003). In contrast, there are no significant increases in GLU efflux in the medial prefrontal cortex (Stephans and Yamamoto, 1996b) and nucleus accumbens (Abekawa et al., 1994), regons relatively resistant to the DA-depleting effects of METH.

Although GLU appears to be important in mediating METH toxicity, how METH increases GLU is unknown. No evidence indicates that METH directly increases the release of GLU in the striatum. However, METH

appears to increase striatal GLU via a polysynaptic pathway involving the D1 receptor in basal ganglia output structures (Mark et al., 2004; see Progress Report). Further studies are needed to evaluate the mechanistic underpinnings of how *synaptic* GLU could be increased by METH via the activation of this pathway. Since the vesicular GLU transporter-1 (VGLUT1) is responsible for the sequestration of GLU into vesicles for release (Bellachio et al., 2000), studies are needed to evaluate the synthesis and expression of VGLUT1 that are associated with neuronal presynaptic terminals after exposure to METH **(Specific Aim 1A**).

The increase in GLU observed after METH is delayed and prolonged (Nash and Yamamoto, 1992, 1993; Stephans and Yamamoto, 1994). The mechanisms contributing to this temporal profile are unknown but could involve the differential contribution of D1 (Mark et al., 2004) and D2 receptors in the striatum and substantia nigra. D2 receptors are located on corticostriatal terminals (Hersch et al., 1995; Wang and Pickel, 2002) and negatively modulate striatal GLU (Yamamoto and Davy, 1992; Bamford et al., 2004). D2 receptors in the substantia nigra can also attenuate stimulated GABA release (Matuszewich and Yamamoto, 1999). Therefore, the delay in the METH-induced and D1 mediated increases in corticostriatal GLU can occur by two mechanisms: (1) a D2 mediated inhibition of corticostriatal GLU release or (2) a D2 inhibition of GABA release in substantia nigra that in turn, increases nigrothalamic GABA and inhibits thalamocortical and corticostriatal GLU projections. The prolonged elevation in GLU in striatum or SN that occurs after the delayed increase could be due to the eventual depletion of DA stores by METH that subsequently decreases D2-mediated inhibitory tone on striatonigral GABA and corticostriatal terminals. Regardless of the pathway involved, striatal GLU would be disinhibited to cause a *persistent* elevation in extracellular GLU concentrations **(Specific Aim 1B)** that is critical to the manifestation of METH toxicity**.** The increase in GLU may in turn, have excitotoxic consequences.

Excitotoxicity is a consequence of high concentrations of GLU (Olney, 1980). This is typically reflected by cell loss produced by GLU-induced increases in intracellular calcium and the activation of calcium-dependent proteases such as calpain I. Calpain in turn, degrades spectrin, the structural component of neurons, into specific molecular weight breakdown products of 145-150 kDa fragments. Consequently, spectrin proteolysis has been used as a reliable and specific marker of GLU-dependent neurotoxicity (Siman et al., 1989). Therefore, we posit that METH produces excitotoxicity as evidenced by the increased breakdown of spectrin (See preliminary data, Fig. 6) in basal ganglia structures such as the caudate and substantia nigra that receive a substantial GLUergic input from the cortex/thalamus (Albin et al., 1989) and subthalamic nucleus/cortex (Smith et al., 1996), respectively **(Specific Aim 2A)**.

The possibility that METH may increase protein breakdown products while increasing ubiquitin inclusions and immunoreactivity (Fornai et al, 2004) suggests that the ubiquitin-dependent proteolytic process may be impaired such that proteins tagged with ubiquitin aggregate rather than being degraded by the proteasome. It has been shown that nitrosative oxidative stress decreases the ubiquitin-proteasome pathway (Yao et al., 2004). Thus, it is possible that METH (through oxidative stress; see below) also inhibits this pathway. We hypothesize that this could subsequently affect GLU receptor function. PSD-95 (post synaptic density protein) plays an important role in AMPA receptor surface expression. Inhibition of ubiquitination of PSD-95 decreases AMPA internalization by NMDA (Colledge et. al., 2003). Therefore, an impairment of ubiquitination and proteasomal activation may prevent GLU receptor internalization and/or degradation and lead to an enhanced GLUergic response. **Specific Aim 3 will examine the activity of the proteasome and ubiquitin-dependent proteolysis after METH and/or chronic stress.**

Oxidative Stress, DA, and Glutamate

As noted above, METH increases the extracellular concentrations of both DA and GLU. A common mechanism underlying the toxic effects of both DA and GLU is the production of oxidative stress. Oxidative stress is defined as the cytotoxic consequences of reactive oxygen species (e.g., $^{\bullet}O_2^-$, $^{\bullet}OH$) generated as byproducts of oxidative metabolism. The targets of free-radical mediated damage are proteins (protein nitration), lipids (lipid peroxidation), and DNA (nucleotide oxidation).

The mechanism underlying DA-induced cytotoxicity may involve the generation of free radical species and quinones (Olanow, 1992) which attack thiol-containing proteins (Fornstedt et al., 1989). In fact, the intrastriatal injection of high concentrations of DA results in neurotoxicity and in the formation of protein bound, cysteinyl adducts of DA; both of which are prevented by the co-administration of antioxidants (Hastings et al., 1996). Furthermore, free radicals, and possibly DA quinones, can decrease DA transporter function (Berman et al.,

1996; Fleckenstein et al., 1997). Therefore, the massive increase in the extracellular concentrations of DA, such as that produced by METH, could result in the production of hydroxyl free radicals, oxidative stress, and eventual damage to DA terminals. Along these lines, there is strong supportive evidence by Fleckenstein and co-workers that altered sequestration of DA by the vesicular monoamine transporter, VMAT2, may contribute to DA-derived oxidative stress in the cytosol of DA terminals (for review, see Fleckenstein and Hanson, 2003). Furthermore, METH also produces oxidative stress as evidenced by increases in the production of the $^{\bullet}$OH-salicylate adduct, 2,3-dihydroxybenzoic acid (Giovanni et al., 1995; Fleckenstein et al., 1997; Yamamoto and Zhu, 1998), as well as lipid peroxidation, and protein carbonyl formation in both the striatum and hippocampus (Yamamoto and Zhu, 1998; Gluck et al., 2001). Conversely, antioxidants acting as free radical scavengers attenuate the depletion of striatal DA and 5HT in response to METH (DeVito and Wagner, 1989; Cappon et al., 1996; Yamamoto and Zhu, 1998).

As stated previously, GLU increases calcium influx leading to the activation of calpain and the eventual lysis of spectrin into specific breakdown products. Based on the GLU and calcium dependency of spectrin proteolysis by calpain, spectrin breakdown has been used as a specific marker of excitotoxicity (Siman et al., 1989). In addition, GLU and GLU receptor activation also can cause neuronal death through oxidative stress mechanisms via the activation of nitric oxide synthase, the production of nitric oxide and superoxide and the consequent formation of highly reactive peroxynitrite (Lafon-Cazal et al., 1993). Conversely, enhanced metabolism of superoxide through the overexpression of the human ZnCu-superoxide dismutase (SOD) gene results in a resistance to the DA-depleting effects of METH (Cadet et al., 1994). Similarly, neuronal nitric oxide synthase deficient mice that presumably produce little nitric oxide radical and peroxynitrite are also resistant to the toxic effects of METH (Itzhak et al., 1998).

Despite this wealth of evidence linking METH and oxidative processes, it is unknown what specific cellular elements are damaged by METH. Recent findings however, show that METH produces an oligomerization of the DA transporter (Baucum et al., 2004) in a pro-oxidative manner that could involve peroxynitrite-induced damage to the DA transporter (Park et al., 2002). Additionally, our published and preliminary data collected during the previous funding period indicate that the activities of complexes II and IV of mitochondria and VMAT2 are decreased (See Preliminary Data). Therefore, **Specific Aims 2B and 3A will examine the oxidation/nitration and nitrosylation of mitochondrial electron transport chain proteins and VMAT2.**

Metabolic/Bionergetic Stress and Methamphetamine

Impaired energy metabolism also may contribute to METH-induced neurotoxicity. The results of early experiments in the 1970's showed that low doses of amphetamine and METH increased metabolism in cerebral cortex or whole brain as measured by formation of lactate and changes in high energy substrates such as ATP and phosphocreatine (e.g. Sylvia et al., 1977).

The pattern of glucose utilization following systemic administration of amphetamine or METH reveals that brain energy utilization increases in a region-specific manner. The increase appears to be greatest in those regions most susceptible to the toxic effects of METH. METH increases the extracellular concentrations of lactate in the striatum but not in the prefrontal cortex, the latter area being relatively resistant to the long term DA depleting effects of METH (Stephans et al., 1998). Therefore, the regional selective effect of METH-induced energy consumption may be due to GLU release, oxidative stress, and the long-term depletions of DA.

The increase in energy consumption by neurotoxic amphetamines may be due, in part, to the sodium-dependent reversal of the amine transporter by which these drugs facilitate the efflux of DA (Raiteri et al., 1979, Fisher and Cho, 1979). Under steady state conditions, neurons have been estimated to expend more than half of their ATP supply solely to maintain ion (e.g. Na^+) homeostasis (Siesjo, 1978). Therefore, the continued reversal of the transporter increases intracellular sodium and prolongs the activation of the ATP-dependent Na^+/K^+ ATPase. Thus, METH may enhance energy utilization indirectly through the disruption of transmembrane ion gradients.

METH depletes striatal ATP content (Chan et al., 1994) and compounds that block energy production cause neuronal damage similar to METH. Malonate, a reversible inhibitor of succinate dehydrogenase in complex II of the mitochondria, depletes ATP and produces an accumulation of lactate (Beal et al., 1993a; 1993b). Malonate also potentiates the depletion of DA produced by METH (Albers et al., 1996; Burrows et al., 2000b). Moreover, DA terminals in the striatum are selectively more vulnerable than GABA neurons to malonate and inhibition of the mitochondrial electron transport chain (Zeevalk et al., 1995; 1997). These findings suggest that a constitutive metabolic vulnerability is inherent to DA versus other neurotransmitter

systems (Marey-Semper et al., 1993). Consequently, perturbations in energy metabolism may contribute to the selective damage to DA neurons produced by METH.

Our recent studies during the previous funding period showed that indeed, the activities of complexes II and IV of the mitochondrial electron transport chain are decreased early after METH (Burrows et al, 2000a; Brown et al., 2005; See Progress Report). Interestingly, the decrease in complex II activity is blocked by the NMDA antagonist MK801, as well as by a peroxynitrite scavenger and suggest that activation of nitric oxide synthase and the production of nitric oxide through the activation of GLU receptors may produce oxidative/nitrosative damage to complex II. **To investigate this possibility, the alterations in activity and nitration and nitrosylation of complex II protein will be examined in Specific Aim 2B**.

In summary, GLU, DA, bioenergetic compromise, and hyperthermia (Burrows et al., 2001) appear to be necessary for METH neurotoxicity but they appear to be insufficient when altered independently. The local perfusion of METH under conditions that produce an equivalent increase in extracellular DA as that observed after the systemic administration of METH does not produce long-term depletions of striatal DA tissue content (Burrows et al., 2000). This finding is consistent with the conclusion that increases in extracellular DA alone do not correlate with the induction of oxidative damage (LaVoie and Hastings, 1999) after METH. In addition, local METH perfusion does not increase GLU or produce hyperthermia (Burrows et al., 2000). Hyperthermia alone does not cause DA depletions or inhibition of mitochondrial oxidative phosphorylation and the direct application of METH to mitochondria does not inhibit complex II activity (Brown et al., 2005). Thus, a convergence of multiple factors appears necessary for METH toxicity. Consequently, it is important to consider increased GLU (**Specific Aims 1-2**), DA (**Specific Aims 1A and B, 2A**) and bioenergetic compromise (**Specific Aim 2B**) independently and collectively in a coordinated manner (**Specific Aims 1-2**).

Role for Environmental stress

There are parallels between stress and amphetamine and their ability to alter GLU, DA, and glucocorticoid secretion. Moreover, stress plays an important role in both drug abuse and relapse (Sinha, 2001). Environmental stress and amphetamine increase DA and GLU release in the striatum (Abercrombie et al., 1989; Gresch et al., 1994; Moghaddam, 1993; Stephans and Yamamoto, 1996b) and increase circulating glucocorticoid concentrations (Antelman and Chiodo, 1983). Stress also predisposes the brain to neurotoxic insults. Chronic stress causes an imbalance between pro-oxidants and antioxidants as evidenced by increases in xanthine oxidase activity and decreases in superoxide dismutase activity in the brain (Kaushik and Kaur, 2003). Glucocorticoid release induced by stress can compromise neuronal viability such that the neurotoxicity induced by ischemia, seizures and hypoglycemia is exacerbated by glucocorticoids and attenuated by adrenalectomy (McEwen and Gould, 1990; Sapolsky and Pulsinelli, 1988). These insults appear to be mediated via the NMDA receptor and excessive levels of excitatory amino acids. More recently, our studies have also shown that chronic stress prior to METH enhances METH toxicity, as evidenced by an enhancement of METH-induced striatal DA depletions (Matuszewich and Yamamoto, 2004). However, it is not known how stress exacerbates METH toxicity and if stress can augment GLU-mediated excitotoxicity produced by METH. **Specific Aim 3 will directly examine the interrelationship between excitotoxicity, environmental stress, and METH**.

Model of Chronic Unpredictable Stress

Several different paradigms using rats have been employed to model stressful experiences (e.g. social aggression, maternal separation, footshock, tail pinch and restraint). One paradigm that has been widely used to mimic repeated but mild stressful events is chronic unpredictable stress (CUS). In this paradigm, the type and time of stress exposures are varied (Katz et al., 1981; Willner et al., 1992; Ortiz et al., 1996). The advantage of this paradigm over other chronic stress models is that the unpredictable nature of the exposures to varied stressors mimics the exposure to unexpected stressful life events. Moreover, the paradigm is not confounded with learning and adaptation. This stress paradigm has also been shown to cause a persistent elevation in plasma levels of corticosterone (CORT) (Herman et al., 1995; Araujo et al., 2003) whereas repeated predictable stress results in diminished CORT responsiveness with time (Bielajew et al., 2002; Lopez et al., 1998). Most importantly, CUS produces neurochemical changes in the mesolimbic DA system, increases place preference for cocaine at lower doses, and enhances cocaine-induced DA effects (Haile et al, 2001). Conversely, chronic (predictable) restraint stress is without effect on these measures (Ortiz et al., 1996; Haile et al., 2001). In contrast to the findings that CUS alters DA-mediated behaviors, it produces

marginal or no changes in physiological measures such as body weight gain, adrenal and spleen weights (Bielajew et al., 2002), or abnormal cell numbers in the hippocampus (Sousa et al., 1998). Thus the CUS regimen is mild and does not have any obvious debilitating effects on physiology or morphology. Based on the above evidence, CUS will be used throughout this proposal as an mild stress model to examine the interactions between chronic stress and METH-induced neurotoxicity.

Effects of METH and Stress on the Hippocampus

METH produces depletions of 5HT (Ricaurte et al., 1980) and oxidative damage in the hippocampus (Gluck et al., 2001). Despite evidence of cognitive deficits in humans who abuse METH (Thompson et al, 2004) and that the hippocampus is intimately involved in learning and memory functions, surprisingly little is known about how METH is toxic to the hippocampus (HIPP).

In addition to the vulnerability of the HIPP to METH, the HIPP is also vulnerable to stress. The HIPP has some of the highest densities of CORT receptors in the brain and is a main target of glucocorticoid action (Aronsson et al., 1988; Reul and de Kloet, 1985). In view of the fact that stress activates the HPA axis and increases the release of CORT (Munck et al., 1984) and GLU (Lowy et al., 1994), stress may damage the HIPP (Stein-Behrens et al., 1994; McEwen, 2001). This damage may occur through an increase in cytosolic calcium load on hippocampal neurons that exacerbates the response to an insult directly via postsynaptic effects and indirectly through increased GLUergic tone impinging on the hippocampal neuron (Sapolsky, 2000).

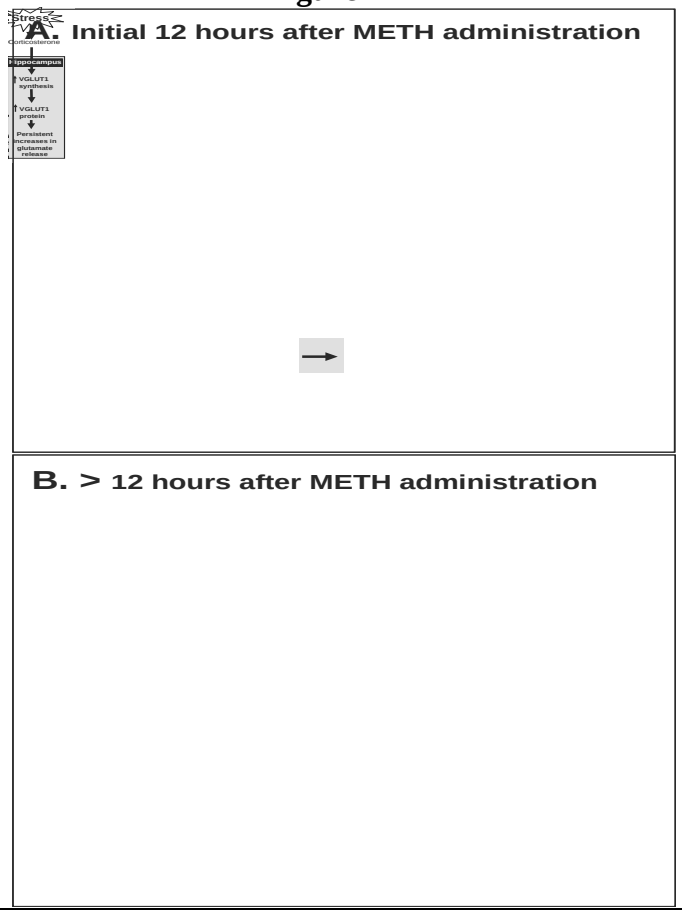
In fact, several parallels exist between METH and stress that could intersect when administered in combination to affect the HIPP. METH or stress increases CORT (Szumlinski et al., 2001; Munck et al., 2004), decreases brain glutathione levels, and increases lipid peroxidation in the HIPP (Madrigal et al., 2001; Gluck et al., 2001). Therefore, the combined exposure to stress and METH may be deleterious to the HIPP through GLUergic and oxidative mechanisms. **Specific Aim 3 will examine the contribution of stress to the toxic effects of METH on the HIPP and striatum by evaluating the GLU-dependent mechanisms elucidated in Specific Aims 1 and 2.** Along these lines, our preliminary data indicate that CUS and METH synergize to augment hippocampal 5HT depletions (See Preliminary Data, Fig. 18)).

Summary and Hypothetical Model

Based on mounting evidence that GLU is important in mediating the toxic effects of METH and stress, it is important to examine the mechanisms that mediate the increase in GLU after METH and how these factors are affected by prior exposure to stress. The proposed experiments are designed to elucidate the determinants and consequences of enhanced GLU release after neurotoxic doses of METH and will examine the extent to which GLU contributes to METH-induced oxidative and metabolic stress. The underlying general hypothesis is that when both DA and GLU are increased by METH and/or chronic stress, they may add or synergize to destroy metabolically compromised DA and 5HT axon terminals and perhaps striatal and hippocampal soma that have been exposed continually to GLU.

Figure 1 shows that **(A)** In <12 hrs, METH and stress converge to (1) increase CORT and/or (2) activate a D1/GABA-dependent polysynaptic path in the basal ganglia to increase VGLUT1 synthesis in hippocampus and striatum, respectively. **(B)** In > 12 hrs, VGLUT1 synthesis will increase VGLUT1 protein in striatum and hippocampus to produce a persistent increase in extracellular GLU resulting from the disinhibition of inhibitory D2 control. This increase would eventually result in oxidative damage to VMAT2 protein and the

Figure 1



mitochondrial ETC. Damage to these cellular elements will lead to excitotoxicity to terminals and soma in hippocampus and striatum. Please note that this hypothetical model is simply the basis for the proposed studies and does not represent all of the factors involved in METH toxicity.

SIGNIFICANCE

Abuse of METH is a major health concern as evidenced by the report from the Community Epidemiology Work Group (CEWG) meeting in 2003 (www.drugabuse.gov/Infofax/methamphetamine.html) revealing high levels of abuse in the U.S that are spreading throughout the entire nation at an unparalleled rate. In addition, local legislatures have begun efforts to restrict the sale of precursors (e.g. ephedrine/pseudoephedrine) typically used by clandestine labs to manufacture METH. The proposed studies are relevant to the mechanisms responsible for the deleterious consequences of METH and address issues that could be relevant to the etiology of several neurodegenerative diseases associated with excitotoxcity, the co-morbidity of stress and drug abuse, or the enhanced vulnerability of chronically stressed individuals (e.g. PTSD) to METH and related behavioral disorders (cognitive deficits, psychosis, movement disorders). Additional evidence is needed to understand how the cellular mechanisms described above integrate to damage the striatum and hippocampus under *in vivo* conditions. More specifically, further information is needed to elucidate the compromised cellular processes produced by GLU and DA that are determinants of METH neurotoxicity

The investigation of enhanced drug-induced behavioral and biochemical effects by stress on brain DA and GLU systems may provide insight into reasons for the high co-morbidity of substance abuse with post-traumatic stress disorder (Keane et al., 1988; Brown et al., 1995). The converse may also be relevant such that prior exposures to stimulants may enhance the acute or long-term effects of environmental stress on motor and cognitive functions. The proposed experiments are novel in that they will address the determinants of how METH alone or chronic stress will produce consequences reflective of excitotoxicity, impaired cellular energetics, and oxidative stress via the integrated and time-dependent alterations in DA and GLU.

PROGRESS REPORT/PRELIMINARY STUDIES

Funding Period (6/1/2000 – 9/1/2005)

This grant is in a no-cost extension period (6/1/05 to 5/31/06) due to the delays resulting from my relocation from Case Western Reserve University to Boston University in September, 2001.

During the most recent funding period, studies directly related to this project have resulted in 17 publications in peer-reviewed journals, 1 book chapter, and 1 manuscript submitted for peer-review. In addition, 35 abstracts for poster/slide presentations at international and national meeting have been presented. The P.I. also was invited to speak on topics directly related to this project on 36 different occasions.

The Specific Aims were to identify the neural pathways and processes that mediate enhanced striatal GLUrgic activity to produce long-term DA depletions after METH. The processes hypothesized to be involved in mediating METH toxicity were excitotoxicity, oxidative stress and compromised bioenergetics. In addition, new factors were identified that include environmental stress and stimulant sensitization.

All of the original Specific Aims were addressed and resulted in the following key published findings:

Summary of Key Findings

A. METH decreased the activity of Complex IV as assessed histochemically. These decreases were in the most prominent DA projection areas. (European Journal of Pharmacology, 400:99-102, 2000).
B. METH also decreased the activity of Complex II but not Complex I in the striatum (J. Neurochemistry, 95: 429-436, 2005).
    a. The decrease in Complex II activity was assessed ex vivo in an isolated mitochondrial fraction.
    b. The decrease was blocked by the NMDA antagonist, MK801 or the peroxynitrite scavenger, Fe-TPPS and suggests that GLU and peroxynitrite contribute to the early decrease in complex II activity.
    c. The decrease was not due to a direct effect of METH on the mitochondria or hyperthermia and was selective for the striatum compared to the hippocampus.
C. METH synergizes with the central administration of malonate to deplete striatal DA (Journal of Pharm. and Exptl. Therapeutics, 292: 853-860, 2000).
    a. The central administration of METH did not deplete striatal DA but only when co-administered with the complex II inhibitor, malonate.

   b. The depletion of DA compared to the depletion of 5HT was more sensitive to the combined effects of METH and malonate.

D. METH increased both reduced glutathione and oxidized glutathione in the striatum but did not alter vitamin E or vitamin C (European Journal of Pharmacology, 400:99-102, 2000).
   a. METH was selective for the brain glutathione system and support a role for oxidative stress in METH toxicity

E. Chronic stress enhanced the toxic effects of METH on DA and 5HT (Neuroscience 124: 637-646, 2004).
   a. Chronic unpredictable stress enhanced METH-induced DA release and long-term DA and 5HT depletions in the striatum.
   b. Chronic unpredictable stress also enhanced the lethality associated with METH.
   c. Chronic unpredictable stress did not increase the concentrations of METH in the brain.

F. Chronic unpredictable stress increased produced long-lasting hyperthermic responses to a 5HT2 agonist, DOI (Psychopharmacology 169: 169-175, 2003)
   a. DOI-induced hyperthermia was augmented by prior exposure to 10 days of chronic unpredictable stress when examined at 8, 30 or 60 days after the last stressor.
   b. The enhancement of hyperthermia was evident even after the stressor was removed and absent for more than 8 weeks.

G. METH activates the striatonigral pathway to increase GLU release in the striatum (Journal of Neuroscience 24: 11449-11456, 2004)
   a. METH increased GABA release in the substantia nigra that was blocked by the D1 antagonist SCH23390.
   b. METH increased GAD65 mRNA expression in the striatum.
   c. Blockade of GABA-A receptors with bicucullilne in the substantia nigra blocked the decrease in GABA release in the ventromedial thalamus and the long-term depletions of DA in the striatum.

H. Lobeline attenuated the decreases in VMAT2 immunoreactivity and long-term DA content after METH but did not block METH-induced DA release (J. Pharm. and Expt.l Therapeutics 312: 160-169, 2005).
   a. The attenuation of METH-induced decreases in VMAT2 immunoreactivity and DA content were both temperature-dependent and independent.
   b. The temperature independency of lobeline on METH toxicity was shown by the findings that lobeline blocked the VMAT2 decreases and long-term DA content when administered 5 hours after METH when elevated body temperatures have returned to normal.

I. Subchronic exposure to low doses of METH, that have been shown to produce behavioral sensitization, attenuated stress-induced DA release and increased DA transporter immunoreactivity in the nucleus accumbens shell (Psychopharmacology, 181, 467-476, 2005).
   a. Stress-induced DA release in nucleus accumbens shell was attenuated by prior exposure to low doses of METH. There were no changes in stress-induced DA release within the nucleus accumbens core.
   b. Subchronic treatment with METH increased DA transporter but not norepinephrine transporter immunoreactivity in the nucleus accumbens but not in the striatum or medial prefrontal cortex.

J. Cortical lesions attenuated the METH-induced increases in striatal GLU and blocked the long-term depletions of DA in the striatum (In: "*Glutamate and Addiction*", Humana Press, 2001, pp 211-228). In addition, this study also showed that a Ca^{2+}-free medium with TTX blocked the increase in GLU and further attests to the impulse-dependency of the microdialysis measures of extracellular striatal GLU.

K. Extracellular DA and GLU were monitored during the injections of METH in the striatum and for 24 hrs after the last injection of METH. Figures 2 and 3 show that the increases in DA release during METH (only the 4 hr and 8 hr timepoints are shown but METH was injected 4 times, once every 2 hrs over 8 hrs) was significantly lower at 16, 18 and 32 hrs (i.e. 8, 20 or 24 hrs after the last injection of METH) compared to baseline concentrations. Extracellular also GLU increased, peaked at 12 hrs (4 hrs after the last METH injection), and remain elevated for the next 24 hrs (32 hr timepoint). *$p<0.05$ compared to baseline.

Figure 2

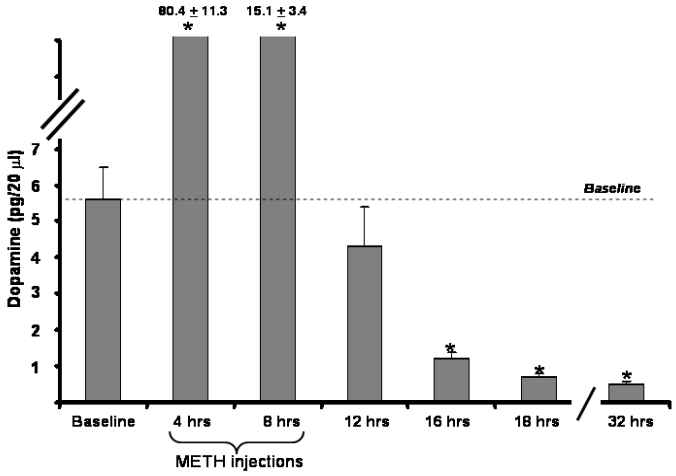
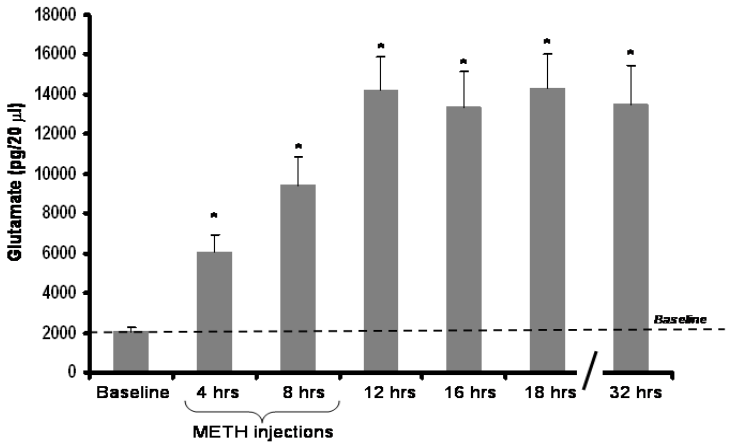


Figure 3



L. METH increases spectrin breakdown as indicated by an increase in the 145 kDa protein that is the product of the action of calcium dependent protease, calpain. AMPA antagonism blocked spectrin proteolysis. (J. Neurochemistry 96: 1267-1276, 2006).

M. To test if peroxynitrite can degrade VMAT2 and increase s-nitrosylation, peroxynitririte was incubated in vitro with synaptosomes, after which VMAT2 or s-nitrosyl-cysteine immunoreactivity was assessed (Fig. 4A). Peroxynitrite but not METH decreased VMAT2 immunoreactivity in a concentration-dependent manner and increased s-nitrosyl-cysteine immunoreactivity. Degradation occurs presumably by the proteasome within the synaptosome since direct application of peroxynitrite onto the blot did not change VMAT2 immunoreactivity with the antibody. Figure 4B shows that incubation of vesicles with peroxynitrite produces an overall smearing, indicative of s-nitrosylation of cysteine residues on multiple proteins.

Figure 4A

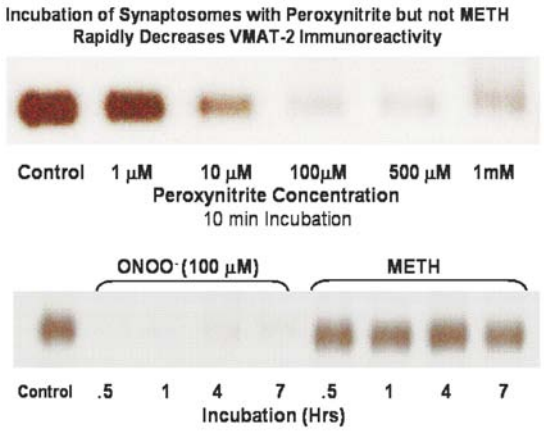
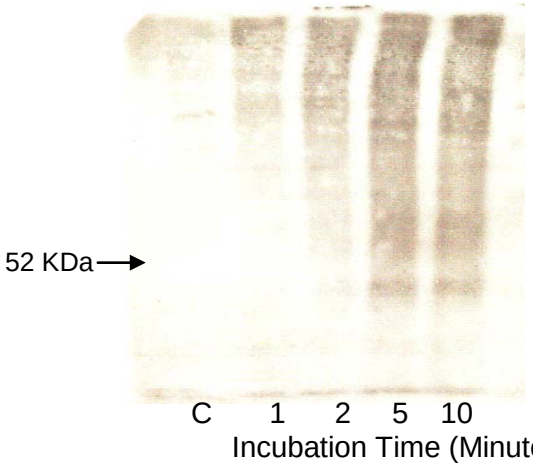


Figure 4B

Incubation with Peroxynitrite Increases S-Nitrosyl-Cysteine Immunoreactivity of Vesicles



Summary of Results

During the previous funding period, significant progress was made toward the testing of the hypothesis that METH increases corticostriatal GLU indirectly via the striatonigral path in a D1 and GABA-A dependent manner to disihinibit (via a decrease in GABA) the thalamocortical pathway. Additional neuroanatomical studies were performed to further validate the importance of the corticostriatal GLUergic path by showing the lesions of the cortex not only attenuate METH-induced striatal GLU release but also blocked the long-term depletions of DA in the striatum. Biochemical mechanisms mediating the increase in GLU were also identified. These include findings of NMDA and peroxynitrite dependent decreases in Complex II of the mitochondria, a decrease in Complex IV, and increased spectrin proteolysis in a calpain-dependent and AMPA receptor dependent manner. In addition, it was shown that METH compromises the antioxidant capacity of the brain selectively through an increase in oxidized glutathione without affecting vitamin C or E.

New studies were performed as well that provide the rationale for the proposed experiments. Chronic stress enhances the toxicity of METH. We plan to extend these studies in **Specific Aim 3** to uncover the mechanisms underlying this enhanced effect. The sustained rise in GLU may be mediated by a lack of DAergic tone for 24 hrs after METH. The delayed and sustained rise in GLU will be evaluated in Specific Aim 1. In addition, increases in striatal GAD 65 mRNA shows that the striatonigral system is activated after METH. This system appears critical to the increase in corticostriatal GLU and thus, will be a focus of the proposed studies in **Specific Aim 2** that will elucidate within this system, the causes and consequences of METH

Additional new preliminary data that provide a strong rationale for the proposed studies are included in the Experimental Design section where appropriate. In brief, some of these results include: VGLUT1 protein is increased by METH, VMAT2 is decreased and indicative of oxidative damage to this protein after METH, cell loss in the substantia nigra pars reticulata is modulated by D2 receptors, striatal GLU release in striatum and hippocampus is enhanced by METH in chronically stressed rats, 5HT depletions are enhanced by METH in chronically stressed rats, and METH decreases Complex II protein.

Abstracts and Publications Funded by the award during 6/1/2000 to 6/30/2006

A. Abstracts

35 poster or slide presentations have been presented.

B. Peer-Reviewed Publications

17 peer-reviewed papers have been published.

Burrows, K., Nixdorf, W., and Yamamoto, B.K. Central administration of methamphetamine synergizes with metabolic inhibition to deplete striatal monoamines. J. Pharm. & Exptl. Therapeutics, 292: 853-860, 2000

Burrows, K.B., Gudelsky, G.A. and Yamamoto, B.K. Role of Metabolic Inhibition in Methamphetamine and MDMA Toxicity: Evidence for Decreased Mitochondrial Function following Drug Administration. European Journal of Pharmacology 398: 11-18, 2000.

Harold, C., Wallace, T., Friedman, R., Gudelsky, G.A., and Yamamoto, B.K. Methamphetamine selectively alters brain antioxidants. European Journal of Pharmacology, 400:99-102, 2000.

Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. Enhancement of MDMA neurotoxicity by the energy inhibitor malonate. J. Neurochemistry 77: 647-654, 2001

Tor-Agbidye, J., Yamamoto, B.K. and Bowyer, J.F. Seizure activity and hyperthermia potentiate the increases in DA and serotonin extracellular levels in the amygdala during exposure to d-amphetamine. Toxicological Sciences 60: 103-111, 2001.

Matuszewich, L, and Yamamoto, B.K Long-lasting effects of chronic stress on DOI-induced hyperthermia. Psychopharmacology 169: 169-175, 2003.

Matuszewich, L, and Yamamoto, B.K. Chronic stress augments the acute and long-term effects of methamphetamine. Neuroscience 124: 637-646, 2004.

Soares, J, Kliem M.A., Betarbet, R., Greenamyre, J.T., Yamamoto, B., and Wichmann, T. Role of external pallidal segment in primate parkinsonism: Comparison of the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism and lesion of the external pallidal segment. J. Neuroscience 24: 6417-6426, 2004.

Mark, K.A., Soghomonian, J-J, and Yamamoto, B.K. High-Dose Methamphetamine Acutely Activates the Striatonigral Pathway to Increase Striatal GLU and Mediate Long-term DA Toxicity. J. of Neuroscience 24(50): 11449-11456, 2004.

Eyerman, D and Yamamoto, B.K. Lobeline attenuates methamphetamine induced changes in VMAT-2 immunoreactivity and monoamine depletions in the striatum. J. Pharm. & Exptl Ther. 312: 160-169, 2005.

Brown, J. and Yamamoto, B. Psychostimulants and mitochondrial function. Pharmacology and Therapeutics 99: 45-53, 2003.

Matuszewich, L, and Yamamoto, B.K. Effects of chronic stress on methamphetamine-induced DA depletions in the striatum. Ann N Y Acad Sci. 1032:312-4, 2004.

Broom, S.L. and Yamamoto, B.K. Effects of Subchronic Methamphetamine Exposure on Basal DA and Stress-induced DA Release in the Nucleus Accumbens Shell of Rats. Psychopharmacology, 181:467-76, 2005.

Brown, J.M., Quinton, M.S. and Yamamoto, B.K. Methamphetamine-Induced Inhibition of Mitochondrial Complex II: Roles of GLU and Peroxynitrite. J. Neurochemistry 95: 429-436, 2005.

Staszewski R and Yamamoto, B.K. Methamphetamine-Induced Spectrin Proteolysis in the Rat Striatum. J. Neurochemistry 96: 1267-1276, 2006

Hatzipetros, T and Yamamoto, B.K. Dopaminergic and GABAergic Modulation of Glutamate Release from Rat Subthalamic Nucleus Efferents to the Substantia Nigra. Brain Research, 1076: 60-67, 2006.

Quinton, M.S. and Yamamoto, B.K. Causes and Consequences of Methamphetamine and MDMA Toxicity. Am. Association of Pharmaceutical Sciences Journal 8: E337-E347, 2006.

Book Chapter

Burrows, K.B. and Yamamoto, B.K. Roles of corticostriatal glutamate and oxidative stress in methamphetamine neurotoxicity. In: "Glutamate and Addiction", Humana Press, 2001.

Submitted

Tata, D and Yamamoto, B.K. Interactions between Methamphetamine and Psychological Stress: Roles of Glutamate, Oxidative Stress and Mitochondrial Dysfunction. Submitted to *Addiction*

EXPERIMENTAL DESIGN

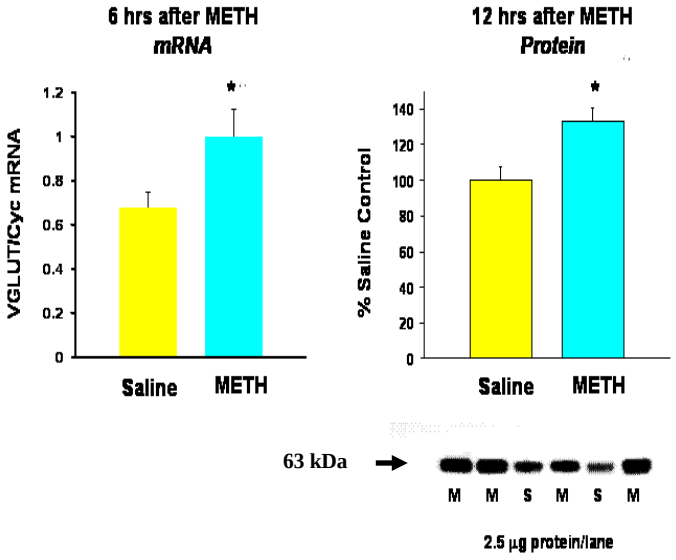
Rectal temperatures will be monitored hourly in all of the proposed studies during the administrations of saline and METH and for 6 hrs thereafter where appropriate. This extended timecourse of temperature monitoring is based on our findings that METH induced hyperthermia persists for 5 hrs after the last injection of METH (Eyerman and Yamamoto, 2005) but returns to basal values within 5 hrs. Therefore, we will assess if any drug treatments during or after METH will affect METH hyperthermia and METH-induced toxicity.

Specific Aim 1: The determinants and time-dependency of the METH-induced increases in GLU transmission will be evaluated. The specific hypotheses are (A) METH will increase GLU release in the striatum through the initial activation of substantia nigra D1 receptors and the increased expression of the vesicular GLU transporter (VGLUT1) as well as increased vesicular GLU uptake, EAAT3 expression, and (B) the initial activation of GLU transmission will be followed by a more protracted disinhibition of corticostriatal GLU transmission through diminished D2-mediated inhibitory tone.

Rationale: METH increases extracellular GLU in the striatum (Nash and Yamamoto, 1992; Stephans and Yamamoto, 1994; Abekawa et al., 1994). The increase in GLU remains elevated for at least 32 hrs after the last injection of METH (Nash and Yamamoto, 1993; Fig. 3). Moreover, we have shown that cortical ablation blocked the increase in GLU and blocked the long term depletion of DA in the striatum (Burrows and Yamamoto, 2001). These data indicate that GLU originates from corticostriatal terminals and contributes to the long-term depletions of DA after METH. Moreover, we have shown that antagonism of the D1 receptor in the substantia nigra blocks the METH-induced increase in corticostriatal GLU presumably through the direct inhibition of GABA release in the SN, a resultant disinhibition or increase in nigrothalamic GABAergic activity, and a decrease in thalamocortical and corticostriatal GLU activity (Mark et al., 2004).

Figure 5

METH Increases Cortical VGLUT-1 mRNA and Striatal Protein Immunoreactivity



VGLUT1 is responsible for the regulated accumulation of GLU into synaptic vesicles for exocytotic release (Naito and Ueda, 1985; Bellocchio et al., 2000). It has also been reported that VGLUT1 protein is only localized to nerve terminals, and not cell bodies or dendrites (Fremeau, Jr. et al., 2004). Moreover, VGLUT1 expression strongly influences the strength of GLU transmission, since GLUergic neurotransmission, specifically quantal size of GLU release, is significantly reduced in VGLUT1 knock out mice (Wojcik, et al., 2004). Wojcik et al., (2004) also reported that this reduction in GLUrgic transmission can be rescued and enhanced by over expression of VGLUT1. Furthermore, other studies have reported that the actual number of vesicular transporters can directly regulate the extent of GLU release (Wilson, et al., 2005). Specifically, Wilson et al.,

(2005) reported that increasing the number of VGLUT1 expressed at excitatory synapses increases the amount of GLU released per vesicle.

Corticostriatal terminals express the vesicular GLU transporter-1 (VGLUT1) (Kaneko et al., 2002) and D2 receptors (Wang and Pickel, 2002). D2 receptors inhibit corticostriatal GLU transmission (Yamamoto and Davy, 1992; Bamford et al., 2004). Therefore, the expression of the vesicular GLU transporter (VGLUT1), vesicular GLU uptake, and their regulation by substantia nigra D1 receptors and striatal D2 receptors at specific timepoints after the repeated injections of METH will be examined. Support for the role of VGLUT1 in the effects of METH is illustrated by Figure 5 showing that METH increased VGLUT mRNA by 22% and vesicular VGLUT1 protein by 33% at 6 and 12 hrs after METH, respectively. mRNA was measured by RT-PCR. No changes were observed in EAAT2 and vesicular VGLUT2 protein (Fig. 6). Furthermore, depolarization-induced GLU release is augmented by METH pre-exposure (Fig. 7) suggesting vesicular GLU release is elevated by and after METH.

D2 receptors in the substantia nigra can also attenuate stimulated GABA release (Matuszewich and Yamamoto, 1999). Therefore, the delay in the METH-induced and D1 mediated increases in corticostriatal GLU can occur by two mechanisms: (1) a D2 mediated inhibition of corticostriatal GLU release or (2) a D2 inhibition of GABA release in substantia nigra that in turn, increases nigrothalamic GABA and inhibits thalamocortical and corticostriatal GLU projections. The prolonged elevation in GLU in striatum or SN that occurs after the delayed increase could be due to the eventual depletion of DA stores by METH that subsequently decreases D2-mediated inhibitory tone on striatonigral GABA and corticostriatal GLU terminals. Regardless of the pathway involved, striatal GLU would be disinhibited to cause a persistent elevation in GLU that is critical to the manifestation of METH toxicity via excitotoxic mechanisms.

Figure 6

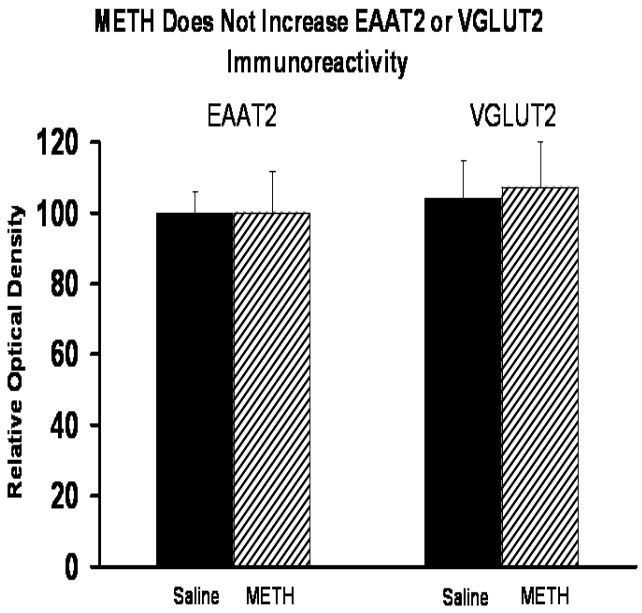
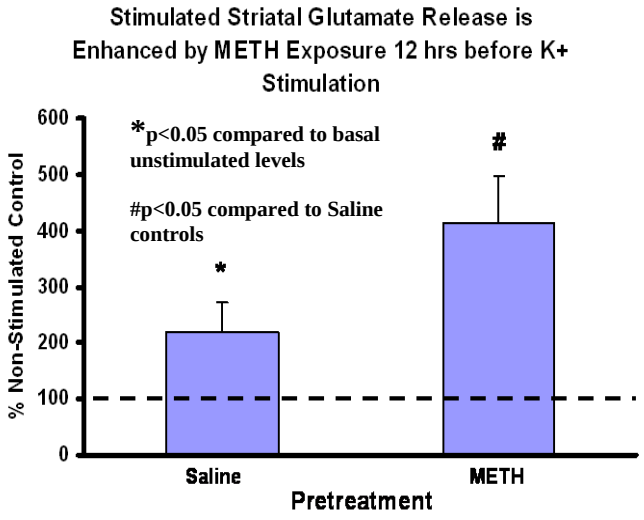


Figure 7



Design:

Microdialysis probes will be implanted into the substantia nigra pars reticulata and the ipsilateral striatum of each rat as previously described (Mark et al., 2004). Dialysis probes will be used to simultaneously measure extracellular GABA or DA in the substantia nigra pars reticulata (SNr) and GLU in the striatum while also permitting the perfusion of the GABA-A antagonist biccuculline (10 μM) (Mark et al.., 2004), the D1 antagonist SCH23390 (10 μM) (Mark et al., 2004), the D2 antagonist haloperidol (100 μM) (See and Berglind, 2001), or the D2/D3 agonist quinpirole (10 μM) (Abarca et al., 1995) into the SNr.

Three days after the stereotaxic implantation of the microdialysis probes, the dialysis experiments will commence. After a 2 hr equilibration/pre-baseline period, 3 baseline samples will be collected over 1.5 hrs. Immediately after the collection of the third baseline sample, saline or METH (10 mg/kg ip) will be injected every 2 hr over 8 hr for a total of 4 injections. Dialysates (30 min samples) will be assayed for DA and GABA.

Specific Aim 1A - To examine the role of SN D1 and GABA-A receptors on VGLUT1 expression, the perfusion (reverse dialysis) of either the D1 antagonist or the GABA–A antagonist into the SNr will begin

simultaneous with the injection of saline or METH and continue for the remainder of the systemic injections of saline or METH. Six and 12 hrs later (times that presumably precede the increase VGLUT1 protein), the rats will be killed. VGLUT1 mRNA will be assayed in cortex and striatum and VGLUT1 protein and uptake will be measured in striatum as well as EAAT3 protein. Ten rats will be needed per condition X 2 drug injections (saline or METH) X 3 SNr perfusions (aCSF, SCH23390 or bicucculline) X 2 timepoints (6 and 12 hrs) = 120 rats

Specific Aim 1 B

Experiment 1: To examine the site-specific role of D2 receptors in modulating VGLUT1 and EAAT3 expression, haloperidol will be perfused into the SNr or striatum during the injections of saline or METH. Extracellular GLU will be measured in the striatum during the timecourse of the dialysis perfusion. All rats will be killed 6 and 12 hours later and VGLUT1 mRNA will be assayed in cortex and striatum and VGLUT1 protein and uptake will be measured in striatum. Ten rats will be needed per condition X 2 drug injections (saline or METH) X 2 SNr perfusion conditions (aCSF or haloperidol) X 2 brain regions (SN or striatum) = 40 rats.

Experiment 2: Based on the timecourse of extracellular striatal and SNr DA in Experiment 1, systemic quinpirole injections (0.5 mg/kg ip) (Lomanowska et al., 2004) will commence when the elevated extracellular concentrations of DA in the SNr have returned to or below basal values (e.g. @ 6 hrs after the last/4th METH injection). This should permit the maintenance of striatal and nigral D2 activation in the face of declining DA concentrations. The injections will be once every 4 hrs over 8 hrs.

Extracellular GLU in the striatum will be measured every 30 min during the entire timecourse of the experiment. All rats will be killed at 6 and 12 hours after the last/4th METH injection and VGLUT1 mRNA will be assayed in cortex and striatum and VGLUT1 protein and uptake will be measured in striatum. Ten rats will be needed per condition X 2 drug injections (saline or METH) X 2 D2 agonist conditions (saline or quinpirole) X 2 timepoints (6 and 12 hrs post METH) = 80 rats.

Experiment 3: To examine the site specific role for D2 modulation of extracellular GLU in the striatum, the local perfusion of quinpirole in the SNr and/or striatum will be used. Based on the timecourse of extracellular striatal and SNr DA in Experiment 1, quinpirole perfusion will commence when the elevated extracellular concentrations of DA in the SNr and striatum have returned to basal values. The perfusion will continue for another 18 hrs. Extracellular GLU in the striatum will be measured every 30 min during the entire timecourse of the experiment.

Twelve hrs after the termination of the perfusion of quinpirole, rats will be killed and their cortex and ventromedial thalamus (VMT) will be assayed for VGLUT1 mRNA and the striatum assayed for VGLUT1 protein. Ten rats will be needed per group X saline or METH X striatal or nigral perfusion X aCSF or quinpirole = 40 rats.

Discussion, Anticipated Results, and Potential Pitfalls

These experiments will test the hypothesis that the initial increase in extracellular GLU is D1 dependent and is delayed by D2 receptor activation. The persistence of the increase in GLU is hypothesized to be due to an increase in VGLUT1 synthesis and expression. These increases are posited to be a consequence of a loss of D2 tone resulting from the eventual depletion of DA stores over the next ~24 hrs that in turn, disinhibits corticostriatal GLU at the level of the SN and striatum. Although this disinhibition of GLU produced by a lack of D2 stimulation may be transient (although see Burrows and Meshul, 1999 for enhanced GLU immunoreactivity that persists for 4 weeks after METH) due to the typical partial lesion of the DA system produced by METH, it could initiate a cascade of events that could eventually lead to longer-term excitotoxicity that will be investigated in Specific Aim 2. Therefore, antagonism of the D1 receptor relatively early during the administrations of METH and maintaining D2 tone with a D2 agonist later after METH when DA levels are low or depleted, should block the increases in VGLUT1 and extracellular GLU in striatum.

Based on preliminary data (Fig. 5), VGLUT1 protein in the vesicle fraction should be increased in the striatum after METH. This should be preceded by an increase in VGLUT1 mRNA in cortex and VMT since both regions contain GLUergic cell bodies that project to the cortex and striatum, respectively. This outcome would support the conclusion that the synthesis and expression of VGLUT1 protein and consequently, vesicular GLU uptake are increased after METH. An increase in GLU uptake by vesicles would be consistent with the finding that the level of expression of VGLUT1 determines the amount of GLU that is loaded into synaptic vesicles (Wojcik et al., 2004). In addition, increased expression of VGLUT1 may enhance excitatory synaptic transmission via increases in the amount of GLU released per vesicle (Wilson et al., 2005). These changes together would account for the increase in corticostriatal GLU release observed previously. Moreover, the experiments in this aim are designed to evaluate how this increase might occur. Since we have shown that the METH-induced increase in striatal GLU is attenuated by D1 or GABA-A antagonism in the SN, increases in VGLUT1 mRNA and protein as well as

vesicular GLU uptake in cortex/thalamus and striatum, respectively, should also be attenuated. It is interesting to note that although D1 antagonism in the SN attenuated the METH-induced increase in striatal GLU and the long-term depletion of DA, it did not block it. This result suggests another factor is involved. It is posited that not only is it important to block the initial increase in GLU, it is also equally if not more critical to block the sustained elevation of GLU.

One possible mechanism for the delayed and protracted increase in extracellular GLU is the modulation by the D2 receptor. METH should increase the extracellular concentrations of DA in the SNr that presumably acts via D1 receptors and a polysynaptic nigrothalamocortical path, to increase GLU release from corticostriatal terminals (Mark et al., 2004). However, it is hypothesized that the increase in GLU (via D1 receptor activation) is delayed because it is countered initially by the simultaneous activation of D2 receptors in the SN or striatum. Thus, D2 antagonism in the SN and/or striatum with haloperidol during the administration of METH should immediately enhance the increase in nigral GABA and disinhibit striatal GLU such that the increase will be observed much earlier than the typical 2-3 hr delay observed (Specific Aim 1B, Experiment 1). It is unclear if D2 antagonism in the SN and/or striatum will augment the increases in VGLUT1 mRNA, protein, and/or uptake but future studies can examine if these changes occur at earlier timepoints. It is predicted that the combined effects of D2 antagonism in the striatum and SN on extracellular GLU will occur even earlier than the effects of D2 antagonism in either region alone.

In contrast, the systemic administration (Specific Aim 1B; Experiment 2) or local perfusion of the D2/D3 agonist, quinpirole (Specific Aim 1B; Experiment 3) at times when extracellular DA has returned to basal concentrations or is depleted, should block the increase in VGLUT1 synthesis and protein as well as extracellular GLU within the striatum. These data suggest that maintenance of D2 tone in the striatum and/or SN is necessary to dampen corticostriatal GLU that otherwise would be disinhibited to produce a persistent elevation of extracellular GLU. Subsequently, the effects on METH-induced decreases in DA content and DAT immunoreactivity will be examined. It is not clear at what timepoints and for how long the administration of quinpirole should take place; however, the measures of extracellular DA at later times after METH should provide important information along these lines. The feasibility of these experiments will be assessed initially be using a protocol of repeated systemic administrations of quinpirole (after the administration of METH) that produce a persistent D2 activation with no desensitization (Lomanowska et al, 2004).

The thalamus also sends GLUergic projections to the striatum; however, these projections contain VGLUT2 and not VGLUT1 protein (Kaneko et al., 2002). Thus, if VGLUT1 is not changed, changes in VGLUT2 will be examined. If VGLUT2 is increased, it would indicate that the thalamostriatal projection is affected preferentially by METH. We expect that although this path may be involved, the role of the cortex is critical since cortical ablation blocks the increase in striatal GLU and the long-term depletions of DA (Burrows and Yamamoto, 2001) The maintenance of D2 tone may be important not only for the dampening of corticostriatal GLU but it may also inhibit subthalamonigral GLU transmission as well (Figure 9; Hatzipetros and Yamamoto, 2006). In addition, disinhibition of GLU transmission in the striatum and SN may produce excitotoxicity to terminals and soma as well as GLU-derived oxidation of cellular elements affected by METH (e.g mitochondria) (for review, see Brown and Yamamoto, 2003). Therefore, Specific Aim 2 will assess evidence of excitotoxicity in the striatum and SN.

Specific Aim 2: The excitotoxic and biochemical consequences of METH-induced increases in GLU transmission will be studied. METH is posited to produce excitotoxicity in the striatum and substantia nigra pars reticulata (SNr) via diminished D2-mediated inhibition. METH will decrease energetic function as assessed by decreases in mitochondrial Complex II activity and protein through GLUergic oxidative processes.

Rationale: No conclusive evidence links high concentrations of GLU produced by METH with excitotoxicity that is indicative of structural damage and proteolysis. Along these lines, a few recent studies have indicated neuronal inclusions within cell bodies of the striatum and substantia nigra of mice (Fornai, 2004) and one report of an increase in markers of apoptosis in the rat (Jayanthi et al., 2005). Regardless, no studies have linked evidence of oxidative stress and bioenergetic compromise after METH and damage to specific cellular components. Preliminary data indicate that METH produces spectrin proteolysis in the striatum as evidenced by the appearance of specific molecular weight breakdown products of spectrin that are typically produced by the calcium dependent protease, calpain. This proteolysis appears to be mediated by the AMPA GLU receptor since the proteolysis is blocked by the AMPA antagonist GYKI52466 (Staszewski and Yamamoto, 2006). Furthermore,

we have shown that complex II-III activity of the mitochondria in the striatum is decreased by METH in a NMDA and peroxynitrite dependent manner (Brown et al., 2005).

Our most recent preliminary data indicate that METH decreases complex II protein in striatal homogenates and VMAT2 protein in all subcellular fractions (synaptosomal, membrane-associated and vesicular). Importantly, immunoprecipitated synaptosomal VMAT2 protein from METH (M) compared to saline (S) treated rats is oxidized by s-nitrosylation of cysteine residues as evidenced by nitrosylcysteine immunoreactivity (Fig. 8). Therefore, these studies will be extended to examine how DA receptor modulation of corticostriatal GLU as described in Specific Aim 1 affects these markers of excitotoxicity. We also will expand upon our findings to show that complex II protein is oxidatively damaged via nitrosylation of cysteine residues in a GLU-dependent manner.

The SNr also receives a substantial GLUergic innervation. This innervation arises from the subthalamic nucleus and cortex (Kita and Kitai, 1987; Smith et al., 1990). This subthalamonigral GLUergic path is activated when striatal DA levels are depleted as in Parkinson's disease or after 6-OHDA lesions (Gajendiran et al., 2005; Yokoyama et al., 1998; Kreiss et al., 1997). We hypothesize that a lack of DA tone on D2 heteroreceptors located on asymmetric excitatory synapses (Wang and Pickel, 2002) leads to enhanced GLU release and excitotoxic damage to cell bodies in the SNr. This possibility is significant in light of the fact that antipsychotic D2 antagonist drugs are used to treat acute psychosis produced by METH overdose (Richards et al, 1998). Figure 9 shows that raclopride (RAC) infusion into the SNr enhanced carbachol (CARB) stimulated GLU release from subthalamonigral efferents and further supports the conclusion that the microdialysis measures of GLU are impulse-dependent. New preliminary data indicate that excessive levels of GLU in the SNr are produced when D2 receptors are antagonized after the administration of METH (Fig. 10).

In addition, subchronic haloperidol (0.5 mg/kg) administration for 5 days after METH produces a selective cell loss (measured by decreased NeuN staining of neuronal nuclei) (Fig. 11) by 32% (Fig. 12) in the SNr but not in the SNc. We propose to verify these potentially important findings by continuing to examine NeuN staining in addition to using spectrin proteolysis analysis. Importantly, we plan to identify the phenotype of these damaged cells by taking advantage of the co-

Figure 8

VMAT-2 Protein is Oxidized by Nitrosylation within 1 hr after Repeated METH

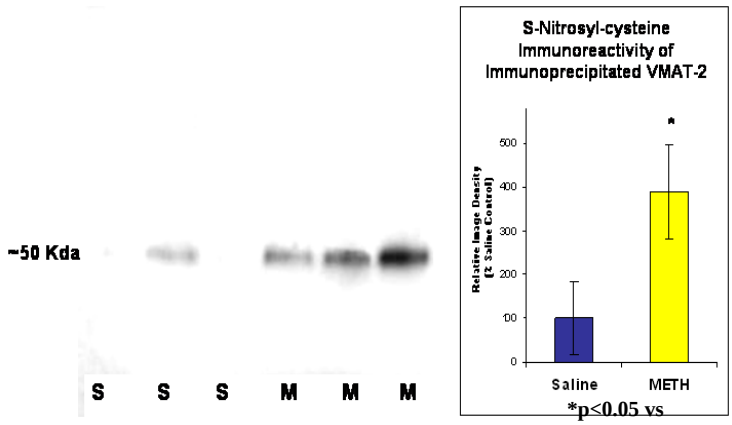


Figure 9

RACLOPRIDE PERFUSED INTO THE SN ENHANCES GLUTAMATE RELEASE PRODUCED BY CARBACHOL STIMULATION IN THE SUBTHALAMIC NUCLEUS

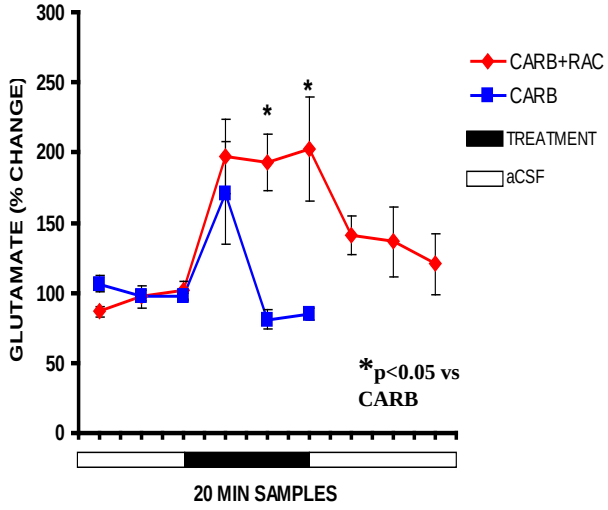
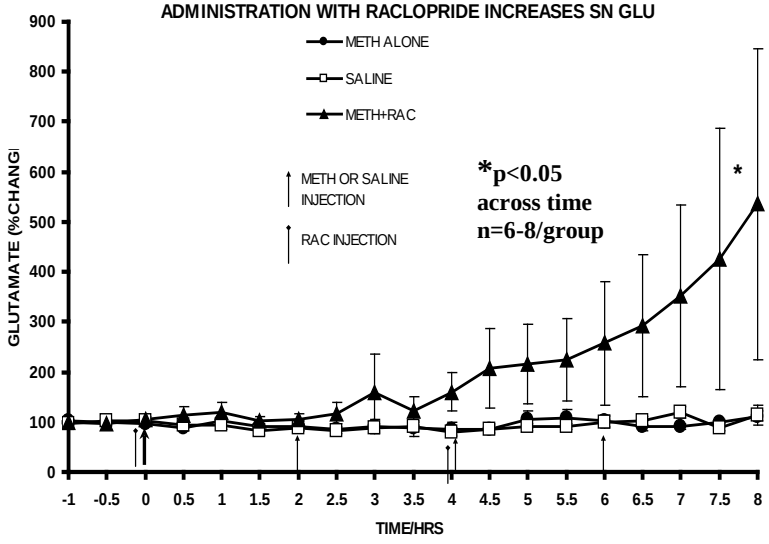


Figure 10

METH ALONE DOES NOT INCREASE GLU IN THE SN. CO-ADMINISTRATION WITH RACLOPRIDE INCREASES SN GLU

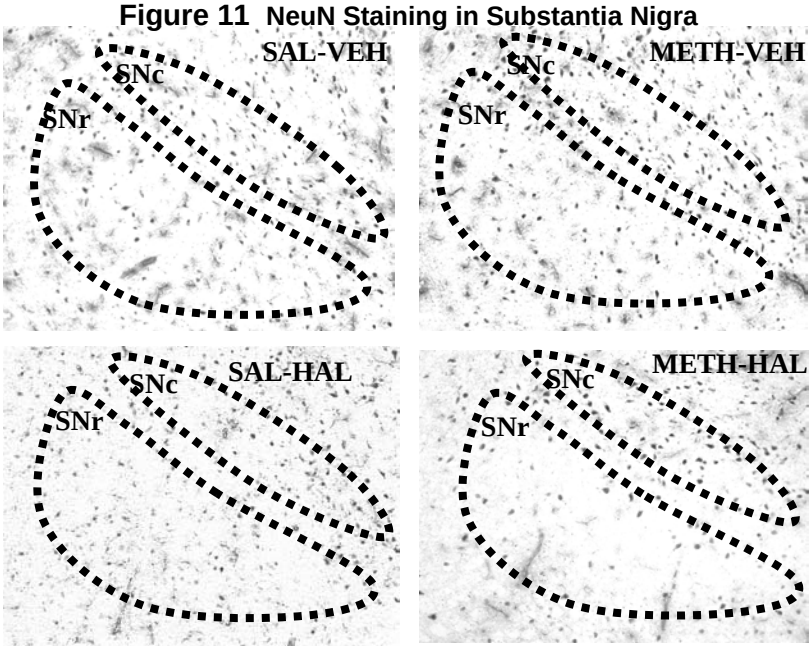


SN=Substantia nigra; RAC = raclopride (0.5 mg/kg ip) ; *p<0.05 from saline controls. METH (10 mg/kg, ip) alone did not increase glutamate

localization of Fluorojade-C as a marker of dying cells with other neuronal markers (Schmued et al, 2005). To assess the role of GLU in cell death in the SNr, the NMDA antagonist will be administered during the systemic administrations of haloperidol to examine if there is an antagonism of the increases in Fluorojade-C staining, spectrin proteolysis, and loss of mitochondrial enzyme activity in the SNr.

Design:

Figure 11 NeuN Staining in Substantia Nigra



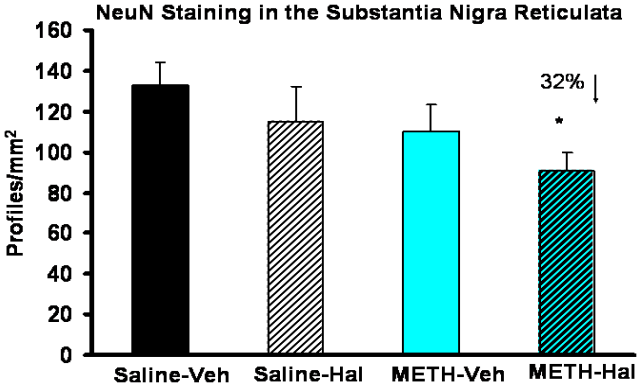
Experiment 1: This experimental design will accomplish 4 goals: (1) assess if changes in mitochondrial enzyme activity and protein at early timepoints that precede (2) evidence of excitotoxic proteolysis, and (3) dying cells/cell loss after METH while evaluating (4) if D2 blockade enhances these effects.

Saline or METH (10 mg/kg q 2 hr X 4) will be injected into rats. Body temperatures will be measured hourly during the administration of METH or saline.

After the administration of saline or METH, haloperidol (0.5 mg/kg, ip) or tartaric acid vehicle (0.5 M; pH to 5.3, 0.1% bodyweight) will be injected daily for 5 days. Rats will be killed on the 1st, 3rd and 5th days of haloperidol treatment and on the day after the termination of haloperidol treatment (6th day).

Spectrin Proteolysis: Striatum and substantia nigra will be dissected from frozen coronal sections and assayed for spectrin breakdown products (See General Methods). There will be 2 METH groups (saline or METH) X 2 haloperidol groups (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Figure 12
METH Followed by Haloperidol Decreases NeuN Staining in the Substantia Nigra Reticulata



*p<0.05 vs Saline

Mitochondrial Enzyme Assays: Separate groups of rats will be used to measure Complex I and Complex II activities in purified mitochondrial preparations from bilateral striatum and substantia nigra (See General Methods). There will be 2 METH treatments (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Mitochondrial Protein: Complex I and II proteins will be detected via western blot analysis. There will be 2 METH treatments (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Mitochondrial protein immunoprecipitation: Complex I and II proteins will be immunoprecipitated and analyzed by western blot for s-nitrosylcysteine and nitrotyrosine immunoreactivity. There will be 2 METH treatments (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Fluorojade-C immunohistochemistry: Separate groups of rats will be treated as above, killed and perfused with 0.4% paraformaldehyde. Frozen coronal sections will be prepared for double staining for Fluorojade-C and GAD65 (or GAD67). Alternate sections will be double stained for Fluorojade-C and tyrosine hydroxylase or parvalbumin staining to detect GABA interneurons. There will be 2 METH treatments (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

NeuN and GAD staining: Additional groups of rats will be treated, killed and perfused. Brains will be sliced into coronal sections and stained with the neuronal nuclei stain NeuN and counted. MK801 treatment: A different group of rats will be treated with saline or METH as above and then with either tartaric acid vehicle or haloperidol. Saline or MK801 will be administered daily, 15 minutes before each of the tartaric acid or haloperidol injections.

Rats will be killed one day after the termination of the 5 day treatment. NeuN and GAD65 and 67 positive cells will be examined. There will be 2 METH groups (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 2 MK801 treatments (saline or MK801) X 10 rats/group = 80 rats

VMAT2 and plasmalemmal DA transporter (DAT): Fleckenstein and co-workers have reported decreases in VMAT2 activity and protein after METH. We will assess VMAT2 protein in striatal subcellular fractions (vesicular, synaptosomal and membrane-associated fractions) after METH to examine if there is a general loss of VMAT2 protein after this injection paradigm of METH, with and without haloperidol. Our preliminary data suggest a general loss of VMAT2 protein in all fractions. On this basis, we will examine if VMAT2 protein is oxidized (i.e. nitrated/s-nitrosylated) as evidenced by nitrotyrosine/nitrocysteine immunoreactivity of immunoprecipitated VMAT2 (see Fig. 10). We also will examine DAT protein in the synaptosomal fraction. There will be 2 METH treatments (saline or METH) X 2 haloperidol treatments (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Ubiquitin-dependent proteolysis: The 19s regulatory cap of the proteasome that is responsible for ubiquitin-dependent proteolysis will be examined in striatal and SN homogenates. There will be 2 METH treatments (saline or METH) X 2 haloperidol groups (vehicle or haloperidol) X 4 timepoints X 10 rats/group = 160 rats

Experiment 2: This experiment is designed to test the hypothesis that the maintenance of D2/D3 tone during the times immediately after the injections of METH when DA levels are depleted (albeit transiently) will block changes in the above markers of cell loss/damage and excitotoxicity. The above groups will be duplicated except that quinpirole (0.5 mg/kg ip) will be injected initially after METH at a time determined in Specific Aim 1B, Experiment 2, and at 6 and 12 hrs after METH. Body temperatures will be measured during these times to examine if quinpirole affects core temperature. There will be 2 METH treatments (saline or METH) X 2 quinpirole treatments (vehicle or quinpirole) X 3 time points X 10 rats/group = 120 rats

Experiment 3: This experiment will assess if the early activation of the D1 receptor in the SNr during METH is responsible for the oxidative and excitotoxic changes in the striatum. All of the above measures in Experiment 1 will be evaluated but in rats with dialysis probes implanted in the SNr for the local perfusion of the D1 antagonist SCH23390 or artificial CSF during the administrations of METH. There will be 2 METH treatments (saline or METH) X 2 D1 antagonist treatments (vehicle or SCH23390) X 3 timepoints (1, 3, and 7 days after METH) X 10 rats/group = 120 rats

Discussion, Anticipated Results, and Potential Pitfalls

These studies will evaluate the time-dependent events associated with excitotoxicity to mitochondria, cell membranes, and soma in striatum and SNr. It is predicted that decreases in mitochondrial enzyme activity/protein or VMAT2 protein will occur before increases in spectrin proteolysis or cell loss. The results would suggest that damage to mitochondria via GLU-dependent oxidative stress leads to a disruption of cellular energetics, decreased sequestration of DA into vesicles, and followed by cellular oxidative stress and eventual damage to the cell. Based on our preliminary data, METH alone should increase spectrin proteolysis in the striatum. It is unclear if NeuN staining will be decreased in the striatum after METH alone although there is some evidence of inclusion bodies within cells of the mouse striatum (Fornai et al., 2003). However, previous studies have shown no Fluorojade staining indicative of dying cells in the rat striatum after METH (Eisch and Marshall, 1998). Therefore, no cell loss in the striatum or the SNr is expected with METH only (Fig. 12) and would indicate that the maintenance of extracellular DA, despite the significant loss of DA terminals after METH alone is sufficient to maintain D2 activation and dampen any enhanced corticostriatal GLU release that could damage cell bodies. This would be consistent with the majority of studies indicating that METH toxicity is limited to monoamine terminals. A different scenario may occur with METH + post-METH haloperidol. An augmented decrease in mitochondrial enzyme activity and/or protein and VMAT2 protein in striatum may occur at earlier timepoints than the decreases in NeuN or increases in proteolysis. Furthermore, if the decrease in NeuN staining in the SNr is mediated by excitotoxic events, spectrin proteolysis should be evident in the SNr, which in turn, should be blocked by quinpirole through a D2-mediated inhibition of GLU release from terminals in striatum and SNr, as well as VGLUT1 expression in striatum (Specific Aim 1). Alternatively, these changes may be blocked directly by MK801 antagonism of NMDA receptors on soma. Regardless, there may be decreases in NeuN staining and increases in Fluorojade-C staining in the striatum of METH + haloperidol post-METH rats as a consequence of disinhibited corticostriatal (Specific Aim 1) and subthalamonigral GLU release (Figs. 9 and 10).

Fig. 13 shows that haloperidol administered after METH increases GLU in the SNr and persists for at least 3 hrs. Therefore, elevated GLU may lead to this cell loss.

Decreases in Complex I and II activities and protein within the striatum and SNr should also be observed after METH. Preliminary data indicate that Complex II protein subunits are decreased 1 hr after METH (Fig. 14). Unlike our previous studies, we will improve our previous approach to specifically examine Complex I or II independent of Complex III. Although the predicted early decreases should be enhanced by haloperidol and attenuated or blocked by quinpirole, it will be difficult to associate changes in mitochondrial enzyme function/protein with specific cell types since mitochondrial proteins are ubiquitous and not associated with particular cells. Should changes in Complex I or II protein occur, a double labeling approach using antibodies to the subunits of these complexes with Fluorojade-C staining will be used.

Figure 13

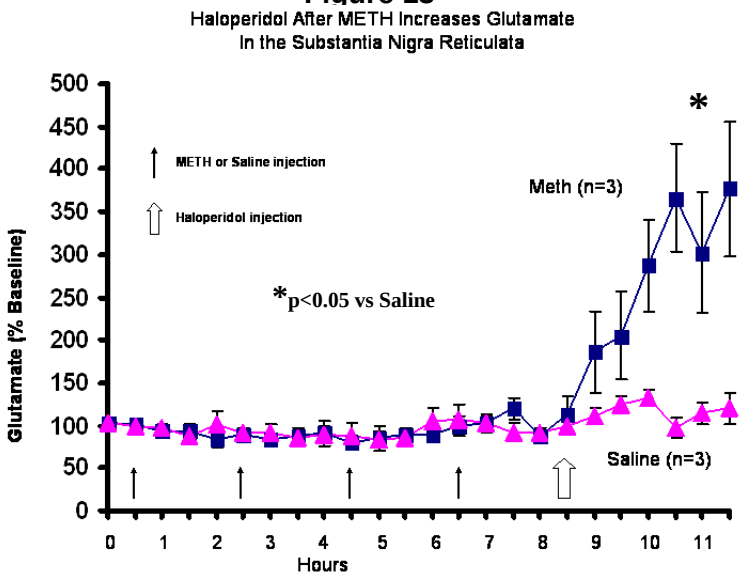
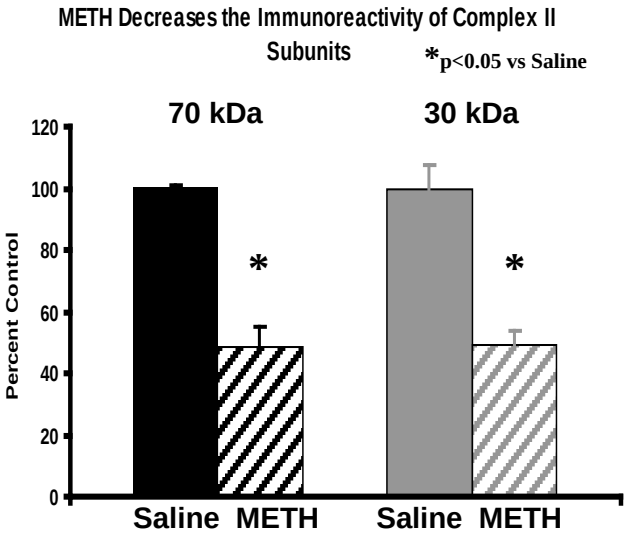


Figure 14



Decreased NeuN staining after METH+haloperidol may be due to a loss of GABA cells in SNr or striatum since GABA interneurons receive a dense GLU input from the cortex. This would be reflected by a co-localization of Fluorojade-C staining with GAD65 or 67 in GABA cells. Since GABA interneurons comprise only 10% of the population of striatal neurons, specific changes in GAD65 or 67 may be difficult to observe. Therefore, we will use parvalbumin staining to label all GABA interneurons. Cholinergic cell bodies in the striatum also have GLU receptors. If there are no changes in GAD65, 67 or parvalbumin staining, choline acetyltransferase staining with Fluorojade-C will be examined. To assess if the NMDA GLU receptor is involved, MK801 will be used to block the effects. Should MK801 be ineffective, we will use the AMPA antagonist GYKI52466 as this blocks METH-induced increases in spectrin proteolysis (Staszewski and Yamamoto, 2006).

Nitrosyl-cysteine immunoreactivity of immunoprecipitated complex I, II and VMAT2 proteins will indicate nitrosylation and oxidation via nitric oxide and/or peroxynitrite. Nitrosyl-cysteine antibody will be used instead of a nitrotyrosine antibody because of the high cysteine content of these proteins. Although unlikely based on our preliminary data (Fig. 4), nitrocysteine immunoblotting may be problematic. Regardless, we will also use the nitrotyrosine antibody. The peroxynitrite scavenger FeTPPS has been used by us and others to block changes in enzyme activity and nitration of proteins (Brown et al., 2005; Imam et al., 2000). Should changes be observed in nitrosylation of mitochondrial proteins and VMAT2, FeTPPS will be used to block the effects of METH.

The 26S proteasome is a major site of protein degradation. The proteasome is composed of two major regions: the 20S holoenzyme, the site of proteolytic activity, and the 19S cap, an ATP utilizing region necessary for ubiquitin-dependent proteolysis. Interestingly, immunohistochemistry done by Fornai et al., (2004) shows the presence of ubiquitin inclusions, both in vitro in PC12 cells, and in vivo in the mouse striatum and substantia nigra after METH. Our preliminary results show that ubiquitin immunoreactivity is increased by 20% in the rat hippocampus 7 days after METH (Johnson et al, 2005) and would suggest an impairment of the ubiquitin-dependent proteolytic process. These data support the conclusion that proteins tagged with ubiquitin appear to aggregate rather than degrade after METH. Therefore, an increase in ubiquitin inclusions and immunoreactivity may suggest an impairment of 19S, ubiquitin-dependent proteolysis that is mediated by nitrosative oxidative stress (Yao et al., 2004). Impairment to this system may prevent GLU receptor internalization via PSD95

ubiquitination and/or 19S proteasome mediated degradation (Rezvani et al., 2003; Colledge et. al., 2003) and lead to an enhanced GLU receptor surface expression and GLU response. Taken together, these results support the proposed hypothesis of a decrease in ubiquitin-dependent proteolysis and enhanced GLU receptor mediated excitotoxicity.

D1 antagonism may attenuate but not block the early decreases in mitochondrial enzyme activity and protein, and the subsequent increases in spectrin proteolysis in the striatum. This would be consistent with our previous finding that D1 antagonism did not block but only attenuated the long-term depletion of DA in the striatum (Mark et al., 2004). It is hypothesized that the remaining deficit that is not blocked by the D1 antagonist is due to the lack of D2 stimulation during the depletion of DA that occurs hrs after the administration of METH. Future studies will examine the early administration of a D1 antagonist during METH in addition to the administration of a D2 agonist after METH to completely block changes in the proposed markers produced by METH.

The rationale for the administration of a D2 agonist after METH is to counter the lack of D2 tone produced by the acute depletion of DA stores. The depletion of DA stores and decreased D2 activation could disinhibit or activate the corticostriatal path, to increase VGLUT1 synthesis and protein, disinihibit GLU release, and produce additional damage to DA terminals and VMAT2. While the increase in GLU release is sufficient to damage an already metabolically and oxidatively compromised DA terminal due to the cytosolic accumulation and reverse transport of DA, it may not be sufficient to damage cell bodies. However, subsequent exposure to a D2 antagonist should further disinihibt GLU release at the level of the striatum and SNr to enhance extracellular GLU) (Specific Aim 1) and produce damage to terminals and soma in these regions via GLU receptor activation (Specific Aim 2). The significance of D2 antagonist exposure after METH is based on the treatment of METH overdose and acute psychosis with D2 antagonist antipsychotic drugs in hospital emergency rooms (Richards et al., 1998). If cell damage or loss is observed in the striatum and SNr in the proposed studies, these findings could have significant clinical implications for the vulnerability of treated individuals to the development of movement disorders such as Huntington's disease.

Overall, these series of experiments will use multiple markers of oxidative and excitotoxic damage to cellular elements in the striatum and SNr after METH to examine how these changes are modified by DA receptors in a time-dependent manner (see Fig. 1). The effects of METH may not be limited to the augmentation by a subsequent and subchronic pharmacological treatment (i.e. D2 antagonist). It is equally possible that the neurotoxic effects of METH can be enhanced by a prior exposure to a chronic environmental challenge such as psychological stress. This will be investigated in Specific Aim 3.

Specific Aim 3: The effect of chronic stress on the excitotoxic effects of METH to the striatum, substantia nigra (SN) and hippocampus (HIPP) will be examined.

Specific Aim 3A: It is hypothesized that chronic unpredictable stress will enhance the excitotoxicity in the striatum and HIPP produced by METH as evidenced by augmented decreases in DArgic markers (striatum), serotoninergic markers (5HT transporter. 5HT content), oxidation/nitration of vesicular monoamine transporter (VMAT2) protein, increased spectrin proteolysis, proteasomal inhibition, and the presence of Fluorojade-C staining. The enhanced markers of excitotoxicity in the striatum will be paralleled by increased D1 receptor protein in substantia nigra. These changes will be blocked by NMDA, AMPA and/or D1 receptor antagonism and chemical adrenalectomy.

Rationale: Mesolimbic and mesocortical DAergic neurons are sensitive to psychological stress (Piazza et al., 1996; see also review by Marinelli and Piazza, 2002). Similarly, stress enhances DA activity in the striatum (nigrostriatal neurons) as evidenced by increases in extracellular DA (Abercrombie et al., 1989; Keefe et al., 1990; Pawlak et al., 2000), DA metabolism (Dunn and File, 1983), and a compensatory up-regulation of the DA transporter (DAT) (Copeland et al., 2005). Stress also increases GLU release acutely and chronic stress produces evidence of oxidative damage (Madrigal et al., 2001). Interestingly, chronic unpredictable stress enhances METH-induced DA release and DA depletions in the striatum (Matuszewich and Yamamoto, 2004). Since the neurotoxic effects of METH are mediated by increases in both DA and GLU, enhanced striatal DA depletions in chronically stressed rats may be related to DA and GLU release produced by METH.

Similar to the enhancement of METH-induced DA release by stress (Matuszewich and Yamamoto, 2004), preliminary data in Figs 15 and 16 show that chronic stress also enhances METH-induced GLU release in the striatum and enhances the long-term depletion of striatal DA. Therefore, GLU may contribute to the enhanced toxicity observed after METH.

Figure 15

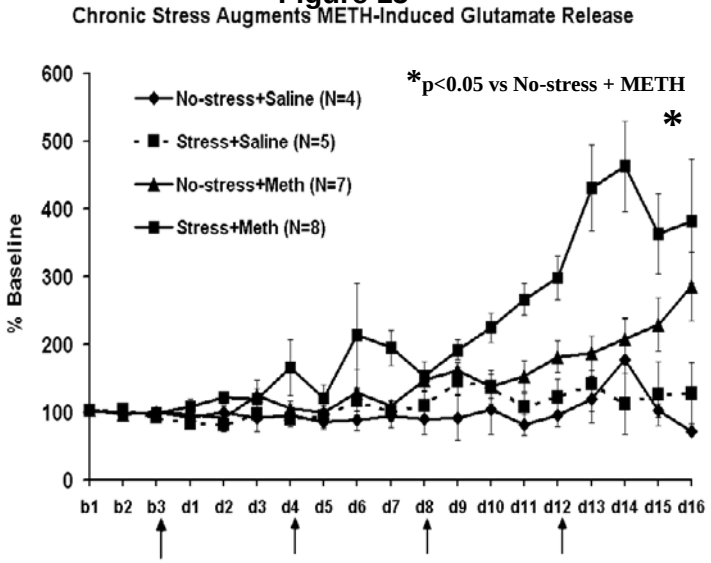
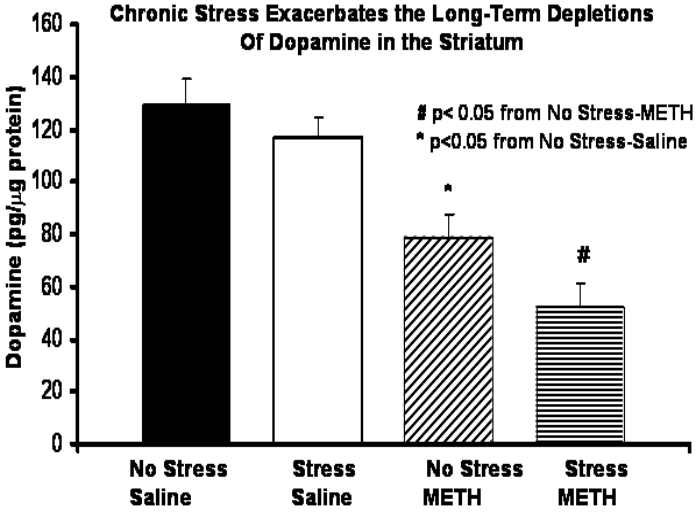


Figure 16



The enhanced METH-associated excitotoxicity after chronic stress may be paralleled by a striatonigral D1 receptor system upregulation. Adrenalectomy decreases the density D1 binding in striatum and substantia nigra, an effect that is attenuated by dexamethasone (Biron et al., 1992). Administration of metyrapone, which blocks a rate-limiting enzyme in corticosterone synthesis, also decreases D1 binding and mRNA in striatum and nucleus accumbens (Czyrak et al., 1997). Conversely, chronic unpredictable stress through elevations of glucocorticoids may increase D1 protein. Since D1 receptor activation facilitates excitatory synaptic transmission mediated by AMPA type GLU receptors (Umemiya and Raymond, 1997; Price et al., 1999), up-regulation of this receptor system may enhance METH-induced DAergic transmission to augment extracellular GLU through the aforementioned polysynaptic pathway and exacerbate toxicity.

Design: Rats will be divided into either a no-stress group or exposed to chronic unpredictable stress (CUS) (see *General Methods for paradigm) for 10 days.*

Specific Aim 3A: This experiment will examine the role of D1 and GLU receptors in mediating the effects of chronic stress on METH toxicity. During the CUS procedure, the D1 antagonist SCH23390, the NMDA antagonist MK801, the AMPA antagonist GYKI52466 or saline vehicle will be injected twice daily (i.e. before each stressor). One day later, rats will receive either saline or METH (7.5 mg/kg ip q 2 hr X 4). A lower dose is required because we found the typical higher dose (10 mg/kg) is the LD80 in chronically stressed rats. Rats will be killed at 1, 3 or 7 days after saline or METH exposure. Striatum, HIPP, and SN will be dissected and biochemical assays identical to those described in Specific Aim 2, Experiment 1 (VGLUT1 and VMAT2 protein, complex I and II, NeuN and Fluorojade-C, and spectrin proteolysis) will be performed. The effect of CUS alone or that followed by METH will be assessed by immunoblot of D1 and DAT.

There will be 2 stress groups (no stress or CUS) X 2 METH groups (saline or METH) X 4 drug antagonists (D1, NMDA, or AMPA antagonist or vehicle) X 10 rats/group = 160 rats/assay. In most cases, different assays can be performed on the same rats. For example, the assays for VGLUT1, VMAT2 and DAT will use the same group of rats. Another group of rats will be for the mitochondrial assays (n=160). A third group of rats will be needed for the immunohistological measures of both NeuN and Fluorojade-C and GAD (n=160). Therefore, 3 separate 3-way ANOVA designs will be needed for the different assays. Three different timepoints (1, 3 and 7 days) after METH will also be evaluated using these designs.

Specific Aim 3B: Chronic unpredictable stress will enhance the excitotoxicity in the HIPP and striatum as evidenced by enhanced GLU overflow, VGLUT1 expression, and spectrin proteolysis. Fluorojade-C staining, decreased GAD65/67 immunostaining, augmented depletions of 5HT and 5HT transporter protein

(hippocampus), DAT (striatum) and decreased activity of the electron transport chain activity will be blocked by metyrapone treatment.

Rationale: The HIPP is particularly vulnerable to and represents one of the main targets of stress as it contains high amounts of glucocorticoid (GC) receptors (Aronson et al., 1988). There are numerous consequences of stress and GC exposure on the HIPP including but not limited to dendritic atrophy, suppression of neurogenesis, and impairments in spatial learning and memory performance (Magarinos and McEwen, 1995). Hippocampal atrophy has also been observed in human subjects with major depression, an effect that is linked with alterations in HPA axis reactivity and stress (Kendler et al., 1999). Reductions of serotonergic activity are also postulated to be involved in the pathogenesis of depression and provide the rationale for treatments focused on the enhancement of 5HT transmission (Graeff et al., 1996). METH also causes a decrease in 5HT markers in the HIPP (Ricaurte et al., 1980) and human METH users report higher self-ratings of depression (Graeff et al., 1996; London et al., 2004). Human METH abusers also display smaller hippocampal volumes, an effect that correlates with impaired memory performance on a word-recall test (Thompson et al., 2004). Taking into account that stress increases the risk of drug abuse and relapse as well as the similarities between the effects of stress and METH on the HIPP, the determination of hippocampal alterations produced by CUS and METH is interesting and important in the context of human drug abuse.

Multiple parallels exist between the neurochemical effects of stress and METH with regard to oxidative damage, bioenergetic compromise, and GLU transmission. Both stress and METH administration increase plasma corticosterone (CORT) (Szumlinski et al., 2001) and GLU in the HIPP (Lowy et al., 1993), impair glucose uptake, and decrease mitochondrial function (Madrigal et al., 1991). Adrenalectomy attenuates MDMA-induced hippocampal 5HT depletions, an effect that is reversed by CORT administration (Johnson et al., 1989). Moreover, MK-801, a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) GLU receptor, protects against METH-induced hippocampal 5HT depletions (Farfel et al., 1992). CORT decreases hippocampal GLU uptake and prolongs NMDA-mediated elevations in Ca^{2+} as well as compromising antioxidant defense mechanisms via decreases in copper/zinc superoxide dismutase and brain glutathione levels (McIntosh et al., 1998) . CORT also enhances ATP loss in response to a hypoxic insult, enhances reactive oxygen species and toxicity produced by the oxygen radical generator adriamycin, and enhances the accumulation of extracellular GLU after kainic acid (Stein-Behrens et al., 1992). Both CORT and stress also enhance kainate-induced structural damage and spectrin breakdown in the HIPP (Stein-Behrens et al., 1994).

Figure 17

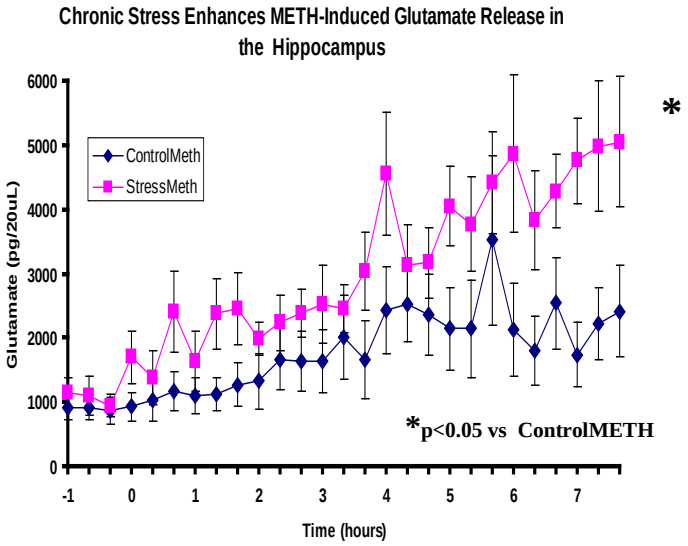
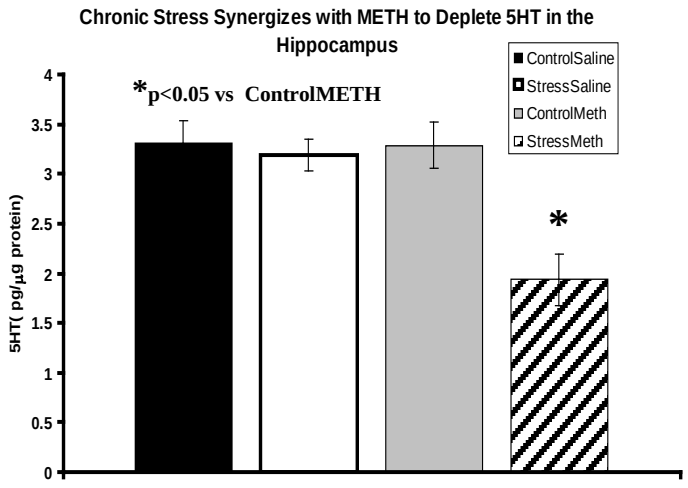


Figure 18



Preliminary data that support the feasibility of these studies indicate prior exposure to CUS enhanced METH-induced increases in GLU within the HIPP (METH=7.5 mg/kg X 4) (Fig. 17; n=8/METH and n=6/saline) and CUS alone increased VGLUT1 protein (Fig. 19; n=8/group). CUS alone also increased basal CORT (Fig. 20) and synergized with a lower dose of METH (7.5 mg/kg X 4) to deplete 5HT in the HIPP (Fig 18, n=10-13) (*p<0.05 from saline controls). It should be noted that this lower dose of METH alone did not deplete 5HT.

Figure 19

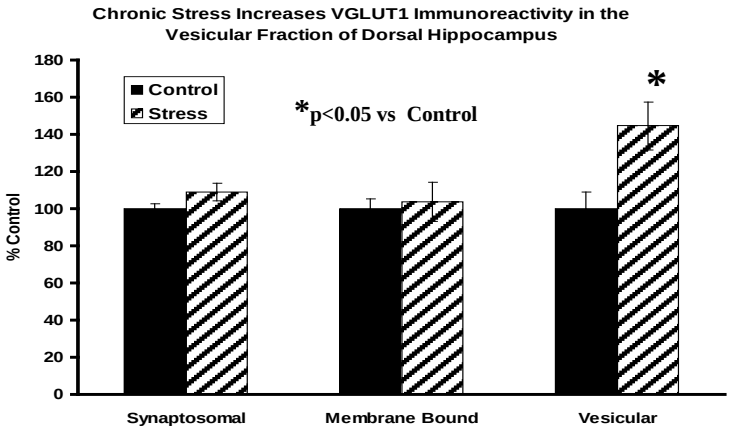
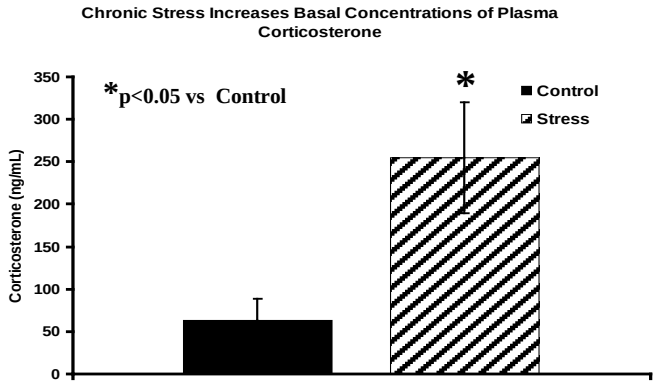


Figure 20



Design: This experiment will assess the glucocorticoid dependency of the CUS effects on extracellular GLU and markers of excitotoxicity in the striatum and HIPP after METH. Rats will be divided into either a no-stress group or exposed to chronic unpredictable stress (CUS) (see General Methods for paradigm) for 10 days. Half of the CUS rats will be injected with metyrapone daily (150 mg/kg, ip) (Czyrak et al., 1997) during the CUS regimen. On the 7th day of the CUS regimen, rats will undergo stereotaxic surgery for implantation of microdialysis probes into the striatum and HIPP to measure extracellular concentrations of GLU.

One day after the last stressor of the CUS regimen, dual probe microdialysis studies in the striatum and HIPP of the same rat will be performed. Rats will receive either saline or METH (7.5 mg/kg ip q 2 hr X 4) and then will be killed at 1, 3 or 7 days after saline or METH exposure. To assess toxicity, striatum and HIPP contralateral to the dialysis probe implantation site, as well as the substantia nigra, will be dissected. Biochemical assays identical to those described in Specific Aim 2- Experiment 1, (VGLUT1 and VMAT2 protein, mitochondrial complex I and II, NeuN, GAD, and Fluorojade-C staining, and spectrin proteolysis) will be performed. In addition, D1, DAT and SERT immunoreactivity in striatum and substantia as well as SERT protein in HIPP will be assessed by immunoblot assay.

There will be 2 stress groups (no stress or CUS) X 2 METH groups (saline or METH) X 2 chemical adrenalectomy conditions (metyrapone or vehicle) X 10 rats/group = 80 rats/assay. As stated above, different assays can be performed on the same rats. The assays for VGLUT1, VMAT2 and DAT will use the same group of rats. Another group of rats will be for the mitochondrial assays. A third group of rats will be needed for the immunohistological measures of both NeuN and Fluorojade-C. Therefore, 3 separate 3-way ANOVA designs will be needed for the different assays. Three different timepoints (1, 3 and 7 days) after METH will also be evaluated using these designs.

Discussion, Anticipated Results, and Potential Pitfalls

Prior exposure to CUS should enhance METH-induced GLU release in the striatum and HIPP as suggested by Figs. 15 and 17. This enhancement should be blocked by metyrapone administration during CUS exposure and will indicate that the protracted increases in basal CORT produced by CUS (see Fig. 20) are necessary for the enhanced responsivity to METH. Future studies could examine the acute role of CORT in mediating the acute METH-induced increases in GLU and long-term toxic effects by administering the glucocorticoid receptor antagonist, RU486 just prior to each injection of METH or saline.

CUS should enhance the toxicity of METH as evidenced by augmented increases in spectrin and ubiquitin-dependent proteolysis and Fluorojade-C/GAD staining, increases in nitration and nitrosylation of complex II and VMAT2, and increases in VGLUT1 protein. Decreases in NeuN staining should also be observed. Furthermore, CUS should enhance the decrease in DAT, SERT, VMAT2 and complex II activity in striatum and HIPP produced by METH. These changes should be blocked by metyrapone treatment during the CUS exposure and indicate that the CUS effects on METH toxicity are mediated by CORT. If metyrapone differentially affects some of these changes (e.g. in HIPP) and not others (e.g. in striatum, substantia nigra), this would indicate that these effects are differentially sensitive to CORT and may be associated with different densities of CORT receptors in these regions. If metyrapone is not effective in blocking the effects of CUS in general, an alternate approach will be to adrenalectomize rats prior to CUS.

The administration of GLU receptor antagonists during CUS should assess the role of GLU and its receptors in mediating CUS-induced vulnerability to METH toxicity. It is likely that the AMPA antagonist may

be the most effective, particularly in the HIPP at blocking the effects of CUS. This would be consistent with the findings that chronic stress itself increases the expression of AMPA receptors or AMPA-mediated currents in the HIPP (Schwedt and Jezova, 2000; Karst and Joels, 2003). It is recognized however, that increases in NMDA receptors also have been noted (Bartamusz et al., 1995; Krugers et al., 1993) and AMPA receptors can facilitate NMDA responses. Depending on which GLU receptor antagonist administered during the stress paradigm is the most effective against the CUS enhancement of METH toxicity, it would be interesting to examine if antagonism of the NMDA receptor acutely during the administration of METH (while maintaining hyperthermia) would block the CUS enhanced toxicities.

Since adrenalectomy has been shown to decrease D1 receptor expression in the striatum and SN (Biron et al, 1992), CUS alone should increase D1 receptors in the striatum and/or SN but not in the HIPP due to the low density of D1 receptors in this latter region. Therefore, D1 antagonism during the CUS regimen should block any changes in the striatum and SN produced by CUS alone or CUS followed by METH as in Specific Aim 1.

The area least affected by CUS may be the SN due to the lower density of GR receptors in this area. Based on our preliminary data (Figs. 11 and 12), no changes in this region are expected with METH alone. However, spectrin proteolysis and decreased NeuN staining should be observed in the CUS+METH group similar to the effects of the combined treatment of METH + haloperidol on GLU release.

Some markers of excitotoxicity and oxidative stress may be differentially affected by CUS alone or CUS followed by METH even though all of the markers chosen for evaluation and manipulation are intimately linked. Regardless of the outcome, new information will be gained on the changes most vulnerable to CUS when rats are subsequently challenged with METH.

It remains to be determined how METH increases the release of GLU in the HIPP (Fig. 17). Nevertheless, METH could increase GLU release through the increase in CORT and activation of CORT receptors on hippocampal pyramidal cells. If this is the case, metyrapone during CUS should attenuate the effects of prior CUS exposure on the exacerbated increase in GLU produced by METH. Future studies could examine the acute effects of RU486 on METH-induced GLU release. We have shown that CUS augments the METH-induced hyperthermia through a $5HT_2$ mediated mechanism (Matuszewich and Yamamoto, 2004). Thus, hyperthermia through $5HT_2$ mechanisms may mediate the enhanced GLU release. Nevertheless, these experiments are beyond the scope of the proposed studies that are needed to establish initially, if stress contributes to the hypothesized METH-induced excitotoxicity to 5HT terminals and/or hippocampal neurons.

These series of studies will be the first to address if prior exposure to stress enhances the vulnerability of the HIPP to METH toxicity. Furthermore, new information will be obtained on how stress may predispose the striatum to the excitotoxic effects of METH. Although the current knowledge on the long-term effects of METH in the HIPP is limited to damage to 5HT terminals, it is unknown if there is other evidence of excitotoxicity based on our findings that METH increases the extracellular concentrations of GLU. Moreover, the scope of toxicity may extend beyond damage to 5HT terminals and encompass cell bodies of the HIPP. This will have significant implications for cognitive deficits associated with stress and drug abuse, as well as hippocampal dysfunction/abnormalities linked to major depression.

Statistical Analyses – For all experiments, a minimum of 10 rats will be used for each treatment group as assessed by a power analysis (power = 0.8, $p<0.05$). Data from the dialysis experiments will be analyzed with an ANOVA with repeated measures followed by post hoc analysis with Student-Newman-Keuls test. Tissue concentrations of DA, optical densities of western blots, and mitochondrial enzyme activities will be analyzed by ANOVA with subsequent post hoc analysis of treatment means with the Student-Newman-Keuls test.

TIMETABLE FOR STUDIES: All these studies can be performed simultaneously by the investigators and are not dependent on the other for their conduct.

Specific Aim 1: Years 1-4; **Specific Aim 2:** Years 1—5; **Specific Aim 3:** Years 2-5

GENERAL METHODS

Adult, male Sprague-Dawley rats (175-199 g) will be used for all experiments.

Mitochondrial Enzyme Assays: *NADH Reductase (Complex I)* - Brain regions will be homogenized in ice-cold buffer (30%) w/vol, 2mM HEPES, pH 7.4, containing 220 mM mannitol, 70 mM sucrose and 0.05% bovine serum albumin). The supernatant produced from a 5 min 900 g centrifugation at 4°C will be re-centrifuged at 11,000 X *g* for 10 min. The brown pellet under the synaptosome layer will be resuspended, re-centrifuged and resuspended in fresh 100 mM KCl containing 50 mM KH_2PO_4, pH 7.4. The mitochondrial suspension will be diluted with 50 mM Tris-HCL buffer,

pH 7.5 containing 0.25 M sucrose to a final protein concentration of 0.07 mg/ml. NADH quinone reductase activity will be assessed in this buffer at 25° C. The assay mixture will contain 0.24 mM NADH, 0.1 mM decylubiquinone, and 3 mM potassium cyanide. The reaction will be started by the addition of the decylubiquinone and NADH and the decrease in NADH will be followed spectrophotometrically at 340 nM. *Succinate Dehydrogenase Assay (Complex II)* - Activity will be measured using a modification of the method of Lippold (1982). Tissue will be homogenized in 0.16% Triton X-100 and 32mM sucrose, (pH 7.4). The homogenate will then be added to an incubation buffer containing 50 mM KH_2PO_4, 0.1% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazoliumchloride, 50 mM sodium succinate and 25 mM sucrose, (pH 7.4) in a stoppered glass test tube. Incubation will be carried out for 10 min. at 37°C. The reaction will be stopped by addition of 10% trichloroacetic acid. The solution will be centrifuged and the supernatant discarded. The insoluble formazan product in the precipitate will be solubilized by extraction with 2 ml of ethyl acetate and absorbance read at 490 nm. Results will be expressed as formazan product formed/hr. *For Complex I and II protein determinations*, Western blot analysis will be performed on extracted mitochondria and subsequently transferred to PVDF membranes. Antibodies for the complexes and subnunits will be obtained from Chemicon. Bound antibody will be visualized with anti-rabbit Immunoglobulin antibody (1:2500 dilution) (Amersham Biosciences, Piscataway, NJ; NA934V).

Proteasome activity: Tissue will be homogenized (50mM Tris-HCl, 1mM EDTA, pH 7.5) and sonicated with 30-40 pulses/sample. Proteosome activity will be determined using spectrofluorometry following the addition of various concentrations of substrate *N*-Succinyl-Leu-Leu-Val-Tyr-AMC (7-amino-4-methylcoumarin) (BIOMOL) measuring chymotrypsin-like activity with slight modification (Bulteau et. al. 2001). Proteosomal kinetics will be conducted in a 96-well fluorimetric plate reader (SpectraMAX, Molecular Devices) after the addition of the enzyme substrate. Excitation/emission wavelengths will be 350/440nm. Relative fluorescent units will be taken every 15 sec. over the course of 5 min. and the rate of enzyme activity will be determined for each substrate concentration after adjustments for protein quantification are made (Bradford method). Enzyme kinetics, including Km and Vmax will be determined.

Ubiquitin immunoreactivity: Samples will be prepared according to Vannucci et.al.,(1998). Samples will be homogenized in (PBS in the presence of protease inhibitors: leupeptin, pepstatin, chymostatin, and antipain (1mg/ml) and phenylmethylsulfonyl fluoride (0.2 mM). Protein concentration will be determined according to the Bradford method and equivalent amounts will be run on an 8% gel, transferred to a PVDF membrane (Invitrogen), and probed with an antibody for ubiquitin (Sigma). Ubiquitin immunohistochemistry will also be done, according to methods of Fornai et.al.(2004). Brains will be removed and striatal and hippocampal slices will be taken. Sections will be rinsed in a phosphate buffered saline solution and blocked with 1% bovine serum albumin (BSA) and 20% goat serum in PBS+T to reduce background [Case et. al. 2004]. Sections will be incubated overnight with rabbit antibody I against ubiquitin (Sigma). Secondary fluorochrome antibodies (Chemicon) will be applied and observed using fluorescence microscopy (Nikon Eclipse).

VGLUT1, VMAT2, D1, EAAT3,and Spectrin immunoreactivities: Synaptosomal, membrane-bound, and vesicular fractions will be prepared from whole tissue via differential centrifugation separation as described previously (Riddle et al., 2002) with modifications (Dunah and Standaert, 2001). Briefly, whole tissue will be dissected and homogenized in ice-cold 0.32 M sucrose and centrifuged (800 x g for 12 min; 4 °C) to remove nuclei and large debris. The supernatant will be centrifuged (22,000 x *g* for 17 min; 4 °C) and the pellet retained will be the synaptosomal fraction. This fraction will be subsequently lysed hypo-osmotically with water and centrifuged (22,000 x *g* for 17 min; 4 °C) to pellet a membrane-associated fraction. The remaining supernatant will be the vesicular fraction (non-membrane associated fraction). Protein of each fraction will be determined by the method of Bradford using bovine serum albumin as the standard. Western blot analysis will be performed on all fractions and subsequently transferred to PVDF membranes. The primary VGLUT-1 antibody (1:20,000 dilution) will be purchased from MAb Technologies, Inc (Stone Mountain, GA). Spectrin, D1, EAAT3 and VMAT2 antibodies purchased from Chemicon. Bound antibody will be visualized with anti-rabbit Immunoglobulin antibody (1:2500 dilution) (Amersham Biosciences). Membranes will be visualized via ECL western blotting detection (Amersham Biosciences). Densitometry of the bands will be quantified by using Kodak 1D image analysis software.

Co-immunoprecipitation: Synaptosomal fraction samples will be diluted to 1 mL with DH_20 and 500mL of total synaptosomal homogenate will be used to co-immunoprecipitate VMAT-2 and Complex I and II. Samples will be pre-cleared by addition of 1µg of rabbit IgG (Santa Cruz Biotechnology) and 20µl protein G-PLUS agaraose (Santa Cruz Biotechnology) to each sample for 1hr @ 4°C. Beads will be pelleted by centrifugation at 2,500 X *g* for 1min. Supernatant will be retained and incubated overnight with 1µg primary VMAT-2 antibody (Chemicon AB-1767) or Complex I or II antibodies (Mol. Probes) at 4°C. 20 µl protein G-PLUS will be added and the mixture will be incubated for an additional 24 hrs at 4C. Beads will be pelleted by centrifu gation at 2,500 X *g* for 1min and washed with RIPA buffer. This will be repeated 4 times. Following the final wash and centrifugation, beads will be resuspended in 50µl tris-glycine SDS sample buffer (Invitrogen) and heated for 5 min at 100C. Samples wil l be pelleted by centrifugation at 2,500 X *g* for 1 min. 2 µl of supernatant will be run on 10% tris-glycine gels (Invitrogen) and probed with s-nitrosyl-cysteine (Sigma) and nitrotyrosine (Upstate) antibodies.

Vesicular glutamate uptake: Vesicular GLU uptake in purified synaptic vesicles will be performed in the presence of 2 mM ATP as described by Porciuncula et al., (2004) except that time-dependent decreases in GLU will be measured in the media with dialysis vs appearance in vesicles by HPLC. Vesicles will be assayed subsequently for GLU content.

Real-time PCR (VGLUT mRNA): Total RNA will be extracted from rat cortical slices using a RNeasy® minikit by Qiagen. Real-time reverse transcription (RT)-PCR assays will be performed using the ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, CA). Rat VGLUT1 primers and probes targeting an exon/intron boundary in the rat VGLUT1 gene (NM_053859) will be designed using Primer Express version 1.5a software from Applied Biosystems. All Taqman probes will be synthesized by Applied Biosystems and forward and reverse primers will be synthesized by Oligos Etc. (Wilsonville, OR). Cyclophilin was used as an endogenous control to normalize mRNA levels (Steiger et al., 2004). The forward and reverse primers for rat VGLUT1 will be: 5'-GGATTTATCTGCCAAAAATCGC-3' and 5'-CAACATATTTAGGGTGGAGGTAGC-3'. The forward and reverse primers for rat cyclophilin will be 5'-TGCAGACATGGTCAACCCC-3' and 5'-CCCAAGGGCTCGCCA-3'. The Taqman probes will be synthesized with the fluorescent reporter FAM (6-carboxy-fluorescein) attached to the 5'-end and the quencher dye TAMRA (6-hydroxy-tetramethyl-rhodamine) attached to the 3'-end. The sequence of the VGLUT1 probe will be: 5'-CAACAGGGTCTTTGGTTTGCCATTG-3'. The sequence of the cyclophilin probe was: 5'-CCGTGTTCTTCGACATCACGGCTG-3'. The standard curves for relative quantification will be generated using 0 to 100 ng of total RNA isolated from rat whole brain. Total RNA (20ng) from the rat cortex will be tested. Reactions will be performed in triplicate in a final volume of 50 μl containing Taqman One Step RT-PCR master mixture (Applied Biosystems), 250μM VGLUT1 probe, 900 nM VGLUT1 primers, 200nM cyclophilin primers, and 100nM cyclophilin probes. 2 aliquots of 20 μl/reaction will be loaded in a microplate. Thermocycling will be done under the following conditions: 50 °C for 30 min, 95 °C for 10 min and 50 cycles of 95 °C for 15s and 60 °C for 1 min. The relative amount of VGLUT1 mRNA will be normalized to cyclophilin (internal control).

In situ hybridization: Radioactive-labeled cRNA probes will be produced after *in vitro* transcription from cDNA clones encoding either for the feline GAD67 or the rat GAD65. The cDNAs inserted into bluescript or PSP64/65 vectors will be linearized with restriction enzymes. Transcription of the radioactive cRNAs from the cDNAs will be performed using a Riboprobe kit (Promega; Madison, WI) in presence of 2.5 μM of ^{35}S-UTP (1,000 Ci/mmol; New England Nuclea) and 10 μM unlabeled UTP. Unlabeled CTP, ATP and GTP are added in excess and T3, SP6 or T7 RNA polymerases are used for the reaction. The labeled cRNAs are then purified by phenol/chloroform extraction and ethanol precipitation. The length of the cRNAs will be reduced to 100-150 nucleotides by partial alkaline hydrolysis to improve accessibility of the probe. Brain sections will be dried at room temperature and post-fixed for 5 minutes in 3% paraformaldehyde in PBS (1M; pH 7.2) containing 0.02% Diethylpyrocarbonate (DEPC). Sections will be treated for 10 mins with triethanolamine (0.1 M; pH 8.0) containing 0.25% acetic anhydride and then for 30 min with Tris-glycine (1M; pH 7.0) before being dehydrated and air-dried. Each section will be covered with 3-3.5 ng of radiolabeled cRNA probe and 4 ng of digoxigenin-labeled probe diluted in 20μl of hybridization solution (containing 40% formamide, 10% dextran sulfate, 4xSSC (Sodium Standard Citrate), 10mM dithiothreitol, 1% sheared salmon sperm DNA, 1% yeast tRNA, 1x Denhardt's solution containing 1% RNase-free bovine serum albumin). The sections will be covered with Parafilm™, placed in humidified boxes and incubated for 4 hours at 50°C. Posthybridization washes are in 50% formamide (in 2xSSC) at 52° C for 5 min and 20 min, in RNase A (100μg/ml; Sigma; in 2XSSC) for 30 min at 37° C and in 50% formamide (in 2xSSC) at 52° C for 5 min. Sections will be further rinsed at room temperature for 30 min in 2xSSC containing 0.05% Triton X-100. Sections are washed in 2xSSC for 15 min, quickly dipped in ammonium acetate (300 mM), rinsed in 70% ethanol, and air-dried. Sections will be juxtaposed to Kodak X-OMAT-AR X-ray films (Eastman Kodak) for various lengths of time and then processed for emulsion radioautography. Sections will be dipped in Amersham LM-1 nuclear emulsion, air dried, and stored at 4° C in light-tight boxes in the presence of desiccant. After 4-8 days of exposure, the emulsion radioautographs are developed in Kodak D-19 for 3.5 min at 14° C and mounted in Eukitt™ mounting media

Immunohistochemistry: Perfused brains will be cut on a vibratome. Free-floating sections will be rinsed with 0.02 M KPBS + Triton-X100 for 3x1 min., rinsed in 0.6% H_20_2 in 0.02M PBS+Triton-X100 and rinsed again in 0.02 M KPBS + Triton-X100 for 3x1 min. Sections will then be placed in humid chamber boxes and covered with 5% NGS + KPBS + Triton-X for 60 min.at RT. Sections are covered with the Avidin D Block kit (Chemicon). The sections are then covered with the 1° antiserum solution (Mouse anti-NeuN; Rabbit anti-parvalbumin; Rabbit anti-ChAT, rabbit anti-GAD65/67; Chemicon) + (KPBS + Triton-X) + 1% NGS and incubated for 14–20 hr. at 4°C. The next day, sections are rinsed in KPBS + Triton-X for 3x1 minutes and covered with the 2° antiserum (1:1000) (Goat Anti-Mouse or anti-rabbit IGg-Biotin SP conjugated (Jackson ImmunoResearch) in (PBS + Triton-X) + 1.5% NGS and incubated for 1hr. at RT. Sections are then rinsed in 0.02 M KPBS + Triton-X for 1 min. Labeling will be with a metal-DAB conjugate (Pierce). Sections are then de-hydrated in alcohol and xylene and mounted in Eukitt mounting medium.

Fluoro-Jade C labeling: Sections are dried on a slide warmer at 50 °C for at least 30 min. Slides are immersed in 100% ethanol for 10 min then into 95% ethanol for 3 min. They are rinsed in distilled water for 2 min, and incubated in 0.06% potassium permanganate for 10 min. Slides are washed in ddH_20 for 1-2 min and then transferred in 0.0001% Fluoro-Jade C solution for 7 min. (1 ml of stock Fluoro-Jade C to 99ml 0.1% acetic acid vehicle). Slides are washed with ddH_20 3X1 min and then dried on a slide warmer at 50 °C for 1hr. Slides are immersed in xylene, pulled out and dried for about 30 sec. Fluoro-Jade C labeled sections will be examined under an epifluorescent microscope using a filter for visualizing fluorescein (Ex: 385 nm, Em: 425 nm) or fluorescein isothiocynate (FITC). Staining will be combined with immunohistochemical labeling using a rhodamine-complex secondary antibody (Yu et al., 2004).

Quantitative analyses: Numbers of NeuN, GAD65/67 or parvalbumin cells labeled with the ABC method or labeled by in situ hybrodizaton histochemistry in the different experimental groups will be obtained using the optical fractionator method (West et al., 1991) with the StereoInvestigator software (MicroBrightField, Williston, VT), a Nikon E600 microscope and a color digital camera. The numerical density in the hippocampus, striatum or SNr, defined as the number of objects per mm^3, will be gathered in 8 sections. Quantification in the hippocampus will be carried out in the granule cell layer of the dentate gyrus and the pyramidal cell layer of CA1 and CA3 as well as in the stratum oriens, pyramidale (including the pyramidal cell layer) and radiatum of CA3 and CA1. Cells in the striatum and substantia nigra will be counted in the latero-dorsal and medio-ventral aspects of each structure.

In vivo microdialysis: Rats will undergo stereotaxic surgery under ketamine and xylazine anesthesia for the implantation of stainless steel guide cannulae positioned on dura overlying the anterolateral caudate (+1.2 mm AP from bregma, $\pm$ 3.2 mm lateral to midline), substantia nigra (-5.2 mm AP, 3.0 mm lateral; 15^o angle from vertical), and/or dorsal hippocampus (-3.2 mm AP, $\pm$ 2.0 mm lateral). The rats will recover from surgery for 3 days. Microdialysis probes will be constructed as previously described (Yamamoto and Pehek, 1990). The dialysis surface of the membrane (regenerated cellulose, 210 μm O.D., 13,000 MW cut-off) will be 4 mm in length for the caudate and 1.5 mm for substantia nigra and hippocampus. All probe placements will be verified by Nissl staining. Dialysis probes will be inserted through the guide cannula and connected to an infusion pump programmed to deliver at 1.5 μl/min, a modified Dulbecco's phosphate buffered saline consisting of 138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1.2 mM $CaCl_2$, 0.5 mM d-glucose, pH 7.40. **Biogenic Amines** - Samples will be assayed for DA and 5-HT according to our previously published methods with minor modifications (Donzanti and Yamamoto, 1988a). **Glutamate and GABA** - An aliquot (20μl) of the dialysis sample will be assayed for GLU and GABA by HPLC/EC after precolumn derivatization with *o*-phthaldialdehyde, according our published methods (Donzanti and Yamamoto; 1988b).

Chronic Unpredictable Stress: The 10 day repeated stress paradigm is modeled after Ortiz et al. (1996). Stressed and control rats will be weighed daily to monitor their health during the procedure. *Day 1:* 12 pm, cage rotation for 50 min; 1 pm, swim stress for 4 min. *Day 2:* 11 am, cold room (4°) for 60 min; 6 pm, lights on, overnight. *Day 3:* 12 pm, lights off for 3 hrs; 3 pm, cold isolation (4°) for 15 min. *Day 4:* 6 pm, cage rotation for 50 min, 6 pm food/water deprivation overnight. *Day 5:* 1 pm, swim stress for 3 min; 7 pm isolation housing overnight. *Day 6:* 11 am, restraint stress for 60 min; 3 pm, lights off for 2 hours. *Day 7:* 10 am, swim stress for 4 min; 4 pm, restraint stress for 60 min. *Day 8:* 7 pm, lights on and food/water deprivation overnight. *Day 9:* 10 am, cage rotation for 20 min; 7 pm, lights on overnight. *Day 10:* 7 pm, isolation housing and food/water deprivation overnight.

E. Human Subjects

Not applicable.

F. Vertebrate Animals

1. Adult male Sprague-Dawley rats (200-300 g) will be used in these studies. Approximately 550 rats/year will be used to determine the effects of METH on extracellular concentrations of DA, GLU and GABA in the striatum, substantia nigra and hippocampus (5HT and GLU) of awake, freely moving animals using *in vivo* microdialysis. The experiments involve the systemic administration of drugs and/or the administration of drugs through the dialysis probe. Other groups of rats will be killed by decapitation after drug treatment in order to assess the effects of METH on various neurochemical markers as described in the General Methods.

2. Rats will be used in view of the well characterized neuroanatomy and neurochemical responses of rats. Each experiment requires 10 rats per group.

3. ACLAM board-certified laboratory animal veterinarians within the BUMC Laboratory Animal Science Center will provide veterinary care for animals to be used in the proposed studies. All animals are evaluated daily by animal facility staff trained to monitor the health of laboratory animals. The animal care and use program at Boston University maintains full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care, International.

4 For surgical procedures, the rats will be anesthetized with xylazine and ketamine. Surgery does not commence until a level of anesthesia is attained such that the animals do not respond to a pinch of the paw. No analgesics will be administered as they may interfere with the neurochemical responses during the dialysis procedure.

5 Half of the rats will undergo a mild chronic unpredictable stress procedure (CUS) according to previously published methods (Oritz et al., 1996). CUS does not produce significant changes in physiological measures such as adrenal and spleen weights and has modest effects on body weight gain (Bielajew et al., 2002), indicating that the CUS regimen is mild and does not have any obvious debilitating effects on physiology or morphology. Moreover, CUS has been examined for its face, construct and predictive validity as an animal model of depression (Willner et al., 1992). This has added relevance in light of the reports that depression is known to be a precipitating factor for drug abuse and that substance abuse is highly co-morbid with depression in humans (Kessler, et al., 1994). Rats will be rapidly decapitated and their brains frozen in liquid nitrogen. No anesthetic will be administered due to their confounding effects on metabolism and concentration of neurotransmitters. This method of euthanasia is consistent with the recommendation of the 2000 AVMA Panel on Euthanasia.

6. The method of euthanasia at the end of the experiment is an overdose of anesthetic which is in accordance with the American Veterinary Medicine Association. In most experiments, brain tissue is removed after the rats have been killed by decapitation. The rats will not be given an overdose of anesthetic prior to decapitation, inasmuch as these drugs may interfere with the biochemical assays. Similarly, the heads will not be placed in liquid nitrogen since this procedure prevents the rapid and clean dissection of discrete brain.

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