

CLUB DRUG USE AS SELF-INFLICTED TRAUMATIC BRAIN INJURY

By

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To my family for affording me education and opportunity

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Club drug abuse, such as with methamphetamine (Meth) or 3,4-methylenedioxymethamphetamine (MDMA), is a pervasive and dangerous problem in the United States. Neurological and endocrine toxicity have been reported but would be better understood and treated with the use of simple proteomic biomarkers of pathology. There has already, however, been some success in this in the field of traumatic brain injury (TBI) studies. In brief, TBI has been shown to activate the pro-necrotic calpain and the pro-apoptotic caspase proteases which act to degrade the neural structural proteins α II-spectrin and tau, creating useful markers of disease.

Here, Western blot, immunocytochemistry and immunohistochemistry tools were employed in animal and cell culture models treated with club drugs to search for those same markers in an effort to study the extent of toxicity. Protease inhibitors were also used to help determine mechanism of action. Finally, endocrine hormones were studied for changes in blood concentration using radioimmunoassays.

In brief, Meth and, to a lesser extent, MDMA are found to be neurotoxic, comparable to TBI. In the cortex, co localization with cell death biomarkers is established and is more prominent in the neurons than glia, with particular upregulation in axonal compartments. Cortical cell culture studies showed that club drugs stimulate dual action mechanisms of both calpain and caspase in origin. Furthermore, when either one is suppressed, the other appears to be upregulated. Neuroendocrine studies showed that changes in leptin, ghrelin and neuropeptide-Y may be related to other toxic effects of the drugs.

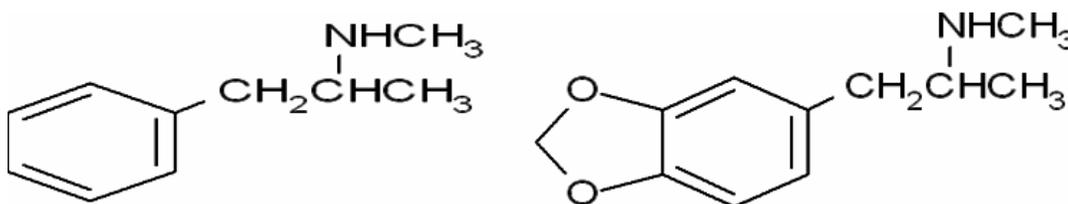
These studies marked the first to identify biomarkers in the brain following club drug use. The tools may be used to further study club drugs or serve as a model for the study of other drugs of abuse. Chronic administration paradigms should be compared to the acute models studied here and human studies need to be conducted to test the application of this work.

CHAPTER 1 BACKGROUND

Club Drugs

Introduction

The term “club drug” includes any of a number of recreational substances consumed in dance clubs or at underground rave parties which may result in brain pathology. Here, we are primarily interested in the neurotoxic effects of two particular stimulants, methamphetamine (Meth or Speed) and the structurally related compound 3,4-methylenedioxyamphetamine (MDMA or Ecstasy).



A) Methamphetamine B) 3,4-Methylenedioxyamphetamine

Figure 1-1. Chemical structure of club drugs. A) Methamphetamine. B) 3,4-Methylenedioxyamphetamine. Both drugs can be found as pills or in white powder form. The only difference in structure is the 3,4-methylenedioxy ligand added to the benzene ring of Meth to make MDMA.

Meth is manufactured in clandestine labs with relatively inexpensive ingredients. It is normally consumed in pill form, but may also be smoked or dissolved into a solution and then injected. Acute users of Meth experience an increase of energy expenditure and loss of appetite and sleep (NIDA 2002). Ecstasy is usually taken as a pill only. Users experience stimulation of mental function, sensory perception, emotion and energy (NIDA 2005b).

These drugs have specific effects on the serotonergic, dopaminergic and, at least with MDMA, norepinephrine neurotransmitter systems. Serotonin is located in virtually all areas of the brain except the cerebellum and is involved in regulating mood, memory, cognition, sleep, aggression, appetite, thirst, sexual function, body temperature and sleep. Dopamine, normally produced by the hypothalamus, plays a role in body movement, cognition, hormone regulation, motivation and pleasure. Norepinephrine is a hormone released by the adrenal glands that functions in attention, impulsivity and activates the sympathetic nervous system.

Epidemiology

Meth was first developed in Japan in 1919 to treat depression and enhance weight loss. It was made a Schedule II controlled substance in 1979, meaning it has a high potential for abuse, but is still available by prescription in low-dose form to treat some conditions such as Attention Deficit Disorder (ADD), narcolepsy or obesity. Recently, police officers ranked Meth as the biggest drug problem they are facing today (NACO 2005). Additionally, what was first seen as a West Coast problem has now moved more towards central and Midwestern states, with dance clubs in the northeast also reporting increased use (NIDA 2006). In 2003, 12.3 million Americans had reported trying Meth at least once, representing 5.2% of the population (SAMHSA, 2004). However, use may be stabilizing among youths. In 2004, 6.2% of high school seniors had reported ever trying Meth in their lifetime, a number that has not changed in a year, while middle school students reported a decrease in use (NIDA 2005a).

Ecstasy was developed in 1912 by the German pharmaceutical company Merck as a parent chemical compound. During the 1970s some psychotherapists began using the drug during sessions, despite the fact it had never been approved by the U.S. Food and

Drug Administration (FDA). It was made a Schedule I controlled substance in 1985 and thus banned from use. Ecstasy, once seen almost exclusively in the club scene, has recently been reported to have increasing use in minority population social settings (NIDA 2006). In 2003, 11 million Americans had reported using MDMA in their lifetime, almost 5% of the population (SAMHSA 2004). However, American youths have recently reported a slight decrease in use and an increase in hazard awareness (NIDA 2005a). This is in contrast to escalating use in Europe (Schifano et al. 2006).

Toxicity

Particularly elevated levels of club drugs can enter the blood stream due to the “binge and crash” pattern of use by addicts trying to eschew tolerance to its effects. The lipophilic nature of the compounds allows them to cross the blood brain barrier and manifest effects on the brain (Nordahl et al. 2003).

Neurotoxicity

The high associated with Meth stems from its effect of causing release of elevated levels of dopamine in the brain (Robbins and Everitt 1999) and its effect on neocortical neurons (Schroder et al. 2003). Meth binds to dopamine transporters (DAT) and blocks the re-uptake of dopamine, overloading the synapse (Baucaum et al. 2004). This activity desensitizes those cells, as well as others containing serotonin, potentially disrupting motivation, pleasure sensation and motor function (Gonzalez et al. 2004). Meth has been shown to cause dopaminergic and, to a lesser extent, serotonergic neurite and nerve terminal degeneration of neurons in the striatum and hippocampus (Larsen et al. 2002). Findings in rat and non-human primates show that Meth depletes dopamine and decreases the activity of tyrosine hydroxylase and 5HT systems (Seiden et al. 1976; Koda and Gibb 1973). Meth has been suggested to reduce the levels of enzymes that synthesize

dopamine and 5HT, leading to toxicity (Ricaurte et al. 1980; Bakhit et al. 1981). Oxidative stress may also help explain the mechanism for toxicity. For example, Meth enhances the creation of an endogenous dopamine toxic metabolite, 6-hydroxydopamine (6-OHDA) (Wagner et al. 1983). Similar findings have been reported related to the generation of 5,6-dihydroxytryptamine (5,6-DHT), a neurotoxin to the serotonergic system (Commins et al. 1987). It has been proposed that Meth-induced redistribution of dopamine from synaptic vesicles to cytoplasmic compartments, where dopamine is oxidized to produce quinones and additional reactive oxygen species, may account for this selective neurotoxicity (De Vito and Wagner 1989; Gluck et al. 2001). Furthermore, it has been reported that Meth exposure, by producing reactive oxygen species, can activate pro-apoptotic genes such as c-Jun, c-myc and L-myc and thus induces neuronal apoptosis directly (Stumm et al. 1999; Deng et al. 2002a; Thiriet et al. 2001). In support of these findings, Meth was found to induce apoptosis in both the CNS-derived catecholaminergic cell line *CSM14.1* (Cadet et al. 1997; Choi et al. 2002) and the immortalized rat striatal cell line *M213* (Deng et al. 2002b). Studies with N-methyl-d-aspartate (NMDA) receptor antagonists also suggest that glutamate release may be an important factoring mediating toxicity when triggered by Meth (Sonsalla et al. 1989).

Over time, a down regulation of dopamine receptors develops in Meth users, resulting in a Parkinson-like syndrome. Other long-term effects have been reported, such as paranoia, hallucinations, mood disturbances, repetitive motor activity and impotence (NIDA 2006). Neurotoxicity from Meth is exacerbated by hyperthermia and damage to blood vessels in the brain from increased heart rate and blood pressure.

High concentrations of MDMA from an “overdose” scenario can also be found among its abusers. It is not uncommon for MDMA users to take multiple tablets to combat tolerance and to compensate for perceived impurity in drug content (Baggott et al. 2000). Furthermore, MDMA inhibits its own metabolism, resulting in an effective drug level that may become neurotoxic. Ecstasy binds to transporters for serotonin, dopamine and norepinephrine, inhibiting their reuptake and increasing their concentration in the synapse. This rapid release of serotonin is followed by a significant depletion and associated deficits (Yamamoto 2001). Additionally, MDMA has been shown to destroy tryptophan hydroxylase, an important enzyme in serotonin function, in multiple areas of the brain including the cortex and striatum (Ricaurte et al. 1985). Other suggestions for mechanisms of MDMA toxicity, without regard to hyperthermia or metabolite effect, include release of 5-HT and its derivatives (Schmidt and Taylor 1987), release of dopamine (Nash and Nichols 1991), increased intracellular glutamate concentrations (White et al. 1994) and possibly free radical damage via nitric oxide (Simantov and Tauber 1997).

Reductions in the numbers of neurons containing serotonin have been reported after MDMA use in rodents and non-human primates (Schmidt and Taylor 1987; Ricaurte 1989; Taffe et al. 2001; Hatzidimitriou et al. 1999; Larsen et al. 2003). Autopsy reports have suggested that these effects also occur in humans (McCann et al. 1998; Kish et al. 2000). The effects on serotonergic and, to a lesser extent, dopaminergic neurons in the brain play a role in disturbing mood, sleep and pain (Reneman et al. 2000). For example, studies have shown MDMA users perform poorly on cognitive and memory tasks (Verkes et al. 2001). Also, due to the additional release of norepinephrine, neurotoxicity

is exacerbated by hyperthermia and cardiovascular injury from increased sympathetic tone (Dafters and Lynch 1998). Interestingly, the neurotoxicity to norepinephrinergic systems seen with MDMA is not seen with Meth (Seiden and Ricaurte 1987). Similar to Meth, however, is the involvement of oxidative stress as a possible mediator of neurotoxicity. Ecstasy disturbs the function of certain antioxidant enzymes, such as superoxide dismutase catalase (Cadet and Thiriet 2001). It has also been associated with increases in the production of hydroxyl radicals, although this effect takes much longer to occur than with Meth (Shankaran et al. 1999). Furthermore, decreases in overall metabolic activity in the brain, particularly the striatum, have been observed in rats after MDMA administration via studies of cytochrome oxidase activity and glycogen breakdown (Yamamoto et al. 1995; Darvesh et al. 2001).

Long term users, subjected to deficits in serotonin function, experience depression, insomnia, paranoia and bipolar symptoms along with a host of others (NIDA 2006). Self-reports indicate that MDMA users score higher than non-users in tests for social anxiety and phobia, although which is the cause and which is the effect has not been established (Parrott 2001). In agreement with acute intoxication studies, long time MDMA users have shown decreased performance on memory tests (Morgan 1999).

Neuroendocrine toxicity

The monoamines released in the brain following club drug use are normally involved in temperature regulation, motor control, and appetite regulation. Therefore, increased concentrations of these monoamines caused by drug use may result in the observed negative effects on these systems (Jaehne et al. 2005; Malpass et al. 1999). An important negative consequence of drug abuse is the development of pharmacologically-induced anorexia. Research has shown that administration of drugs, such as

amphetamines, heroin or cocaine, leads to appetite suppression and development of anorexia (Jonas and Gold 1986; Hseigh et al. 2005). These anorexic effects can be seen with both acute and chronic administration in humans and animals. Acute and chronic use of psychostimulants (such as Meth or MDMA) has been shown to result in decreased appetite, reduced feeding behavior, and drug-induced anorexia (Cho et al. 2001; Ginawi et al. 2005). It has also been shown that rats orally administered MDMA acutely (ranging from 20 mg/kg to 80 mg/kg) had significantly reduced body weight and food intake compared to control groups (De Souza et al. 1996). Rats that were given Meth (2 mg/kg) by intraperitoneal injection also exhibited significant reduction in food intake (Ginawi et al. 2005).

The mechanism for the drug-induced anorexia caused by these substances is mediated by the action of monoamines in the control of food intake. Specific receptor sites in the hypothalamus that control the anorexic action of amphetamine and similar drugs have been discovered (Paul et al. 1982). The biogenic amines released by drug use are believed to act on the paraventricular (PVN), ventromedial, and suprachiasmatic nuclei, which are responsible for energy balance and daily patterns of eating (Ginawi et al. 2005). Excess 5-HT released by psychostimulant administration acts on these nuclei to produce hypophagic effects by decreasing carbohydrate intake in naturally feeding animals thereby disrupting satiety mechanisms (Curzon et al. 1997). Specifically, amphetamine and other drugs were shown to decrease neuropeptide-Y (NPY) levels in the PVN, which resulted in decreased appetite stimulation with corresponding hypophagia (Hseigh et al. 2005; Curzon et al. 1997). Therefore, a proposed mechanism

of drug-induced anorexia is that excess 5-HT released due to drug use decreases production of NPY, inhibiting its normal orexigenic effects.

A significant problem associated with drug abuse is the 'wasting effect' that is seen from drug-induced anorexia in users. About two-thirds of the drug addicts in one study, including heroin, cannabis and ephedrine users, had significantly lowered body mass index compared to the normal population (Islam et al. 2002). Drug users in another study had reported weight loss and anorexia, which the researchers also associated with decreased serum leptin levels (Santolaria et al. 2002). Drug administration causes changes in feeding behavior and body mass through physiological mechanisms, however, more investigation needs to be performed to learn about the relationship between drugs of abuse and their direct effects on metabolic hormones involved in the regulation of feeding behaviors and energy balance (Halford and Blundell 2000; Crowley et al. 2005).

Treatment

Meth users are identified by signs of agitation and increased physical activity. Users also may display pupil dilation, violent behavior and a tendency to clean, sort or disassemble objects. Treatment of Meth is concerned with both addressing both the acute intoxication symptoms and the chronic addiction. Acute overdose of Meth is treated with ice bath and anticonvulsants to combat hyperthermia and seizures. A quiet atmosphere is recommended, with the use of benzodiazepines sometimes used to calm a psychotic patient. Long term Meth addicts are treated with therapy and cognitive behavioral intervention which seek to give the patient skills in coping with life stressors. There are no pharmacological treatments for treating dependence or reversing intoxication. Antidepressants, however, may be useful in combating relapse in abstinent patients.

Ecstasy users may be identified by high blood pressure, faintness, panic attacks or seizures. Acute intoxication is treated with rehydration and balancing of electrolytes. Special care is taken to avoid water intoxication. Similar to Meth, cognitive therapy remains the only treatment for MDMA addiction, since no pharmacological treatments exist.

Traumatic Brain Injury

Introduction

Traumatic brain injury (TBI) is another type of brain pathology and is defined as any sudden trauma which causes damage to the brain. This can be the result of an object piercing the skull to the point that it contacts brain tissue or when the head violently hits an object.

Epidemiology

Every year in the United States, approximately 1.5 million people sustain a TBI (CDC 2001). Of these, 50,000 die and 90,000 can experience an associated lifetime disability. Around 2% of the current US population is living with a TBI-related injury, caused chiefly from vehicle accidents, firearm use, falls and sports (Thurman et al. 1999). The cost to the U.S. healthcare system is estimated at \$56 billion each year (Thurman 2001).

Toxicity

Traumatic brain injury results in overt brain tissue death and the release of a number of amino acids, ions, proteins and enzymes into the blood and cerebrospinal fluid (CSF) (Goodman and Simpson 1996; Graham 2000).

Neurotoxicity

Primary injury is composed of mechanical tearing of neural tissue, either from contusive or counter-coupe injury (Graham et al. 2000). Blunt injury allows for unregulated chemical cascades and inflammatory responses to follow (Gennarelli 1993). Traumatic brain injury changes calcium homeostasis (Graham et al. 1995), which acts to deregulate certain proteolytic enzymes, as well as increase excitotoxicity (Choi and Rothman 1990) and free radical production (Kontos 1989).

Acute symptoms of a TBI include headache, confusion, dizziness with blurred vision, tinnitus, lethargy, changes in sleep patterns or mood and trouble with memory, attention or thinking. More serious TBI can result in nausea and vomiting, seizures, and loss of sensation or balance. Additionally, repeated mild TBIs occurring over time may result in cumulative deficits (CDC 1997).

Neuroendocrine toxicity

Rapid weight loss is also seen in human patients experiencing TBI, exacerbated by the inflammatory response and sympathetic stimulation (Mansoor et al. 1996; Herndon et al. 2001). Altered glucocorticoid production and increasing levels of interleukins have been reported in TBI patients (Mansoor et al. 1996). The catecholamine-mediated hypermetabolic response to some severe injuries causes increased expenditure and muscle-protein catabolism (Herndon et al. 2001).

Treatment

Diagnosis of a TBI includes observation of disorientation, amnesia, extended loss of consciousness, or neurological problems. Because repairing injury is seldom possible, treatment focuses mainly on preventing further injury and learning to cope. This includes radiography and attention to oxygenation and blood flow to the brain as well as sedation,

anti-convulsives and sometimes neuromuscular blockade (Bullock et al. 1995).

Hyperventilation and mannitol diuretic treatments have been used to prevent increases in intracranial pressure (Bullock et al. 1995). Guidelines have also been suggested for monitoring nutrition of trauma patients to prevent nitrogen loss and wasting (Bullock et al. 1995). Once the patient is stabilized, enrollment in physical, occupational, or speech therapy may be necessary to treat neurological consequences of brain damage.

Prognostic indicators, or lack thereof, are important for the consideration of clinical management of TBI patients. The most widely available measure of severity of injury in the clinic, currently, is the Glasgow Coma Scale score (GCS) which objectively measures consciousness (Teasdale and Jennett 1973). However, current guidelines in emergency treatments before arriving at the hospital make the GCS untestable in 44% of patients (Bullock et al. 1995). Another test involves papillary light reflex which may indicate damage to structures in the temporal lobe essential for cognitive function (Sakas et al. 1995). However, direct oculomotor trauma may confound these results (Bullock et al. 1995). Computer tomography (CT) scanning is performed on patients with TBI for the purpose of intracranial pressure monitoring and prognostic significance. Basal cistern injury, hemorrhage, midline shifts, and intracranial lesions can easily be identified by CT scan. However, the cost, speed and availability of the scan are limiting factors in their prognostic value (Levi et al. 1990; Kesler et al. 2000). In fact, despite the prediction of a “good recovery” that may be predicted by some of these tests, many TBI patients continue to display long-term impairment (Marion 1996). As a result, there has been interest in developing biochemical markers for the rapid and accurate assessment or

injury severity, prognosis and evaluation. This may be possible with the use of proteomics.

Proteomics

Proteomics can be defined as the study of global protein expression, structure and function. It is a direct extension of genomics, which is the study of genes, and sheds light on the biochemical interaction of cells with their environment. Of particular interest in proteomics is determining levels of protein expression, how they differ from one organism to the next under different conditions or within the same organism in different body regions or over time. Protein separation and quantification are used to identify specific players during pathological processes.

One goal of proteomics is to identify “biomarkers”, or substances in an organism that may be used to identify some function of health or disease much like how the presence of certain pancreatic islet antibodies help to diagnose the presence of type I diabetes. The most preferable way to detect biomarkers would be via a rapid and simple blood test, although spinal taps and tissue biopsies may be needed for more accurate identification. Additionally, using proteomics to understand the mechanism behind physiologic interactions will help scientists to develop therapies to target or mimic them, thereby treating the disease.

With respect to neurotoxicity, necrosis and apoptosis have been identified to be important cell death processes. Using proteomics to understand their causes and monitoring their presence are important for identifying and treating disease of the brain.

Necrosis versus Apoptosis

Necrosis is a form of accidental or pathological cell death in which the cell plays a less central role on the continuum of cell death. It is most likely to occur when cells

incur conditions such as hyperthermia, toxins, or direct trauma (Willie et al. 1980). Cells are characterized by irreversible swelling of the cytoplasm, massive ion influx and distortion of mitochondria and other organelles. Typically they lyse, releasing their cellular contents and invoking an inflammatory response (Lockshin and Zakeri, 2001).

Apoptosis, first studied in *C. Elegans*, is a form of programmed cell death. It has been conserved evolutionarily to dispose of excess or harmful cells, regulate development, initiate immune responses and maintain homeostasis (Kerr et al. 1972; Ameisen 2002). An example of this is the deletion of cells in the hand stump of a developing fetus for the purpose of producing five distinguishable fingers instead of one (Jacobson et al. 1997). The program has been remarkably conserved from nematodes to humans, suggesting the existence of “core death machinery” in most, if not all, cells (Metzstein et al. 1998). Once apoptosis is initiated, chromosomes condense and move to the periphery. Then, cells shrink in size as the plasma membrane begins to bleb. These contents are then engulfed and digested by neighboring phagocytes or cells (Jacobson et al. 1997). Many biological pathologies can be tied to errors in apoptosis, for instance an absent signal in which cells accumulate or an over expressed signal in which cells are eradicated or division impaired. The calpain and caspase families of proteases act to up regulate necrosis and apoptosis, respectively (Wang 2000; Launay et al. 2005).

Calpains

Calpains are ubiquitous calcium activated cytosolic cysteine proteases with complicated mechanisms regulating their powerful necrotic actions (Banik et al. 1997). Over 14 calpains have been discovered since 1960 (Guroff 1964). Two isoforms, the calpain-1 and calpain-2, are activated by micromolar and millimolar calcium concentrations respectively and are normally involved in membrane fusion, long term

potentiation, platelet activation and cell cycle progression (Carafoli and Molinari 1998; Azam 2001). Activated calpains autolyse and move from the cytosol across the cell membrane (Suzuki et al. 2004). Calpains then have the ability to digest many cytoskeletal substrates (Croall and DeMartino 1991), but are normally inhibited by endogenous calpastatins in the absence of calcium ion influx following injury. A smaller role in apoptosis has also been implicated due to membrane rupture and cathepsin leakage, activating pro-apoptotic caspases (Nath et al. 1996; Yamahsima 2000; Nakagawa and Yuan 2000).

In the general course of protease studies, several inhibitors have been developed to suppress the effects of these enzymes. These tend to react with the cysteine in the catalytic triad in a nonspecific manner (Yuen and Wang 1998) or inhibit by interacting with the calmodulin domain (Shiraha et al. 2002). For example, N-(4-fluorophenylsulfonyl)-L valyl-L-leucinal (SJA6017 or SJA) has been found to act as a general calpain inhibitor in cell culture and *in vivo* by reversibly binding to the active site of calpain-1 (Fukiage et al. 1997; Kupina et al. 2001).

Caspases

Unlike calpains, the pro-apoptotic intracellular cysteine protease caspases are not calcium activated. Normally, caspases reside as inactive zymogens within the cells, requiring pro-domain cleavage to become activated (Springer et al. 1999). Once active, caspase-3, the most abundant member of the caspase family, appears to play the main role in apoptosis in the brain (Chan and Mattson 1999; Slee et al. 2001). Other roles, among over 250 cellular substrates identified to date, include cytokine processing and inflammation (Fischer et al. 2003; Thornberry and Lazebnik 1998). Targets of active caspase-3 enzymes include structural proteins (discussed later), mitochondria, signal

transduction and stress response proteins (Cohen 1997; Chan and Mattson 1999; Earnshaw et al. 1999). Furthermore, digestion of DNA repair proteins prevents recovery and promotes further DNA condensation and fragmentation, driving apoptosis to completion (Woo et al. 1998).

Three general pathways of apoptosis (Figure 1-2) are described involving caspases: the extrinsic, intrinsic and ER pathways (Kaufmann and Hengartner 2001). The extrinsic pathway begins when a transmembrane death receptor is contacted by apoptosis-related ligands such as FAS-L or TNF- α . This results in the mobilization of procaspase-8 or 10 and the activation of the proteolytic caspase-3 or the intrinsic pathway (Hengartner 2000). The intrinsic pathway normally involves mitochondrial release of cytochrome c into the cytosol. This results in the assembly of the apoptosome macromolecule (including Apaf-1, cytochrome c, procaspase-9 and ATP) and the activation of caspase-9. Ultimately, caspase-3 is again involved along with other executioner caspases (Hengartner 2000). The ER stress pathway involves caspase-7 activating caspase-12 independent from intrinsic or extrinsic pathways (Rao et al. 2001; Nakagawa et al. 2000).

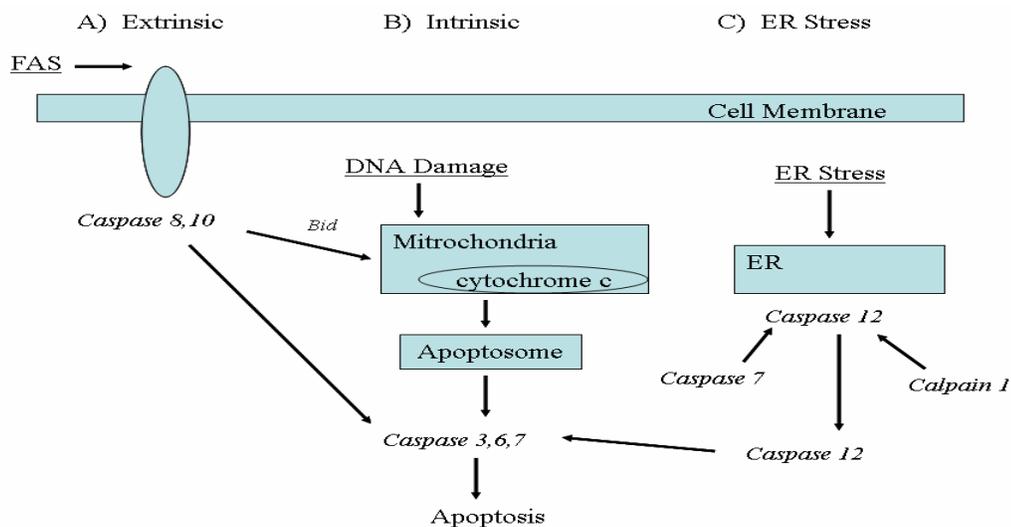


Figure 1-2. Three caspase-associated pathways leading to apoptosis. A) Extrinsic. B) Intrinsic. C) ER Stress.

The interaction between calpains and caspases is complex. Interestingly, active calpains have been shown to inactivate caspase-3 and encourage necrosis at the core of injured sites (Chua 2000). However, they have also been shown to active caspase-12 directly in the ER pathway (Nakagawa and Yuan, 2000).

Similar to calpains, caspase inhibitors have been used to understand injury cascades. For example, benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone (z-VAD-FMK or ZVAD) and carbobenzoxy-Asp-CH₂-OC (O)-2,6-dichlorobenzene (Z-D-DCB or ZD) have been shown to act as general caspase inhibitors (Komoriya et al. 2000; Mashima et al. 1995). The pan-caspase inhibitor ZVAD has been shown to increase the survival of marrow cells after middle cerebral artery occlusion, decreasing apoptosis (Chen et al. 2002). The caspase-3 inhibitor ZD has been shown to reduce TBI related DNA fragmentation (Yakovlev et al. 1997).

Degradation of Structural Proteins

More specifically, increases in calpains and caspases have been shown to be involved with the degradation of two important structural proteins, the cytoskeletal α II-spectrin and the microtubule associate tau (Wang 2000), producing potential biomarkers of toxicity.

α II-Spectrin

Spectrin, also known as fodrin, is an essential component of the cell membrane and cytosolic skeleton. It was first discovered in human erythrocytes in 1968 and was confirmed in nonerythroid cells a decade later (Marchesi and Steers 1968; Goodman et al. 1981). Spectrin is actually an antiparallel heterodimer, however it exists as a tetramer formed by two head-to-head interactions *in vivo* (Shotton et al. 1979). Two genes have been discovered to encode the alpha subunits of spectrin, notably alpha-II which is

known as “brain spectrin” and located mainly in axonal membranes, playing a crucial role in synaptic transmission (Sikorski et al. 1991).

Calpain activation and the presence of calpain-produced α II-spectrin breakdown products (SBDPs) at 150 kDa and 145 kDa are associated with oncotic/necrotic neuronal cell damage and to a lesser extent apoptosis (Siman et al. 1984; Nath et al. 1996; Wang 1998; Wang 2000). Specifically, spectrin is cleaved between the Tyr 1176 and Gly 1177 peptides on the eleventh alpha spectrin repeat near the calmodulin-binding domain, producing two fragments nearly identical in size around 150 kDa. This fragment is then cleaved again by calpain, producing a 145 kDa BDP (Harris et al. 1989). Recently, we have produced antibodies specific to the 150 and 145 kDa calpain-cleaved α II-spectrin protein which can aid in understanding their relative contributions to proteolytic patterns of injury (Roberts-Lewis et al. 1994; Barh et al. 1995; Dutta et al. 2002). Alternatively, caspase-3 overactivation and the presence of caspase-3 products at 149 kDa (sometimes called 150i) and 120 kDa are exclusively associated with apoptotic neuronal death (Nath et al. 1996; Wang 2000). We have also produced antibodies specific to the 120 kDa caspase-cleaved α II-spectrin protein (Nath et al. 2000).

Tau

Tau is a major microtubule-binding protein which is highly enriched in the dendrite portions of neurons. It interacts with tubulin to stabilize and promote microtubule assembly. Phosphorylation of tau results in a disruption of microtubule organization (Taniguchi et al. 2001). Hyperphosphorylation, however, is involved with self-assembly into plaques and associated with tauopathies and Alzheimer’s disease (Alonso et al. 2001).

Like α II-spectrin, tau is also vulnerable to cleavage by both calpains and caspase-3 and results in the production of breakdown products (TBDPs) (Wallace et al. 2003). Tau digestion with calpain yields multiple fragments of about 30-40 kDa in an indistinguishable wide band and a number of more specific smaller fragments (Yang and Ksiezak-Reding 1995; Yen et al. 1999). For example, breakdown products have been seen at 17 kDa after beta-amyloid peptide treatment (Park and Ferreira 2005). Alternatively, tau cleavage by caspase-3 has also been identified in neuronal cells undergoing apoptosis in cerebellar granule neurons (Canu et al. 1998) in culture and in Alzheimer's diseased brains (Rohn et al. 2002). These produce a fragment around 45 kDa (Chung et al. 2001).

By examining the presence of various α II-spectrin and tau fragments in brain regions, one can detect the presence of neuronal toxicity and identify the mechanisms leading to neuronal loss, including whether it is calpain- and caspase-mediated proteolysis (Figure 1-3).

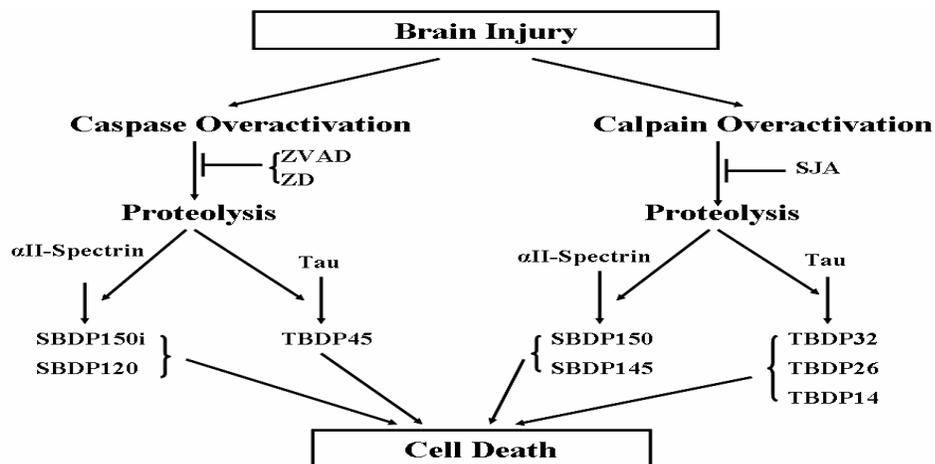


Figure 1-3. Proteolytic caspase and calpain activation following insult and inhibition.¹

¹ Figure 1-3 reprinted from Life Sciences, Vol. 78, Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KKW, Concurrent calpain and caspase-3 mediated proteolysis of α II-spectrin and tau in rat brain after methamphetamine exposure: A similar profile to traumatic brain injury, Pages 301-309, 2005, with permission from Elsevier.

Use of Proteomics in Traumatic Brain Injury

The presence of rapid, definitive biomarker tests to help identify and quantify the extent of neurological pathology would be of great help in the diagnosing and treating TBI. These biomarkers should be readily accessible in biological tissue and CSF or blood, released in a time-locked sequence and have high sensitivity and specificity (Pineda et al. 2004). Accurate information from the biomarkers would help health care professionals determine patient management decisions by assisting conventional brain radiography. The same markers would also provide assistance for major research projects studying mechanism of action or pharmacological treatment. Finally, they could be used as outcome measures in clinical trials.

Because TBI triggers both the necrotic and apoptotic processes (Conti et al. 1998; Clark et al. 2000; Yakovlev and Faden 2001), there has been increasing research into developing calpain and caspase related proteomic biomarkers and treatments for TBI (Pineda et al. 2004). Shortly after a TBI, directly damaged cells begin showing evidence of necrosis including swelling, organelle breakdown and ruptured plasma membranes (Sutton et al. 1993; Dietrich 1994; Denecker et al. 2001). Later, neurons surrounding the immediate injury site begin to also show signs of apoptosis including cell shrinkage, nuclear condensation and blebbing (Conti et al. 1998; Newcomb et al. 1999). In brief, TBI results in the increased activation of the two protease systems: the pro-necrotic calpains and the pro-apoptotic caspase (Yuen and Wang 1998; Nicholson and Thornberry 1997; Wang 2000). Necrosis- or oncosis-linked, calpain-generated SBDP150 and SBDP145 appear to be predominantly observed in brain tissues after early TBI in rats and in humans (Pike et al. 1998; Pike et al. 2001). Smaller amounts of apoptosis-linked, caspase-generated SBDP120 have been reported in certain brain regions (e.g. thalamus)

after TBI (Raghupathi et al. 2000). The same has been seen following ischemic brain injury (Pettigrew et al. 1996) and in PC-12 cells challenged by ceramide (Xie and Johnson 1997). However, both calpain and caspase inhibitors have been shown to decrease neurotoxicity levels following TBI (Kupina et al. 2001; Buki et al. 2003; Knoblach et al. 2004).

Thus, calpain and caspase cleaved spectrin and tau biomarkers are proving useful in the study and management of TBI and its patient population. The same concepts may be applicable to other injuries involving the same proteolytic systems in the brain.

Potential Use of Proteomics in Drug Abuse

Neurotoxicity biomarkers

Currently, no diagnostic or pharmacological therapy exists to diagnose or treat acute Meth or MDMA neurotoxicity (Huber et al. 1997; Martin et al. 2003). The brain derived neurochemical bioamines, dopamine, serotonin and their metabolites (DOPAC and 5-HIAA) are used as blood biomarkers for MDMA neurotoxicity. However, such an approach is nonspecific and does not correlate well with the severity of the brain neurotoxicity (Ali et al. 1991). Similarly, in the case of Meth abuse, blood derived myoglobin is used as a biomarker, also a nonspecific indicator of toxicity, but is more indicative of Meth induced renal failure (Ishigami et al. 2003). Employing traditional biochemical and immunological methods to identify specific biomarkers represents a major challenge due to cost and the need for prior knowledge of the insult mechanisms involved.

Our work aims to characterize neurotoxicity of club drug use by showing that the same proteolytic markers in used TBI studies are present in the rat cortex and hippocampus following acute overdose of Meth or MDMA. The dosages of club drugs

used here are known to be neurotoxic in animals and are squarely in the range of dosages typically used by recreational Meth users after taking into consideration interspecies scaling (Deng et al. 2002b). Although research literature thus far has strongly supported an apoptosis-like cell injury mechanism with Meth neurotoxicity (Deng et al. 2002b), this is in contrast to the mode of cell death in TBI, which has a mixture of oncosis and apoptosis (Pike et al. 1998; Raghupathi et al. 2000), with the former being the predominant mode of cell death. We hypothesize that in the case of Meth or MDMA exposure, brain tissue such as cortex or hippocampus will have detectable levels of SBDPs and TBDPs comparable to that of TBI and that the form(s) of cell death can be examined using the previously mentioned cell death specific biomarkers. This will allow us to characterize the induced cell toxicity as calpain- or caspase-induced and make a direct comparison to that of brain trauma.

Neuroendocrine biomarkers

To study the neuroendocrine toxicity of club drugs and their wasting effect, a number of important hormones have been identified that may prove useful as indices. For example, the physiological interactions between the metabolic hormones leptin and ghrelin have been shown to affect food intake and energy regulation. Leptin is produced mainly by adipose tissue in quantities that are proportional to the amount of adipose tissue in the body. Leptin acts to decrease appetite and increase metabolism by the following mechanisms: (1) stimulating proopiomelanocortin (POMC)/cocaine and (2) amphetamine-regulated transcript (CART) neurons, which suppress food intake, and (3) inhibiting NPY/ Agouti gene-related protein (AgRP) neurons, which stimulate food intake (Kalra and Kalra 2004; Nogueiras et al. 2004; Hosada et al. 2002). Ghrelin, which is principally secreted by the stomach, increases appetite and decreases metabolism.

Ghrelin acts by activating NPY/AgRP neurons, stimulating the release of growth hormone, which stimulates feeding and weight gain (Nogueiras et al. 2004). Research has shown that both leptin and ghrelin hormones interact to mediate appetite (Nogueiras et al. 2004; Hosada et al. 2002). Growth hormone (GH) increases feeding behavior by interactions with hypothalamic pathways that are involved in appetite regulation and energy balance (Egecioglu et al. 2005). Research has demonstrated that GH may mediate its orexigenic effects by interacting with GH receptors on NPY neurons in the arcuate nucleus. This interaction results in a subsequent increase in the production of NPY, leading to a strong appetite-stimulating response (Chan et al. 1996). The four molecules interact together to control appetite (Figure 1-4).

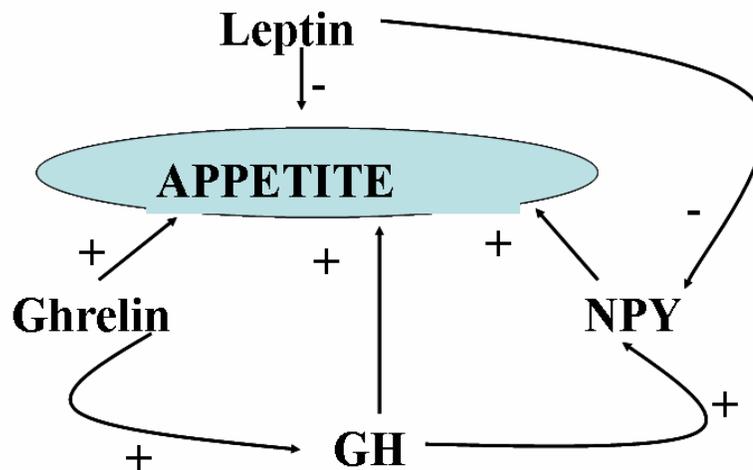


Figure 1-4. Interaction of satiety hormones with appetite.

Ad libitum-fed animals administered Meth have increased serum ghrelin, decreased serum leptin, and increased NPY expression in the arcuate and dorsomedial nuclei compared to saline controls (Crowley et al. 2005). However, with respect to NPY, other research has shown decreased NPY expression subsequent to drug administration (Hseigh et al. 2005; Curzon et al. 1997). These studies suggest that psychostimulant administration may have an effect on metabolic processes involved in weight

maintenance by altering the concentrations of hormones involved in appetite regulation.

Thus, our work also examines of the effects of acute MDMA and Meth administration on the serum concentrations of leptin, ghrelin, growth hormone and NPY to determine if changes in these hormones occur and correlate with weight loss. This has the potential to explain, predict or monitor anorexia resulting from drug use.

CHAPTER 2 METHODS

Animal Treatment

All experiments were performed using male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) that were aged 60 days and weighed between 240 to 270 g. Animals were housed in groups of two per cage and maintained on a 12 h light/dark cycle (lights on 7 AM - 7 PM). Food and water were available *ad libitum*. Appropriate pre- and post-injury management guidelines were maintained and these measures complied with all guidelines set forth by the University of Florida (UF) Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health (NIH) guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*.

Administration of Traumatic Brain Injury

A cortical impact injury device was used to produce traumatic brain injury (TBI) in adult rats (Dixon et al. 1991; Pike et al. 1998). Cortical impact TBI results in cortical deformation within the vicinity of the impactor tip associated with contusion and neuronal and axonal damage largely confined to the hemisphere ipsilateral to the site of injury. Rats were anesthetized with 4% isoflurane (Halocarbon Laboratories) in a carrier gas of 1:1 O₂:N₂O (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues reflected and a unilateral (ipsilateral to the side of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between the bregma and lambda.

The dura mater was kept intact over the cortex. Brain trauma was produced by impacting the right cortex (ipsilateral cortex) with a 5-mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 1.6 mm compression and 150 ms dwell time pressure (compressed N₂) supplied to the pneumatic cylinder, and dwell time were measured by a linear velocity displacement transducer (model 500 HR; Lucas Shaevitz™) that produces an analog signal recorded by a storage-trace oscilloscope (model 2522B; BK Precision). Sham-injured (craniotomy-injured) animals underwent identical surgical procedures but did not receive the impact injury. Naïve animals received no surgery or injury.

Drug Administration

Pharmacologic agents (±)-3,4-Methylenedioxymethamphetamine hydrochloride (MDMA) and (+)-Methamphetamine hydrochloride (Meth) were obtained (Sigma). The drugs were dissolved in 0.9% saline and injected intraperitoneally (IP) at dosage of 5, 10, 20, or 40 mg/kg. For the 20 and 40 mg/kg dosages, 10 mg/kg of drug was administered at once and repeated each hour to achieve the desired dose over time to prevent lethal hyperthermia. Saline was injected at a bolus of 0.3 cc. Animal weight was recorded at 3, 6, 12, 24, and 48 h post final injection.

Cortical Culture Preparations

Primary Cerebrocortical Culture

All cultures were prepared with four replicated wells for each condition. Cerebrocortical cells harvested from a homogenized pool of ten 1-day old Sprague-Dawley rat brains were plated on poly-L-lysine coated 6-well culture plates (Erie Scientific) as previously described (Nath et al. 1998) at a density of 3×10^6 cells/mL. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10%

horse serum in a humidified incubator in an atmosphere of 10% CO₂ at 37°C. After 3 days, the DMEM solution was replaced with DMEM / 10% horse serum containing 1% cytosine arabinoside (ARC). Two days later, the solution was replaced with DMEM/10% horse serum. The cells were cultured an additional 10 days before use.

Neurotoxin Challenges and Pharmacologic Intervention

In addition to untreated controls, the following conditions were used: N-methyl-D-aspartic acid (NMDA) as a positive control for excitotoxicity (300 µM; Sigma), MDMA (1 mM and 2 mM; Sigma), and Meth (1 mM and 2 mM; Sigma) for 24 or 48 h. For pharmacologic intervention, additional cultures were pretreated 1 hour before MDMA or Meth (2 mM) challenge with 30 µM of the calpain inhibitor SJA6017 (Calbiochem) or caspase inhibitors z-VAD-fmk (Bachem) or Z-D-DCB (Bachem) and collected after 48 h. Additional cultures were prepared combining SJA with either z-VAD-fmk or Z-D-DCB.

Quantification of Cell Injury or Death

Lactate dehydrogenase (LDH) release was measured using the commercial kit CytoToxicity 96 (Promega). The kit measures the reduction of nicotinamide adenine dinucleotide (NAD), indicating that primary necrosis and/or secondary necrosis following apoptosis has occurred (Nath et al. 1996; Posmantur et al. 1998).

Cell Lysate Collection and Preparation

The primary neuronal cell culture cells were collected and lysed during a 90 min incubation at 4°C in lysis buffer consisting of 50 mM Tris (pH 7.4), 5 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), and 1x protease inhibitor cocktail (Roche Biochemicals). The cell lysates were then centrifuged at 8000 g for 5 min at 4°C to clear and remove insoluble debris. The supernatant was snap-frozen and stored at -80°C until use.

Western Blot

Tissue Collection

After the appropriate time point, animals were briefly anaesthetized and immediately killed by decapitation. Brains and livers were immediately removed, rinsed with ice cold PBS and entire brain regions (cerebral cortex and hippocampus) were rapidly dissected, snap-frozen in liquid nitrogen, and frozen at -80°C until used. For Western blot analysis, the brain samples were pulverized with a small mortar-pastel set over dry ice to a fine powder. The pulverized brain tissue powder was then lysed for 90 min at 4°C with 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, 1x protease inhibitor cocktail. The brain lysates were then centrifuged at 8000 g for 5 min at 4°C to clear and remove insoluble debris, snap-frozen and stored at -80°C until used. Cell culture positive controls were also used including cortical and hippocampal neurons treated with necrosis-inducing maitotoxin (MTX) (Wang et al. 1996) or apoptosis-inducing staurosporine (STS) (Nath et al. 1996).

Gel Electrophoresis and Electrotransfer

Protein concentrations of tissue lysates were determined by bicinchoninic acid microprotein assays (Pierce Inc.) with albumin standards. Protein balanced samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in two-fold loading buffer containing 0.25 M Tris (pH 6.8), 0.1 mM DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O . Samples were heated for 10 min at 100°C and centrifuged for 2 min at 8000 rpm in a microcentrifuge at ambient temperatures. Twenty micrograms of protein per lane were routinely resolved by SDS-PAGE on Tris/glycine gels (6.5% for spectrin, 10-20% for tau) for 2 h at 200 V. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene

fluoride (PVDF) membranes in a transfer buffer containing 0.192 M glycine and 0.025 M Tris (pH 8.3) 10% methanol at a constant voltage of 100 V for 1 h at 4°C. Blots were blocked for 1 h at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-20. Gels were stained with Ponceau Red (Sigma) to confirm successful transfer of protein and to insure that an equal amount of protein was loaded in each lane.

Immunoblot Analyses of Samples

Blotting membranes containing tissue protein were probed with a primary antibody (mouse monoclonal or rabbit polyclonal) as needed by incubating blots overnight at 4°C in tris-buffered saline with Tween (TBST) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl + 0.05 % Tween 20) with 5% non-fat milk. Primary antibodies included monoclonal anti- α II-spectrin (SBDPs) (Amersham), polyclonal anti-SBDP150 for calpain, polyclonal anti-SBDP145 for calpain, polyclonal anti-SBDP120 for caspase, monoclonal anti-tau (TBDPs) (Cedarlane), polyclonal anti-TBDP45, or monoclonal anti-NF-200. After extensive washing with TBST, the blots were probed with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:5000 dilution)). Following three washes with TBST and 2 h incubation at room temperature with a biotinylated secondary antibody (Amersham), the membrane was incubated for 1 h with Streptavidin-conjugated alkaline phosphatase. Finally, colorimetric development was performed with the one-step BCIP-reagent (KPL). The molecular weights of the intact proteins and their potential breakdown products were measured using rainbow colored molecular weight standards (Amersham). Semi-quantitative evaluation of protein levels was performed via computer-assisted densitometric scanning (NIH ImageJ software). β -actin was displayed to show loading equivalence.

Immunohistochemistry

At the appropriate time point, the animals were anesthetized using 4% isoflurane in a carrier gas of 1:1 O₂/N₂O (4 min), subsequently perfused with 0.9% saline (pH 7.4) followed by 400 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (fix) and then killed by decapitation and brains were removed. A total of 2 h in fix was followed by storage in cryoprotection buffer. Vibratome-cut 40 μM sections were fluorescently immunolabeled with cell type-specific monoclonal antibodies, proteolytic specific polyclonal antibodies and a nuclear counterstain. Briefly, tissue sections were rinsed in phosphate buffered saline (PBS) then incubated for 1 h at room temperature in 2% goat serum/2% horse serume/0.2% Triton-X 100 in Tris-buffered saline (TBS; block) to decrease non-specific labeling. The sections were incubated with two primary antibodies: one of the cell-type specific mouse anti-neuron specific nuclear protein antibody (neuronal nuclei-anti-NeuN) at a concentration of 1:100 (Chemicon), the mouse anti-glial acidic fibrillary protein antibody for astrocytes (anti-GFAP) at a concentration of 1:1000 (Sternberger Monoclonals), the mouse anti-myelin basic protein antibody for oligodendrocytes (anti-MBP) at a concentration of 1:1000 (Chemicon), the mouse anti-microglia-specific antibody for resting/activated microglia (anti-OX42) at a concentration of 1:1000 (Serotec, Inc.) or the mouse anti-neurofilament-200-specific protein antibody (anti-NF200) at a concentration of 1:1000 (Chemicon) and one of either the rabbit anti-calpain-1 at a concentration of 1:500, rabbit anti-SBDP145 at a concentration of 1:500, rabbit anti-caspase-3 at a concentration of 1:1000, or rabbit anti-SBDP120 at a concentration of 1:1000 for 4 days in block at 4°C. After being rinsed in PBS, the tissue sections were incubated with species-specific Alexa Fluor (Molecular Probes, Inc.) secondary antibodies, either Alexa Fluor 485 or 594, at a concentration of 1:3000 in

block for 1 h at room temperature. The sections were then washed in PBS, cover-slipped in Vectashield with 4'-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories), viewed and digitally-captured with a Zeiss Axiovert 200 microscope equipped with a Spot Real Time (RT) Slider high resolution color digital camera (Diagnostic Instruments, Inc.). Sections without secondary antibodies were similarly processed to control for binding of the primary antibodies, as were sections without primary antibodies and also sections with neither primary nor secondary antibodies. On control sections, no specific immunoreactivity was detected.

Radioimmunoassays

After the desired time periods, rats were sacrificed by decapitation and trunk blood was collected. Serum was isolated from collected blood samples by centrifugation (4,000 x g) and stored at -80°C until use. Concentrations of serum leptin, total ghrelin, growth hormone, and neuropeptide-Y (NPY) levels were determined in duplicate with radioimmunoassay (RIA) kits following the manufacturer's protocols: Leptin (RL-83K), Ghrelin (total, GHRT-89HK), and Growth Hormone (RGH-45HK) from Linco and Neuropeptide-Y (RK-049-03) from Phoenix Pharmaceuticals.

Statistical Tests

Western Blot Analyses

Quantitative evaluation of protein levels on immunoblots was performed via computer-assisted 1-dimensional densitometric scanning (Hewlett Packard Scan-Jet 6300) coupled with ImageJ software (NIH). Data were acquired in arbitrary densitometric units and transformed to percentages of the densitometric levels obtained from scans of control samples visualized on the same blots. This procedure was corrected for between-gel differences in film exposure and will allow for comparison of

density values between gels. Changes in any outcome parameter were compared to the appropriate control group. Thus, magnitude of change from control in one model system could be directly compared to magnitude of change from any other model system. Data was acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained on scans from saline administered animals visualized in the same blot. All values are given as mean +/- standard error of measurement (SEM).

Differences among means of treatment groups were compared using a one-way analysis of variance (ANOVA) test at an alpha level of 0.05. Bonferroni or Dunnett post-hoc pairwise comparison tests were used to test for differences between specific treatment groups at an alpha level of 0.05. Student's T-tests were used to compare timepoints.

Radioimmunoassays

All data presented are expressed as mean \pm SEM. Experimental data were analyzed using one-way ANOVA followed by Bonferroni post hoc pairwise comparison tests. Differences were compared among treatment groups at each time point. An alpha level of 0.05 was used to determine statistical significance.

CHAPTER 3
IN VIVO NEUROTOXICITY

Introduction

Club drugs have been shown to cause dopaminergic and, to a lesser extent, serotonergic neurite and nerve terminal degeneration of neurons in the striatum and hippocampus (Larsen et al. 2002). By examining the presence of various α II-spectrin and tau fragments in the brain, one can associate the presence of neuronal toxicity and also identify the mechanisms leading to neuronal loss, that is, calpain- and caspase-mediated proteolysis. Our pilot data has suggested that these same proteolytic markers are present in the rat cortex, hippocampus and cerebrospinal fluid following acute overdose of methamphetamine (Meth) or ecstasy (MDMA). We show that, in the case of Meth exposure, rat cortical tissue has detectable levels of spectrin and tau breakdown products (SBDPs and TBDPs) comparable to that of traumatic brain injury (TBI) (Figure 3-1).

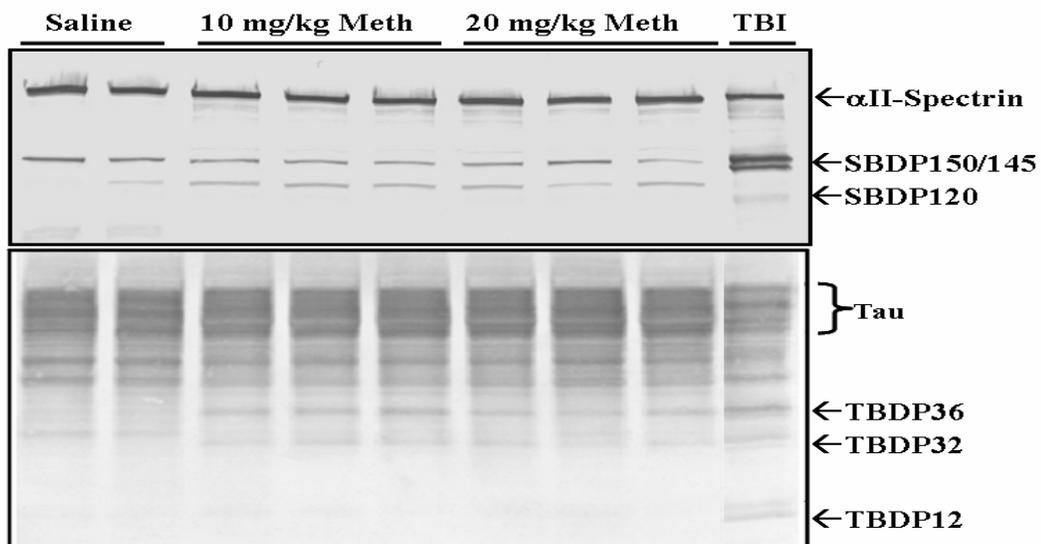


Figure 3-1. Proof of concept that spectrin and tau breakdown products are found in rat cortex 24 h after club drug use.

Immunoblots probed with total α II-spectrin antibody (upper panel) or total tau antibody (lower panel) revealed SBPDs at 150 kDa, 145 kDa and 120 kDa or TBDPs at 36 dKa, 32 kDa and 12 kDa respectively. Similar results were seen after MDMA administration and also for rat hippocampus and cerebrospinal fluid samples (data not shown). This suggests that the predominant form(s) of cell death can be examined given cell death specific biomarkers. Furthermore, this will allow us to characterize the Meth and MDMA induced cell toxicity as calpain- or caspase-induced and make a direct comparison to that of blunt trauma.

Subsequent studies using immunohistochemical (IHC) techniques further explore the role of calpain-1, caspase-3 and their respective α II-spectrin breakdown products (SBDPs) in rat cortex after club drug exposure. Activated calpain-1 and caspase-3 were found to be upregulated and co-localized with neurons after drug use. SBDPs at 145 kDa (from calpain) and 120 kDa (from caspase) were also shown to co-localize with neurons, particularly in the axonal regions. Our findings support the hypothesis of calpain and caspase mediated proteolysis of cortical neuronal proteins following club drug abuse, a finding similar to TBI.

Results

Western Blot Proteomics

Methamphetamine

Analysis of the western blots for α II-spectrin and its breakdown products in the rat cortex and hippocampus (Figures 3-2A and 3-2B, respectively) showed a strong presence of α II-spectrin parent protein in both vehicle and Meth-treated animals.

different ($p < 0.001$). A Bonferroni post-hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).

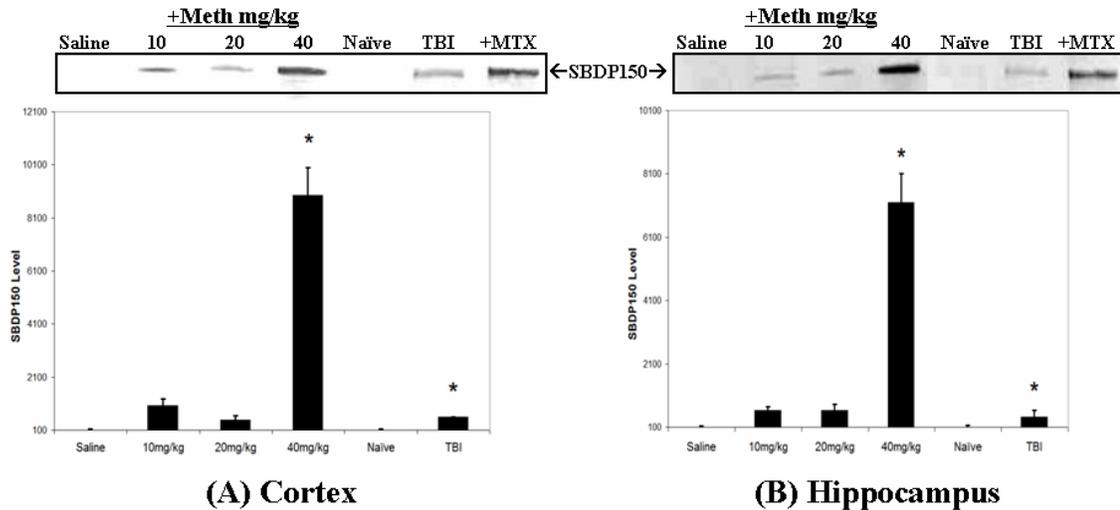


Figure 3-3. Accumulation of α II-spectrin breakdown products at 150 kDa from calpain activation in rat brain 24 h following Meth administration. For saline and Meth treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. A positive control of cells treated with maitotoxin (MTX, a calpain activator) was used for comparison. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) Cortex. B) Hippocampus.

In the hippocampus (Figure 3-3B), similar results were seen. A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post-hoc pairwise comparison test revealed that the 40 mg/kg Meth group is significantly greater than all other groups ($p < 0.05$). For both brain regions, the levels of SBDP150 were greatest for the 40 mg/kg, even more so than the TBI.

The calpain specific α II-spectrin breakdown product at 145 kDa was detected by total α II-spectrin antibody. Data similar to the 150 kDa level were produced. In the rat cortex (Figure 3-4A), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A

Bonferroni post-hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).

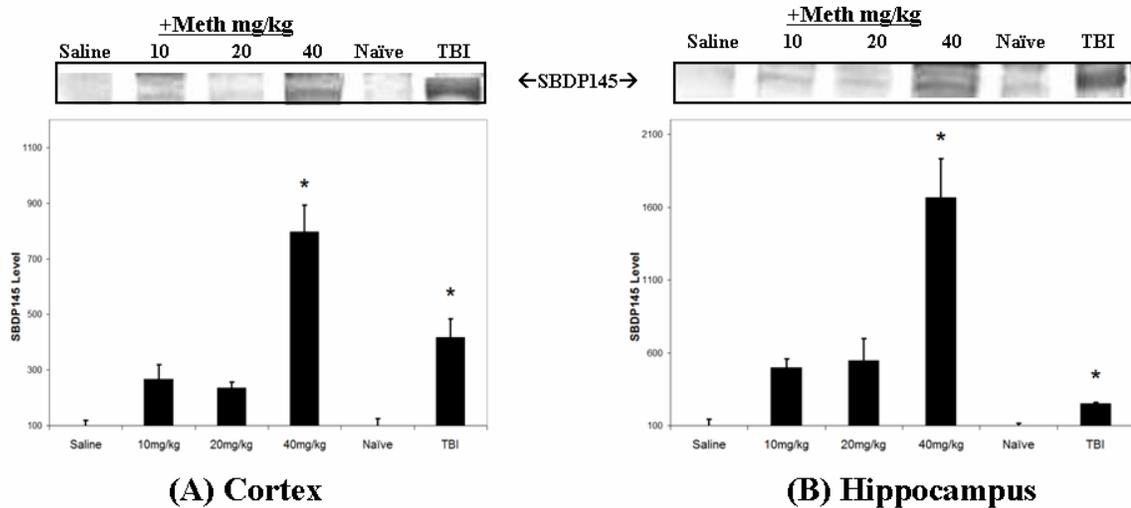


Figure 3-4. Accumulation of α II-spectrin breakdown products at 145 kDa from calpain activation in rat brain 24 h following Meth administration. For saline and Meth treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) Cortex. B) Hippocampus.

In the hippocampus (Figure 3-4B), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post-hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$). In the cortex, the level of neurotoxicity markers after 40 mg/kg of Meth were indistinguishable from those of TBI while they were increased over TBI in the hippocampus.

The caspase specific α II-spectrin breakdown product at 120 kDa, as detected by fragment-length specific antibody SBDP120, was increased after Meth administration. In the rat cortex (Figure 3-5A), evidence was insufficient to show that levels of SBDPs differed, although some difference was suggested.

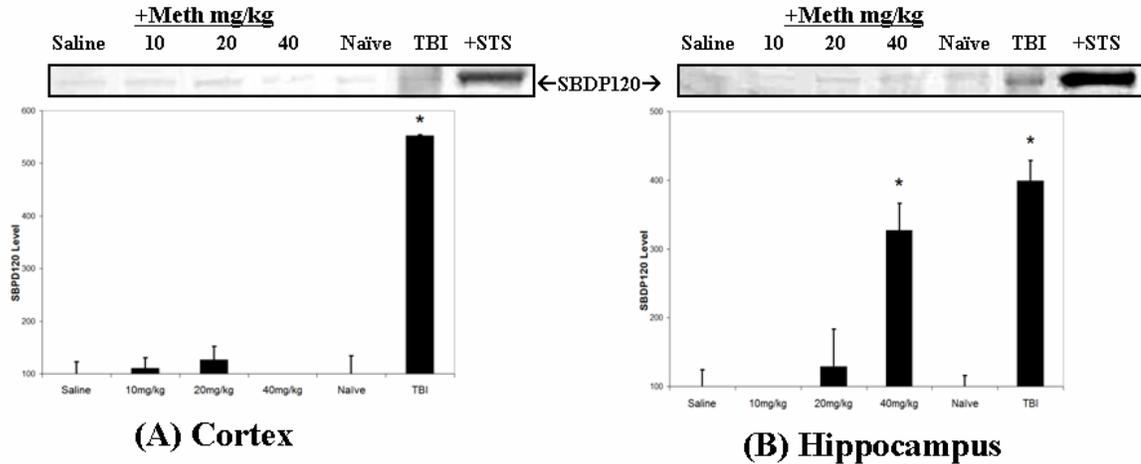
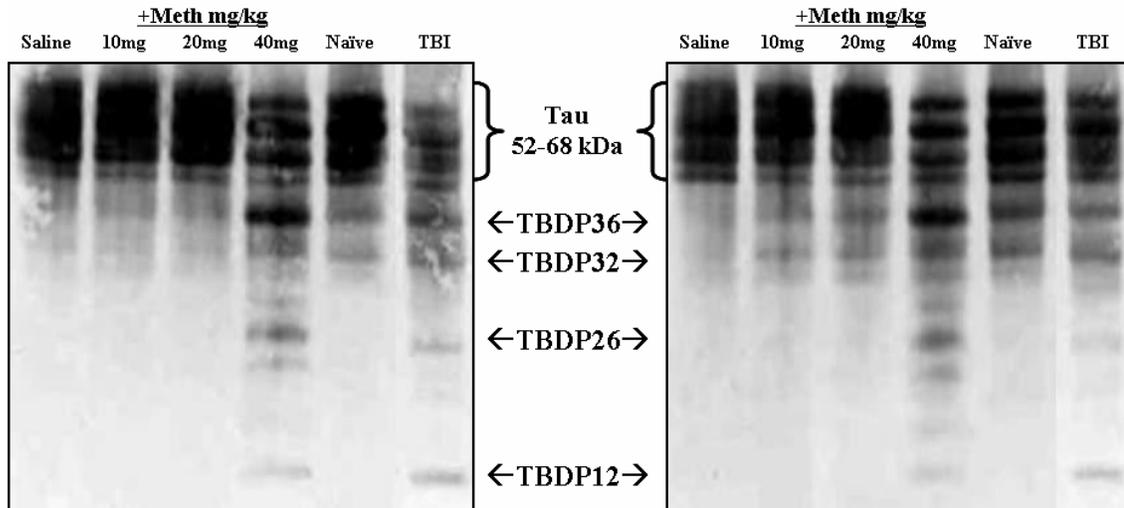


Figure 3-5. Accumulation of α II-spectrin breakdown products at 120 kDa from caspase activation 24 h following Meth administration. For saline and Meth treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. A positive control of cells treated with staurosporine (STS, a caspase activator) was used for comparison in the Caspase specific experiment. $*p<0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) Cortex. B) Hippocampus.

For the hippocampus (Figure 3-5B), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p<0.001$). A Bonferroni post-hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p<0.05$). In the hippocampus, the level of neurotoxicity markers after 40 mg/kg of Meth were indistinguishable from those of TBI.

Western blots for the tau parent protein and its breakdown products in the rat cortex and hippocampus (Figures 3-6A and 3-6B, respectively) showed presence of TBDP at 36 kDa, 32 kDa, 26 kDa, and 12 kDa.

A dose-dependent increase of BDP band intensity seemed evident and the band intensity at the highest doses appeared to be comparable to, if not greater than, that of the TBI counterparts. These TBDPs resulted from calpain-related events, as was demonstrated by cortical neuron culture treated with maitotoxin (MTX) (Figure 3-7).



(A) Cortex

(B) Hippocampus

Figure 3-6. Tau proteolysis in rat brain 24 h following Meth administration. For saline and Meth treatments, n=6. For naïve and TBI (1.6mm), n=3. Easily seen were the Tau parent protein as well as breakdown products at 36 kDa, 32 kDa, 26 kDa and 12 kDa. A) Cortex. B) Hippocampus.

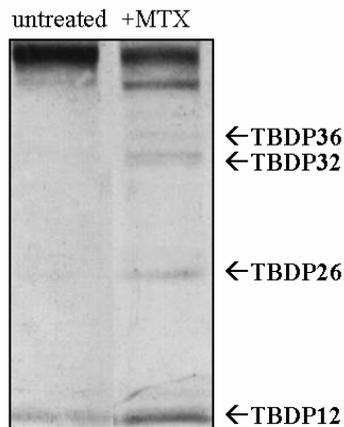


Figure 3-7. Calpain-induced tau proteolysis in rat cortical neuron cultures. Treatment with MTX activated calpains and resulted in TBDPs at 36 kDa, 32 kDa, 26 kDa, and 12 kDa.

Total tau antibody in the rat cortex (Figure 3-8) were studied with one-way ANOVA tests and revealed that means for accumulation of breakdown products among Meth treatment groups were different for three breakdown products ($p=0.057$ for 36 kDa, $p=0.010$ for 26 kDa, and $p=0.008$ for 12 kDa). Bonferroni post-hoc pairwise comparison

tests revealed that the 40 mg/kg Meth group was significantly greater than the saline group for the 36 kDa and 12 kDa BDP ($p < 0.05$) and that it was significantly greater than all other groups for the 26 kDa BDP ($p < 0.05$). In the cortex, the level of neurotoxicity markers after 40 mg/kg of Meth for TBDPs 36 kDa and 32 kDa were indistinguishable from those of TBI.

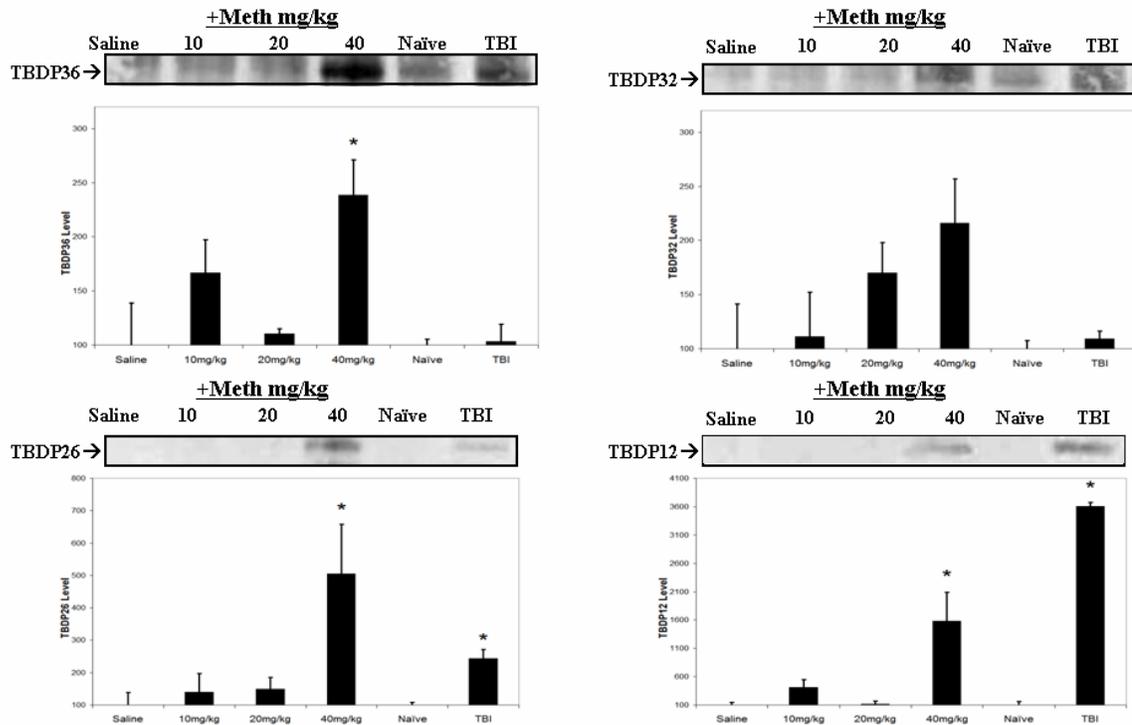


Figure 3-8. Accumulation of tau breakdown products in rat cortex 24 h following Meth administration. For saline and Meth treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control.

Total tau antibody in the hippocampus (Figure 3-9) were studied with one-way ANOVA tests and revealed that means for accumulation of breakdown products among Meth treatment groups were different for three breakdown products ($p=0.003$ for 36 kDa, $p=0.001$ for 32 kDa and $p=0.007$ for 26 kDa). Bonferroni post-hoc pairwise comparison tests revealed that the 40 mg/kg Meth group was significantly greater than the saline group for the 36 kDa, 32 kDa and 26 kDa BDP ($p < 0.05$) and it was also significantly

greater than the 10 mg/kg Meth group for the 26 kDa BDP ($p<0.05$). Bonferroni post-hoc pairwise comparison tests revealed that the 20 mg/kg Meth group was significantly greater than the saline group for the 36 kDa and 32 kDa BDP ($p<0.05$). In the hippocampus, the level of neurotoxicity markers after 40 mg/kg of Meth for TBDPs 26 kDa and 12 kDa are indistinguishable from those of TBI.

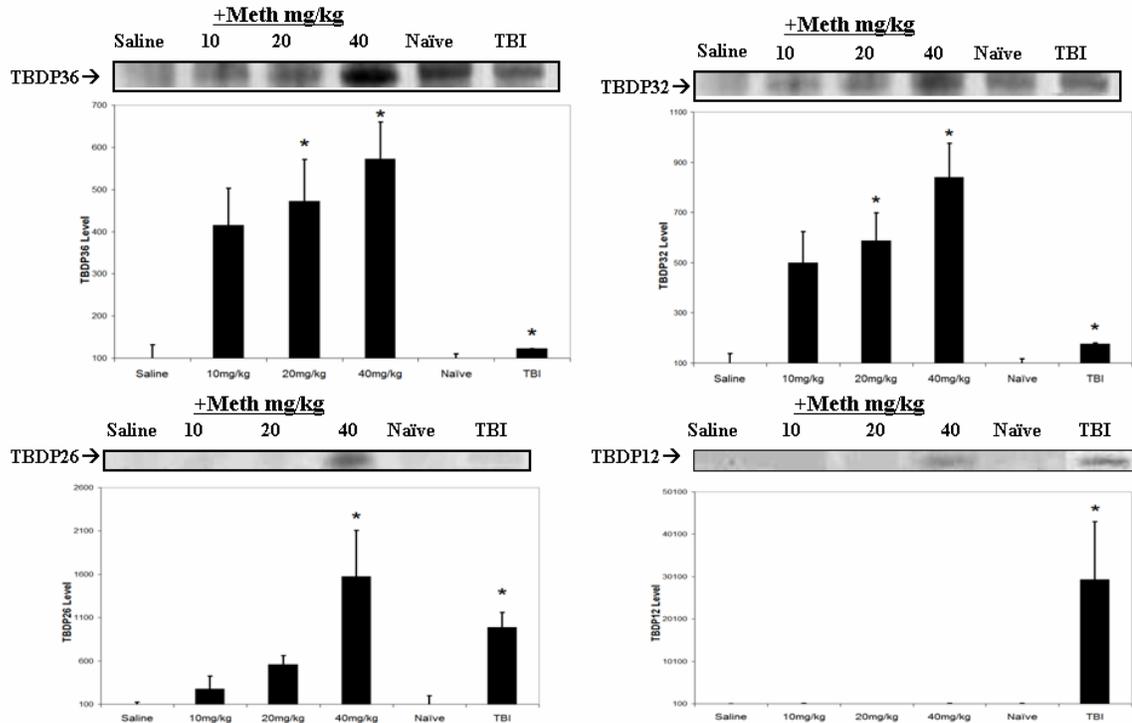


Figure 3-9. Accumulation of tau breakdown products in rat hippocampus increased 24 h following Meth administration. For saline and Meth treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. * $p<0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control.

Degradation of α II-spectrin and tau proteins as a result of 40 mg/kg Meth exposure after 72 h is evident (Figure 3-10). Breakdown products from α II-spectrin appeared to be elevated after Meth exposure whereas the tau breakdown products were not as prevalent. Quantitative analyses of the breakdown products were performed (Figure 3-11). Indeed, for the α II-spectrin (Figure 3-10A), increases were seen for the 40 mg/kg dose of Meth

over saline controls for both the pro-necrotic SBDP150 ($p < 0.01$) and also the pro-apoptotic SBDP120 ($p < 0.01$). Increases were not seen for the hippocampus.

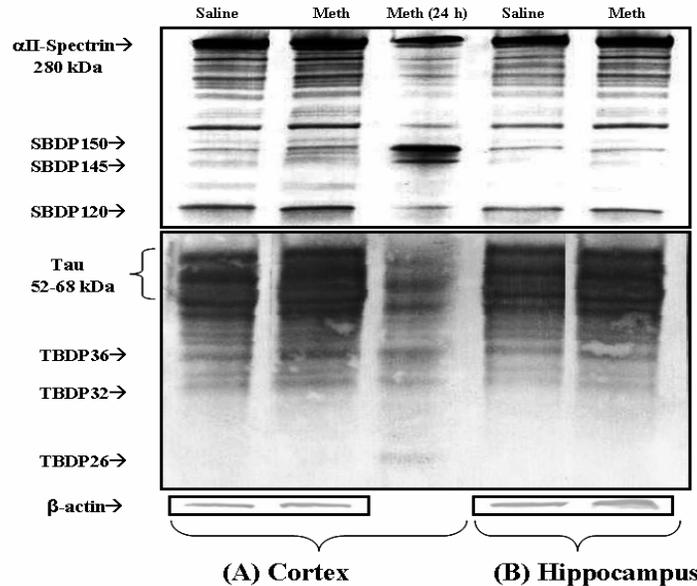


Figure 3-10. Proteolysis of α II-spectrin and tau proteins 72 h after Meth exposure.² Breakdown products of spectrin and tau were seen in the cortex and hippocampus at 150, 145 and 120 kDa for spectrin and 36, 32, and 26 kDa for tau. Breakdown products from Meth were seen with spectrin, including the pro-apoptotic 120 kDa band which was not observed at 24 h. β -actin was shown as an internal standard. A) Cortex. B) Hippocampus.

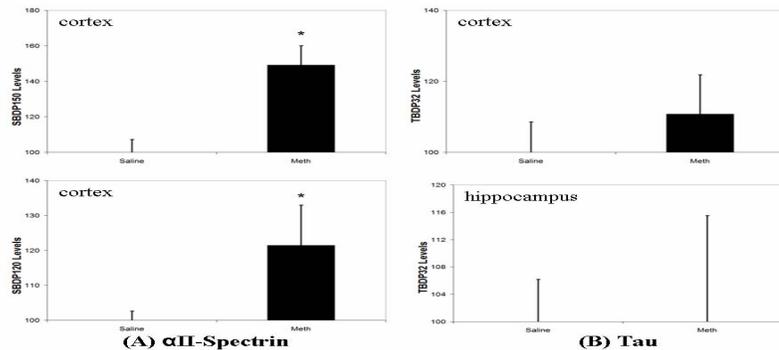


Figure 3-11. Accumulation of α II-spectrin and tau breakdown products in rat brain 72 h following Meth administration. For all treatments, $n = 6$. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) α II-Spectrin. B) Tau.

² Figures 3-10 through 3-15 reprinted from Journal of Addictive Diseases, In press, Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KKW, Ecstasy toxicity: A comparison to methamphetamine and traumatic brain injury, 2006, with permission from The Haworth Press, Binghamton NY. Article copies available from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: docdelivery@haworthpress.com ; <http://www.HaworthPress.com> .

Ecstasy

Degradation of α II-spectrin and tau proteins as a result of MDMA exposure after 24 h is evident (Figure 3-12). Breakdown products following MDMA exposure seemed very weak when compared to Meth or TBI controls.

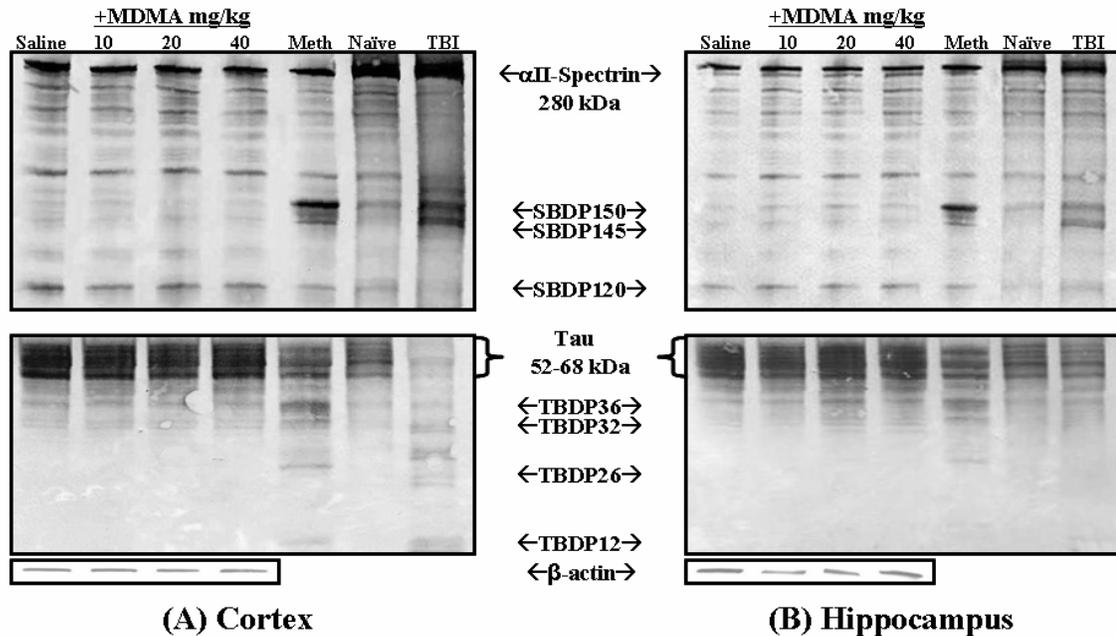


Figure 3-12. Proteolysis of α II-spectrin and tau proteins 24 h after MDMA exposure. Breakdown products of spectrin and tau were seen in the cortex and hippocampus at 150, 145 and 120 kDa for spectrin and 36, 32, 26, and 12 kDa for tau. Total concentration of breakdown products seemed very weak compared to Meth or TBI controls. β -actin was shown as an internal standard. A) Cortex. B) Hippocampus.

Quantitative analyses of the breakdown products were performed (Figure 3-13).

In the cortex (Figure 3-12A), only the pro-necrotic SBDP150 for the 10 mg/kg MDMA dose was significantly increased over saline controls ($p=0.05$). In the hippocampus (Figure 3-12B), the 20 mg/kg MDMA dose showed increases over saline controls for both pre-necrotic bands SBDP150 ($p=0.05$) and TBDP32 ($p=0.03$).

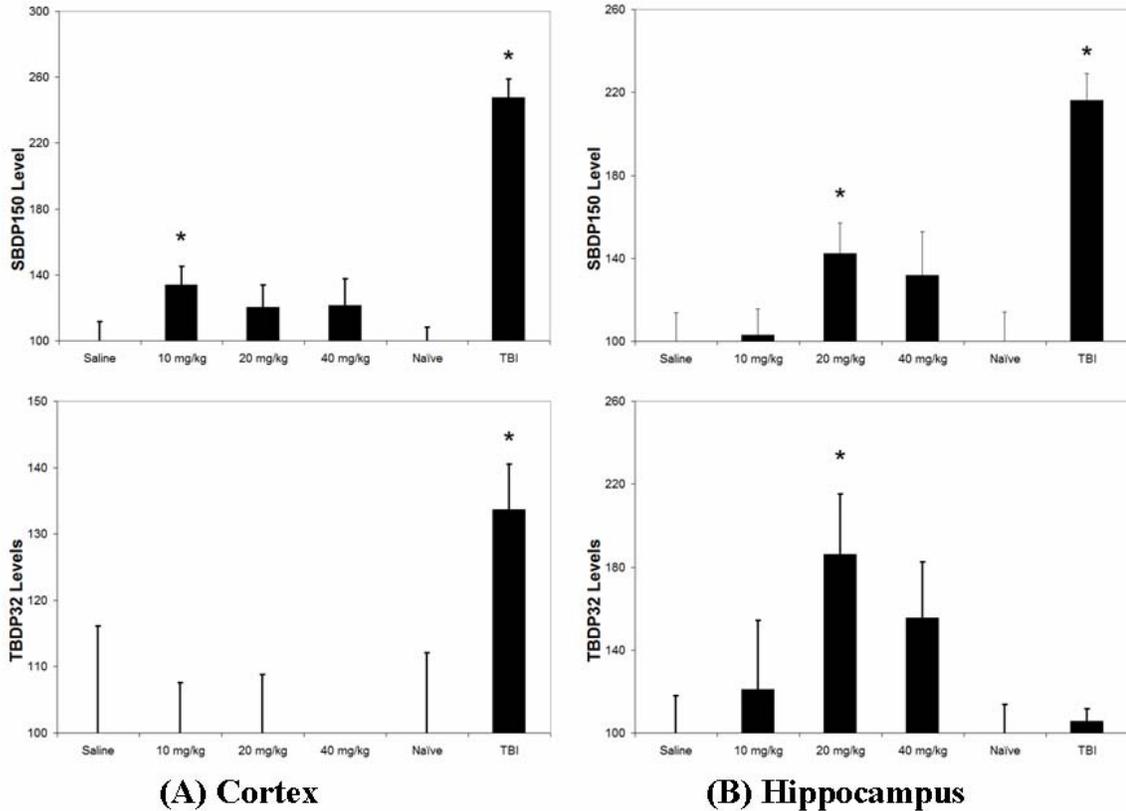


Figure 3-13. Accumulation of α II-spectrin and tau breakdown products in rat brain 24 h following MDMA administration. For saline and MDMA treatments, $n=6$. For naïve and TBI (1.6mm), $n=3$. * $p<0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) Cortex. B) Hippocampus.

Degradation of α II-spectrin and tau proteins as a result of 40 mg/kg MDMA exposure after 72 h is evident (Figure 3-14). Breakdown products from tau breakdown products were more prevalent after MDMA exposure than compared to Meth. Quantitative analyses of the breakdown products were performed (Figure 3-15). For the tau (Figure 3-14B), increases were seen after the 40 mg/kg dose of MDMA over saline controls for the pro-necrotic TBDP32 in both the cortex ($p=0.03$) and also the hippocampus ($p<0.01$).

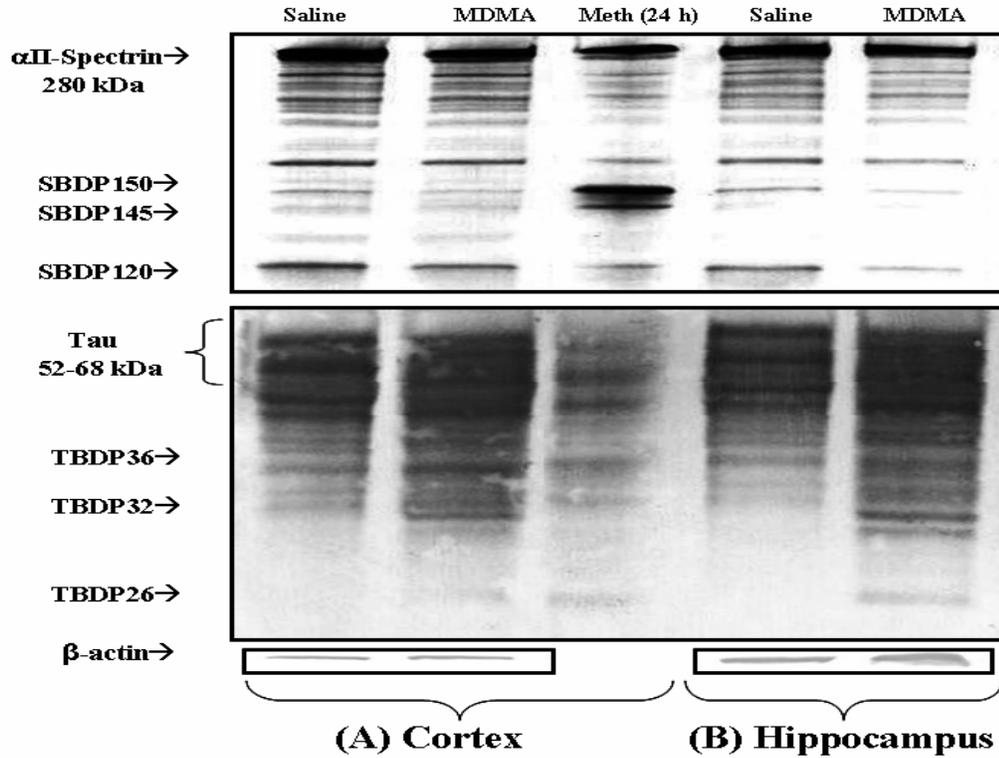


Figure 3-14. Proteolysis of α II-spectrin and tau breakdown products in rat brain 72 h following MDMA administration. For all treatments, n=6. A) Cortex. B) Hippocampus.

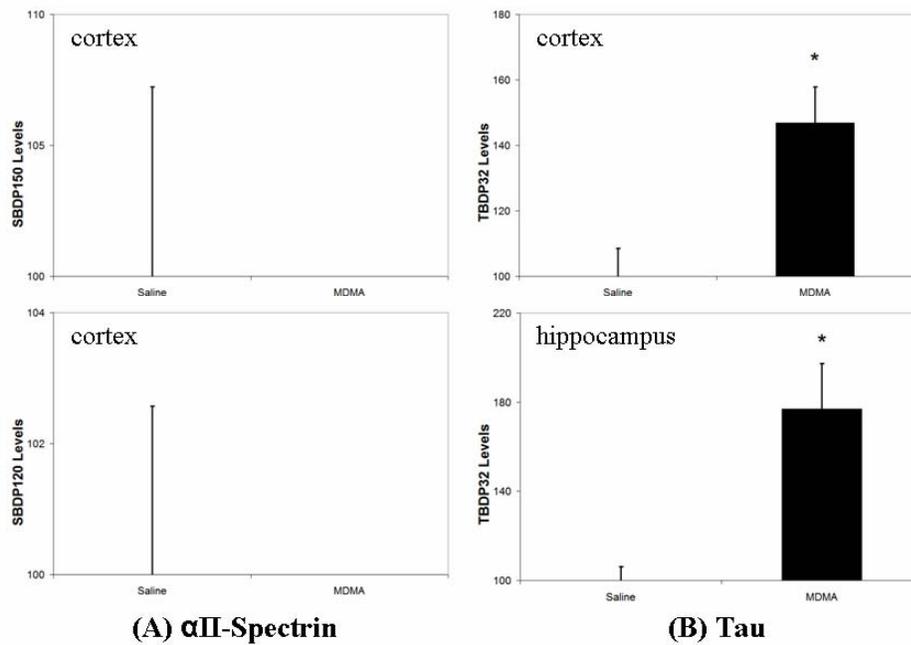


Figure 3-15. Accumulation of α II-spectrin and tau breakdown products in rat brain 72 h following MDMA administration. For all treatments, n=6. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control. A) α II-Spectrin. B) Tau.

Immunohistochemistry Proteomics

In general, proteolytic enzymes and their respective breakdown products were seen to co-localize only with neurons (NeuN). They were not seen to co-localize with astrocytes (GFAP), oligodendrocytes (MBP), or microglial (OX42) (data not shown).

Methamphetamine

The cortices of drug exposed rats revealed increases in calpain-1 expression with Meth treatment (Figure 3-16). The morphology of the brains of drug treated rats with the highest levels of calpain-1 induction had a decidedly disorganized, almost chaotic appearance when compared to saline controls. Calpain-1 immunopositive neurons, when co-localized with NeuN, included cells with apoptotic bodies and evidence of granulation.

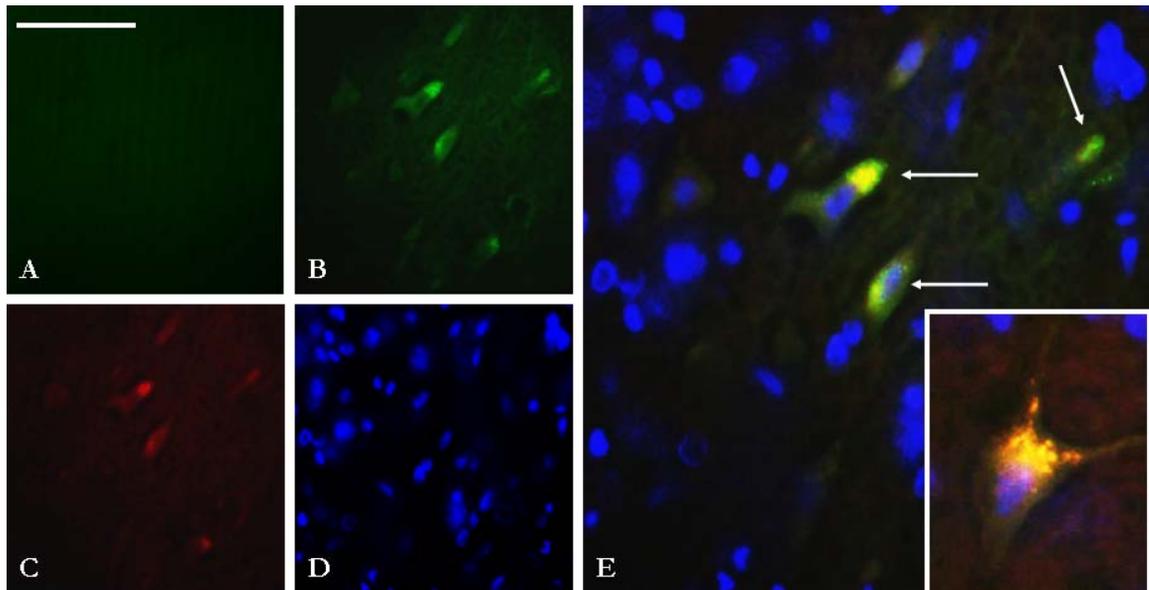


Figure 3-16. Activation of calpain-1 in neurons in the cortex 48 h after 40 mg/kg Meth exposure. Brain tissue from rats treated with saline showed no calpain-1 immunoreactivity (A). In contrast, brain tissue from rats treated with Meth revealed stronger immunopositive calpain-1 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Chromatin was indicated by DAPI staining (D). Calpain-1 co-localized in neurons with NeuN with some granulation (E, arrows, inset). Photomicrographs were at 640x; scale bar 100 μ m, calpain-1 panels A, B, E; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

Similarly, the cortices of drug exposed rats revealed marked increases in the calpain specific cleavage product SBDP145 expression (Figure 3-17). The morphology of the brains of drug treated rats with the highest levels of SBDP145 also had a decidedly disorganized appearance when compared to saline controls. Spectrin BDP145 immunopositive neurons included cells with apoptotic bodies. The network of axons could be visualized using only SBDP145 in some areas, with a “beads on a string” effect seen.

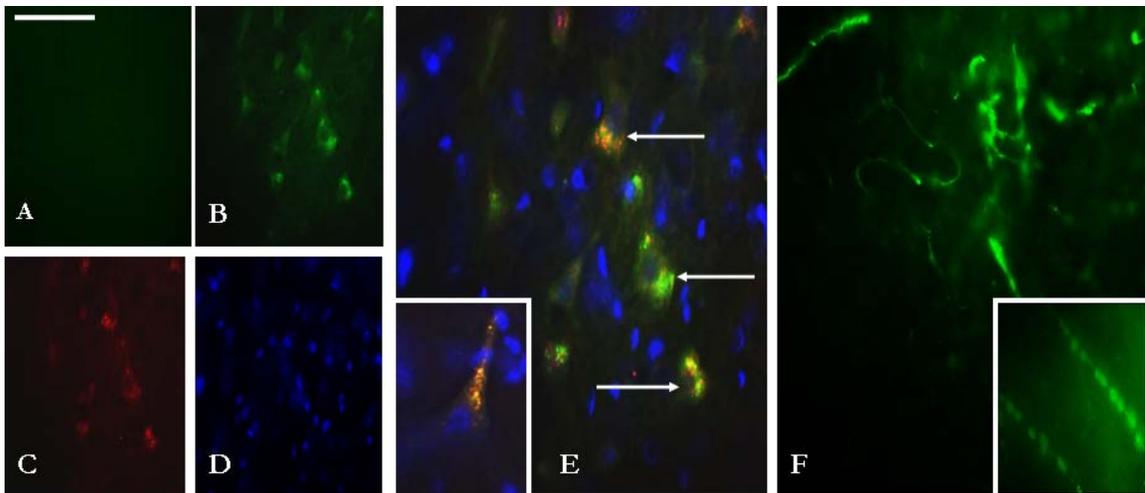


Figure 3-17. Activation of SBDP145 in neurons in the cortex 48 h after 40 mg/kg Meth exposure. Brain tissue from rats treated with saline showed no SBDP145 immunoreactivity (A). Brain tissue from rats treated with Meth revealed immunopositive SBDP145 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Chromatin was illustrated by DAPI staining (D). SBDP145 co-localized in neurons with NeuN (E, arrows, inset). SBDP145 was further revealed in axons and displays a “beads on a string” quality (F, 200x, inset). Photomicrographs were at 640x unless otherwise specified; scale bar 100 μ m, SBDP145 panels A, B, E, F; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

The cortices of drug exposed rats revealed marked increases in caspase-3 expression (Figure 3-18). The morphology of the brains of drug treated rats with the highest levels of caspase-3 induction had a decidedly disorganized, almost chaotic appearance when compared to saline controls. Caspase-3 immunopositive neurons

included cells with apoptotic bodies. Visualization of some axons with caspase-3 antibodies was possible after Meth treatment.

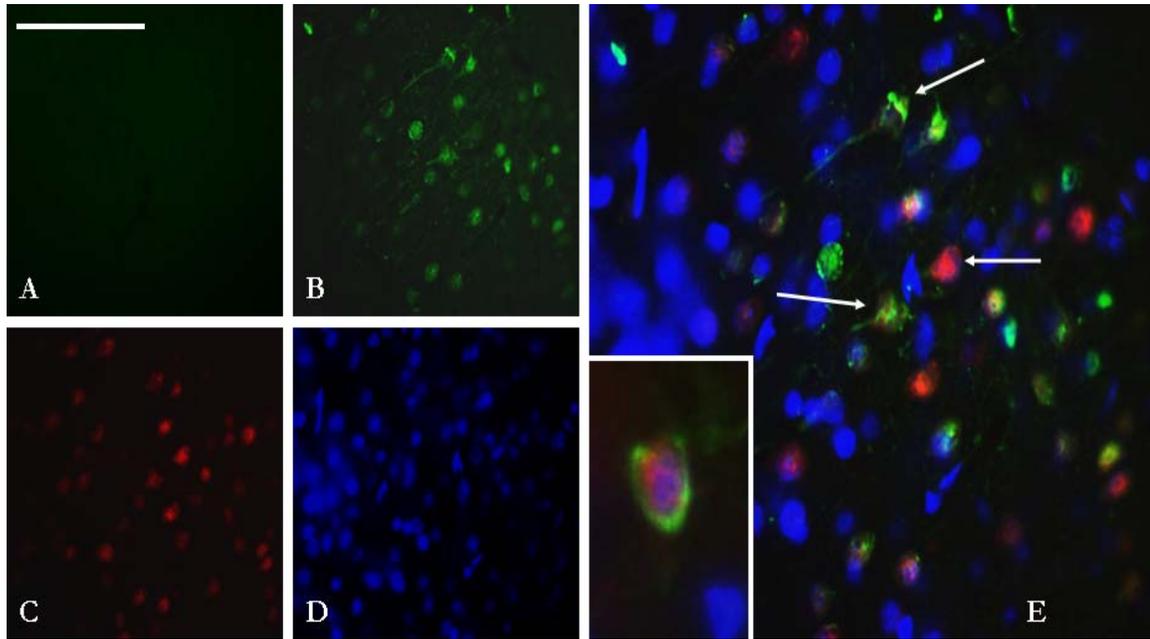


Figure 3-18. Activation of caspase-3 in neurons in the cortex 48 h after 40 mg/kg Meth exposure. Brain tissue of rats treated with saline showed no caspase-3 immunoreactivity (A). Brain tissue from rats treated with Meth revealed immunopositive calpain-1 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Chromatin was illustrated by DAPI staining (D). Calpain-1 co-localized in neurons with NeuN (E, arrows, inset). Photomicrographs were at 640x; scale bar 100 μ m, calpain-1 panels A, B, E; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

Similarly, the cortices of drug exposed rats revealed marked increases in the caspase specific cleavage product SBDP120 expression (Figure 3-19). The morphology of the brains from drug treated rats with the highest levels of SBDP120 also had a decidedly disorganized appearance when compared to saline controls. Spectrin BDP120 immunopositive neurons, when co-localized with NeuN, included cells with apoptotic bodies and evidence of granulation. The network of axons could be visualized using only SBDP120 in some areas, with a “beads on a string” effect seen.

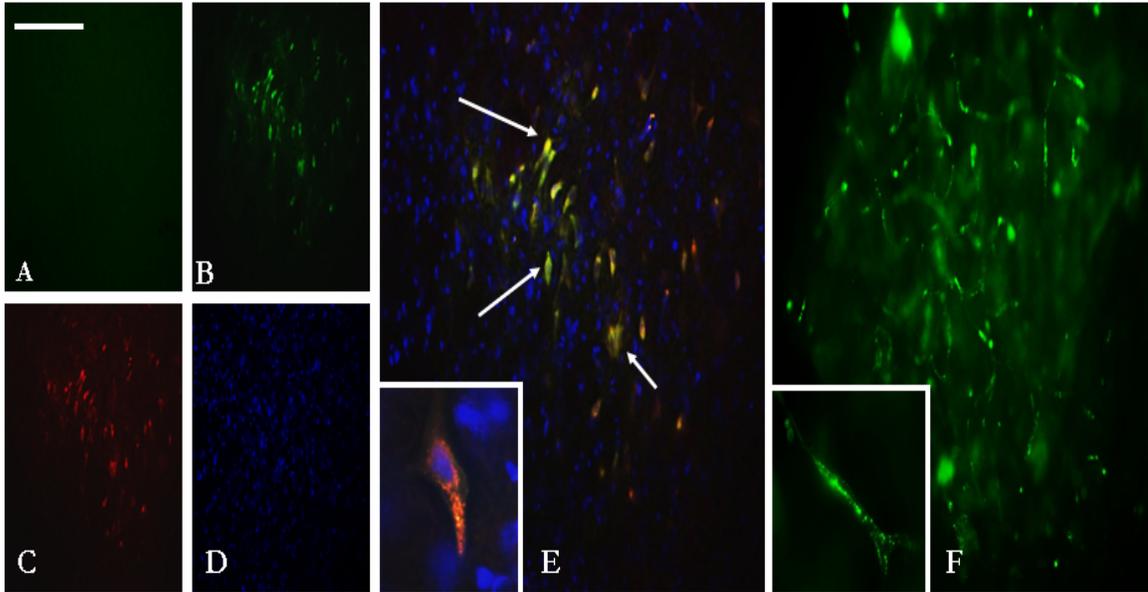


Figure 3-19. Activation of SBDP120 in neurons in the cortex 48 h after 40 mg/kg Meth exposure. Brain tissue of rats treated with saline showed no SBDP120 immunoreactivity (A). Brain tissue of rats treated with Meth revealed immunopositive SBDP120 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Chromatin was illustrated by DAPI staining (D). SBDP120 co-localized in neurons with NeuN with some granulation (E, arrows, inset). SBDP120 was further revealed in axons and displays a “beads on a string” quality (F, 200x, inset). Photomicrographs were at 200x unless otherwise specified; scale bar 300 μ m, SBDP145 panels A, B, E, F; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

Ecstasy

In general evidence of proteolytic enzyme activation and associated breakdown product upregulation were more sparsely populated and difficult to locate with MDMA than with Meth. Background illumination had to be set to higher contrast to discern activation when present. For example, the cortices of MDMA exposed rats revealed increases in calpain-1 expression, though much less evident with Meth (Figure 3-20). The morphology of the brains of drug treated rats with the highest levels of calpain-1 induction were difficult to differentiate from saline controls.

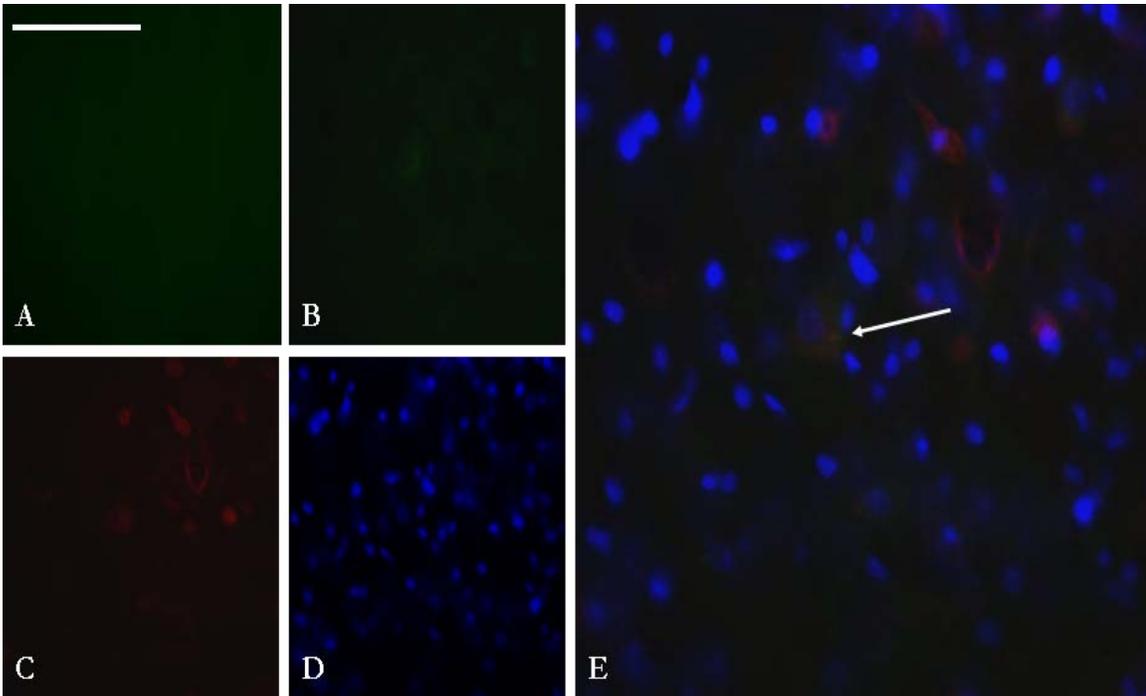


Figure 3-20. Activation of calpain-1 in neurons in the cortex 24 h after 40 mg/kg MDMA exposure. Brain tissue of rats treated with saline showed no calpain-1 immunoreactivity (A). Cortex of rats treated with MDMA showed some weak immunopositive calpain-1 expression (B). NeuN stained neuronal cells (C) show evidence of morphopathology. Apoptotic bodies were clearly present as illustrated by DAPI staining (D). Calpain-1 co-localized in neurons with NeuN (E, arrow). Photomicrographs were at 640x; scale bar 100 μ m, calpain-1 panels A, B, E; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

In contrast, the cortices of drug exposed rats revealed marked increases in the calpain specific cleavage product SBDP145 expression (Figure 3-21). The morphology of the brains from drug treated rats with the highest levels of SBDP145 had a decidedly disorganized, almost chaotic appearance when compared to saline controls. Spectrin BDP145 immunopositive neurons included cells with apoptotic bodies. The network of axons could be visualized using only SBDP145 in some areas, with a “beads on a string” effect seen.

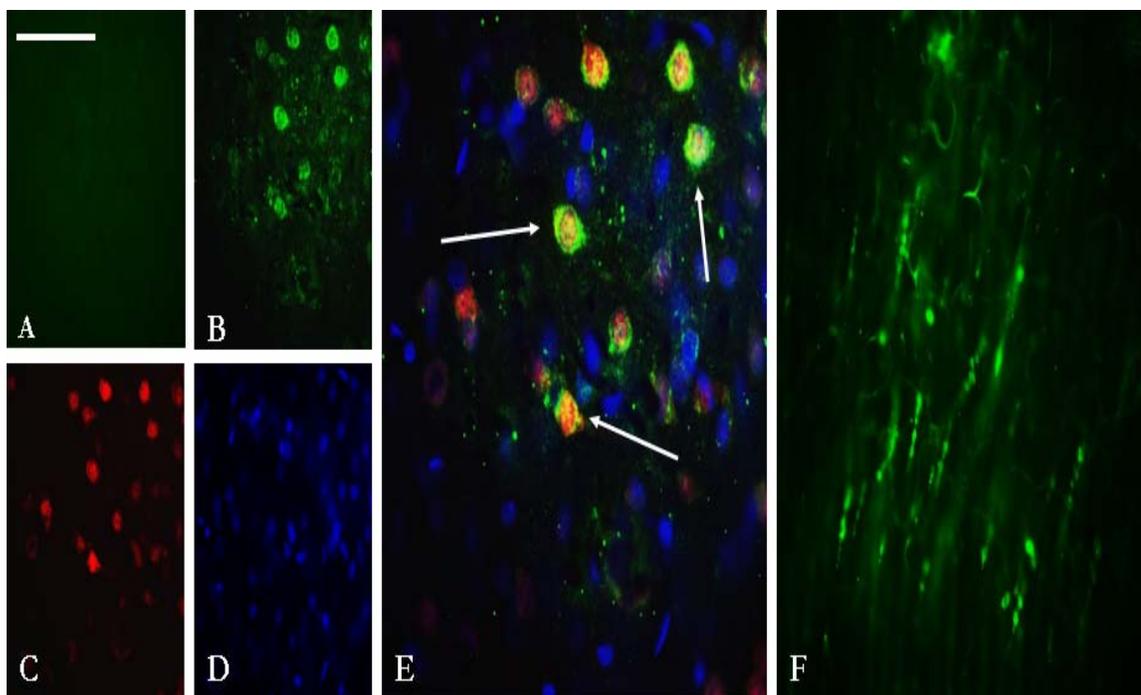


Figure 3-21. Activation of SBDP145 in neurons in the cortex 24 h after 40 mg/kg MDMA exposure. Brain tissue of saline treated rats showed no SBDP145 immunoreactivity (A). In contrast, brain tissue of MDMA treated rats revealed immunopositive SBDP145 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Apoptotic bodies were clearly present as illustrated by DAPI staining (D). SBDP145 co-localized in neurons with NeuN (E, arrows). SBDP145 was further revealed in axons (F, 200x). Photomicrographs were at 640x unless otherwise specified; scale bar 100 μ m, SBDP145 panels A, B, E, F; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

The cortices of drug exposed rats revealed marked increases in caspase-3 expression (Figure 3-22). The morphology of the brains of drug treated rats with the highest levels of caspase-3 induction had a decidedly disorganized, almost chaotic appearance when compared to saline controls. Caspase-3 immunopositive neurons included cells with apoptotic bodies.

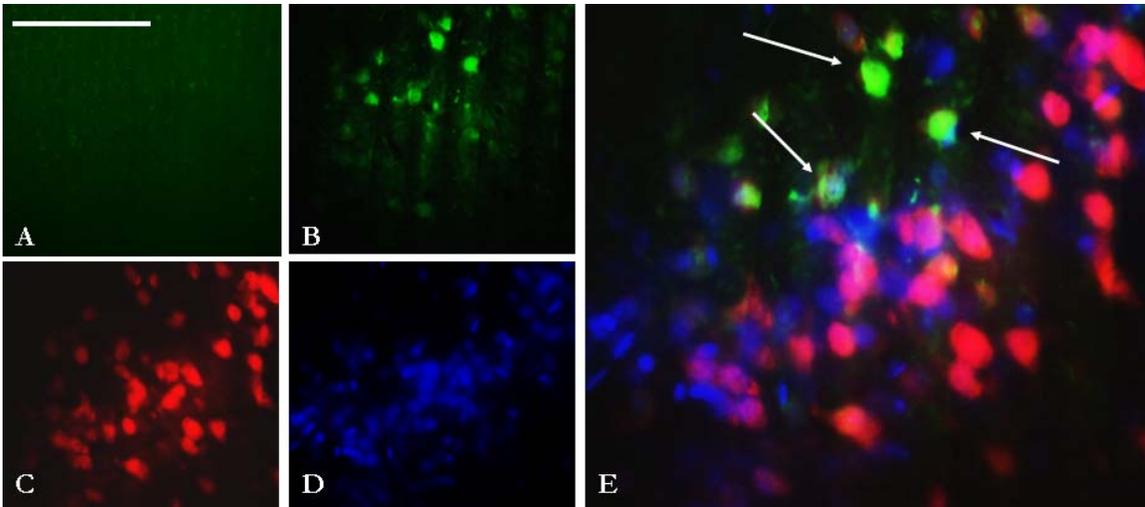


Figure 3-22. Activation of caspase-3 in neurons in the cortex 24 h after 40 mg/kg MDMA exposure. Brain tissue from saline treated rats showed no caspase-3 immunoreactivity (A). In contrast, brain tissue from rats treated with MDMA showed immunopositive caspase-3 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Apoptotic bodies were clearly present as illustrated by DAPI staining (D). Caspase-3 co-localized in neurons with NeuN (E, arrows). Photomicrographs were at 640x; scale bar 100 μ m, calpain-1 panels A, B, E; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

The cortices of drug exposed rats revealed marked increases in the caspase specific cleavage product SBDP120 expression (Figure 3-23). The morphology of the brains of drug treated rats with the highest levels of SBDP120 had a decidedly disorganized, almost chaotic appearance when compared to saline controls. Spectrin BDP145 immunopositive neurons, when co-localized with NeuN, included cells with apoptotic bodies and evidence of granulation. The network of axons could be visualized using only SBDP120 in some areas, with a “beads on a string” effect seen.

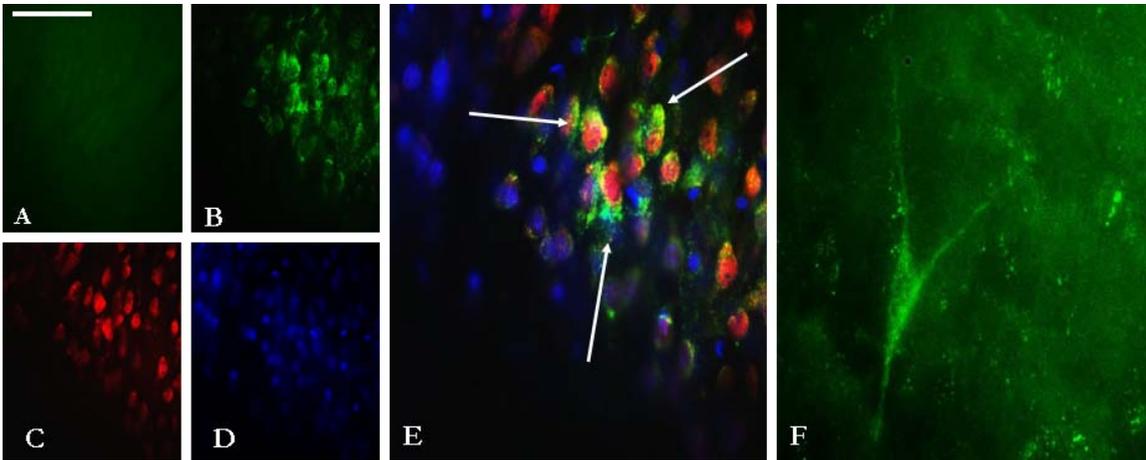
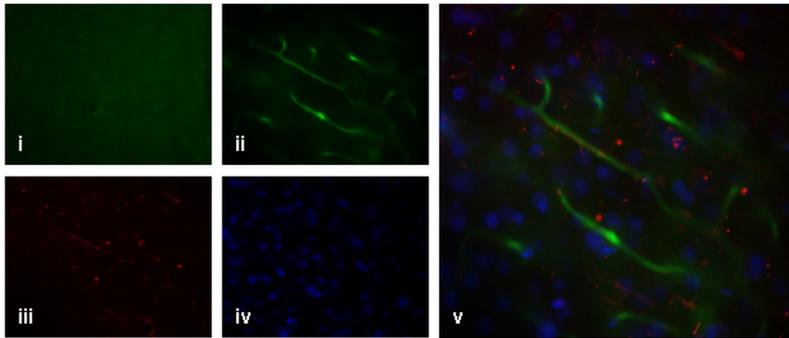


Figure 3-23. Activation of SBDP120 in neurons in the cortex 24 h after 40 mg/kg MDMA exposure. Brain tissue of saline treated rats showed no SBDP120 immunoreactivity (A). In contrast, brain tissue of MDMA treated rats revealed immunopositive SBDP120 expression (B). NeuN stained neuronal cells (C) showed evidence of morphopathology. Apoptotic bodies were clearly present as illustrated by DAPI staining (D). SBDP120 co-localized in neurons with NeuN (E, arrows). SBDP120 was further revealed in axons (F, 200x). Photomicrographs were at 640x unless otherwise specified; scale bar 100 μ m, SBDP145 panels A, B, E, F; NeuN panels C, E; DAPI panels D, E; co-localized images panel E.

Neurofilament-200

Since the SBDPs seemed to outline the network of axons, we wished to determine if the SBDPs would co-localize with heavy chain neurofilaments (NF-200) normally present in high concentrations in vertebrate neuronal axons. The cortices of drug exposed rats indeed revealed co-localization of SBDP145 with NF-200 (Figure 3-24). Western blot analysis showed significant decreases in NF-200 protein after Meth exposure. This co-localization effect was also detectable with SBDP120 (not shown).

(a) SBDP145 with NF-200; 40 mg/kg MDMA, 48 h



(b) SBDP145 with NF-200; 40 mg/kg Meth, 48 h

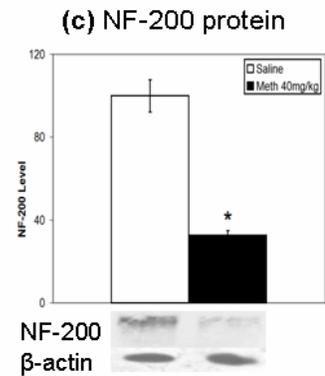
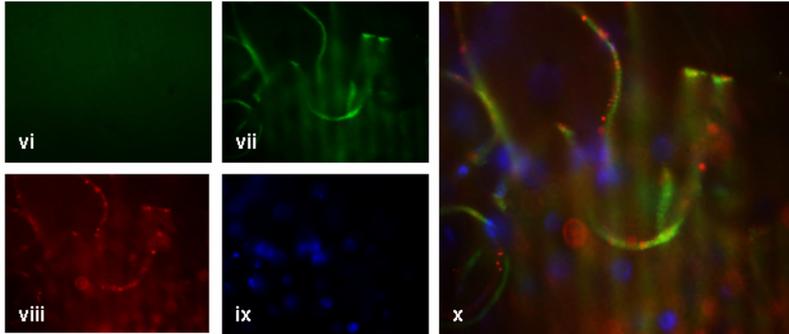


Figure 3-24. Club drug induced colocalization of SBDP145 with NF-200 in the cortex. A) Cortex treated with 40 mg/kg MDMA after 48 h. Brain tissue of saline treated rats showed no SBDP145 immunoreactivity (i). In contrast, cortex from MDMA treated rats showed immunopositive SBDP145 expression (ii). NF-200 stained neuronal axons (iii) were evident. Chromatin was illustrated by DAPI staining (iv). SBDP145 co-localized in axons with NF-200 (v). B) Cortex treated with 40 mg/kg Meth after 48 h. Control with SBDP145 primary antibody but no secondary antibody showed only minor background fluorescence (vi). Brain tissue from rats treated with Meth revealed immunopositive SBDP145 expression (vii). NF-200 stained neuronal axons (viii) were evident. Apoptotic bodies were clearly present as illustrated by DAPI staining (arrow, ix). SBDP145 was seen to co-localize in axons with NF-200 (x). Photomicrographs were at 640x; scale bar 100 μ m, SBDP145 panels i, ii, vi, vii; NF-200 panels iii, viii; DAPI panels iv, ix; co-localized images panels v, x. (C) NF-200 protein in saline (n=5) versus Meth 40 mg/kg treated rats (n=3). Similar results were seen with SBDP120 (not shown).

Discussion

In the analysis of Meth exposure, the level of neurotoxicity in the cortex and hippocampus, as suggested by proteolytic markers, was at least equivalent to our traumatic brain injury model. In both the cortex and the hippocampus, with both α II-spectrin and tau proteolytic markers, the levels of quantified neurotoxicity from Meth

exposure either equaled or exceeded that of TBI. Calpain-associated proteolysis was increased in both the rat cortex and hippocampus as indicated by the increased levels of SBDP150 and SBDP145 (Figures 3-3 and 3-4). At 10 mg/kg and 20 mg/kg of Meth, the levels of breakdown products mimicked that of TBI, while levels for 40 mg/kg of Meth exceeded them. Caspase-associated α II-spectrin proteolysis was clearly established in the hippocampus (Figure 3-5). In the studies of tau, the total tau antibody used did not differentiate the fragments generated by caspase versus calpain. However, caspase-3 tends to produce high molecular weight fragments about 45 kDa whereas calpain produces tau fragments of lower molecular weight (Figure 3-7). Indeed, several of these lower molecular weight tau fragments were observed in rat brain after both Meth administration or TBI suggesting calpain is actively cleaving tau proteins (Figures 3-8 and 3-9). Again, the increase of TBDP after 40 mg/kg of Meth approached or exceeded that of TBI. Given the impurities and unknown concentration of amphetamines in club drugs bought on the streets, some users tend to consume more than one pill in attempts to reach a satisfactory response (Makino et al. 2003).

The results from the α II-spectrin were consistent with a number of studies concerning TBI and other injuries when it was used as the biomarker of choice. Traumatic brain injury in rats resulted in increased SBDPs 150 and 145 in the cortex (Pineda et al. 2004). More specifically, it has been shown that in TBI after 24 h the calpain system was active in the cortex, but not necessarily the pro-apoptotic caspase system (Pike et al. 1998). It is worth noting that calpain is activated in both necrotic and apoptotic cell injury or death (Wang 2000). While our data was insufficient to show unequivocal evidence of caspase activation in rat cortex after just 24 hours, other studies

have suggested evidence of apoptosis in cortex and neocortical neurons following Meth exposure (Deng et al. 2002b; Stumm et al. 1999). There has been, however, evidence of pro-apoptotic caspase activity in the rat hippocampus, thalamus, and striatum following TBI (Pike et al. 2001). Studies on age related cell death in rats also suggests calpain activity in the cortex without concurrent caspase activity (Bernath et al. 2004). Following middle cerebral artery occlusion (MCAO) in rats, both calpain and caspase were evident after 24 h (Pike et al. 2003). Similar club drug use studies have been conducted using methamphetamines and the tau biomarker in which TBDPs were observed after three days in the cortex, hippocampus, and striatum (Wallace et al. 2003). The implication of this is to suggest that a single use of methamphetamine can be as neurotoxic in the cortex and hippocampus as traumatic brain injury. Given the effects of the drug on the cortex and hippocampus, cortical- and memory-related clinical outcomes should be considered when treating a patient with methamphetamine toxicity.

Evidence for neurotoxicity 24 h after MDMA use was much more difficult to elucidate than with our previous work with Meth. Some evidence of necrosis, however, was seen in both the cortex and was slightly more robust in the hippocampus. This is in agreement with previous work (Wallace et al. 2003). The evidence of neurotoxicity being greater for Meth than for MDMA is not surprising, as it correlates with clinical data. Two interesting effects are seen when the club drug treated animals are examined 72 h after exposure. For the Meth, while TBDPs are no longer as robust as they were after 24 h, SBDP levels continue to be strong. Additionally, the pro-apoptotic SBDP120 ceased to be visible after 24 h. Taken with our previous results, this suggests that Meth increases apoptosis within 24 h in the hippocampus while taking as long as 72 h in the

cortex. This is in agreement with current literature which notes that Meth can upregulate pro-apoptotic genes such as c-Jun, c-myc, and L-myc due to its production of reactive oxygen species (Deng et al. 2002a; Stumm et al. 1999; Thiriet et al. 2001). Second, MDMA effects also showed time dependence. Spectrin BPDs seen after 24 h were no longer evident whereas significant tau breakdown products continue to be evident after 72 h.

The results of the IHC studies demonstrate co-localization of both the upregulated calpain-1 and caspase-3 in rat cortical neurons after drug use. Specific spectrin breakdown products at 145 kDa and 120 kDa, respectively, are also found to be increased and this was particularly noticeable in the axons. To our knowledge, this is the first study to show the co-localization of these proteolytic markers and their specific breakdown products in specific neurons of the rat cortex following drug use. However, similar co-localization results were not seen in glial cells such as astrocytes, oligodendrocytes or microglia which support neurons and outnumber them 10 to 1. This is similar to the results found in the upregulation of the lysosomal protease cathepsin-B following spinal cord injury in which it was found to co-localize only with neurons and not glial cells (Ellis et al. 2005). These findings could suggest that the club drugs target only the neurons and not the supportive cells, in agreement with current findings of club drug induced serotonergic and dopaminergic nerve terminal degeneration (Larsen et al. 2002).

Additionally, the time point at which the upregulated proteases and the increase in their cleavage product was most clearly evident was after 24 h of treatment for MDMA and 48 h for Meth. Necrosis is suggested when α II-spectrin is cleaved by calpains to produce a breakdown product at 145 kDa (Harris et al. 1989). Here, calpain-1 was seen

to co-localize with neurons. Also, SBDP145 was evident and most prominent in axonal compartments allowing them to be visualized as a network when illuminated by the immunofluorescent tag. When the axons were more closely inspected swelling could be seen within the axons, displaying a “beads on a string” effect which is morphological earmark of cells undergoing cell death. This supports our hypothesis that neurons are undergoing cell death, specifically, a necrotic form of cell death similar to cells exposed to hyperthermia, toxins or direct trauma.

Moreover, apoptosis is suggested when α II-spectrin is cleaved by caspases to produce a breakdown product at 120 kDa (Nath et al. 1996). Here, caspase-3 was seen to co-localize with neurons. Also, co-localization as seen with SBDP120 was prominent in axonal compartments. This suggests that cells are also undergoing an apoptotic cell death event. Similar findings were reported when mice, treated with 30 mg/kg Meth, had 25% of their neurons found to be apoptotic after just 24 h (Zhu et al. 2005). Taken together, these indicate a dual-attack on neurons after club drug use with both a necrotic and apoptotic component.

To more closely examine the presence of SBDPs in axons, SBDP145 and SBDP120 were counterstained with anti-neurofilament (NF-200), a more specific marker of vertebrate neuronal axons. Upon Western blot examination, NF-200 appeared to be extensively degraded after exposure to Meth when compared to saline controls. However, some filaments that were still intact could be found to co-localize with SBDP145 or SBDP120 in axonal regions of neurons after either Meth or MDMA. Since α II-spectrin is located mainly in the axonal membrane and plays a crucial role in synaptic

transmission, this localization of compartmental damage may help to explain the nerve terminal damage seen with club drug use (Sikorski et al. 1991).

In summary, pro-necrotic calpain-1 and pro-apoptotic caspase-3 were found to be activated and upregulated in rat cortical neurons upon exposure to Meth and MDMA but not astrocytes, oligodendrocytes or microglia. Furthermore, SBDPs at 145 kDa and 120 kDa were found in neurons, particularly in the axonal segment and were further identified via co-localization with neurofilament. These results suggest that the drugs may be selectively targeting neurons and invoking a dual-proteolytic attack via necrosis and apoptosis, particularly in the axonal regions.

CHAPTER 4
IN VITRO NEUROTOXICITY

Introduction

We hypothesize that a dual proteolytic, activity upregulation exists in methamphetamine (Meth) or ecstasy (MDMA) treated cultured neurons suggesting that the cell culture system would provide an excellent model for pharmacological intervention to understand the relative contribution of calpain versus caspase in club drug neurotoxicity. In this study, cultured rat cerebrocortical neurons were challenged with two doses of MDMA or Meth, with and without the presence of protease inhibitors. The results showed increases in the α II-spectrin and tau breakdown products confirming the neurotoxicity of the drugs. Their breakdown profile helps explain the proteolytic activity mechanisms that contribute to the neurotoxicity. We were able to characterize Meth/MDMA induced spectrin breakdown products (SBDPs) and, to the best of our knowledge, are the first to demonstrate the drug induced fragmentation pattern of tau protein (TBDPs) upon treatment by both MDMA and Meth. Furthermore, our work utilizing calpain and caspase inhibitors to attenuate the fragmentation profile observed highlights the major role the cysteine proteases play in the course of Meth and MDMA neurotoxicity and represents a possible therapeutic venue in measuring drug neurotoxicity.

Results

Cell Toxicity

Phase contrast microscopy

Primary cerebrocortical neurons were cultured from rat brain and challenged for 48 h with MDMA and Meth (Figure 4-1).

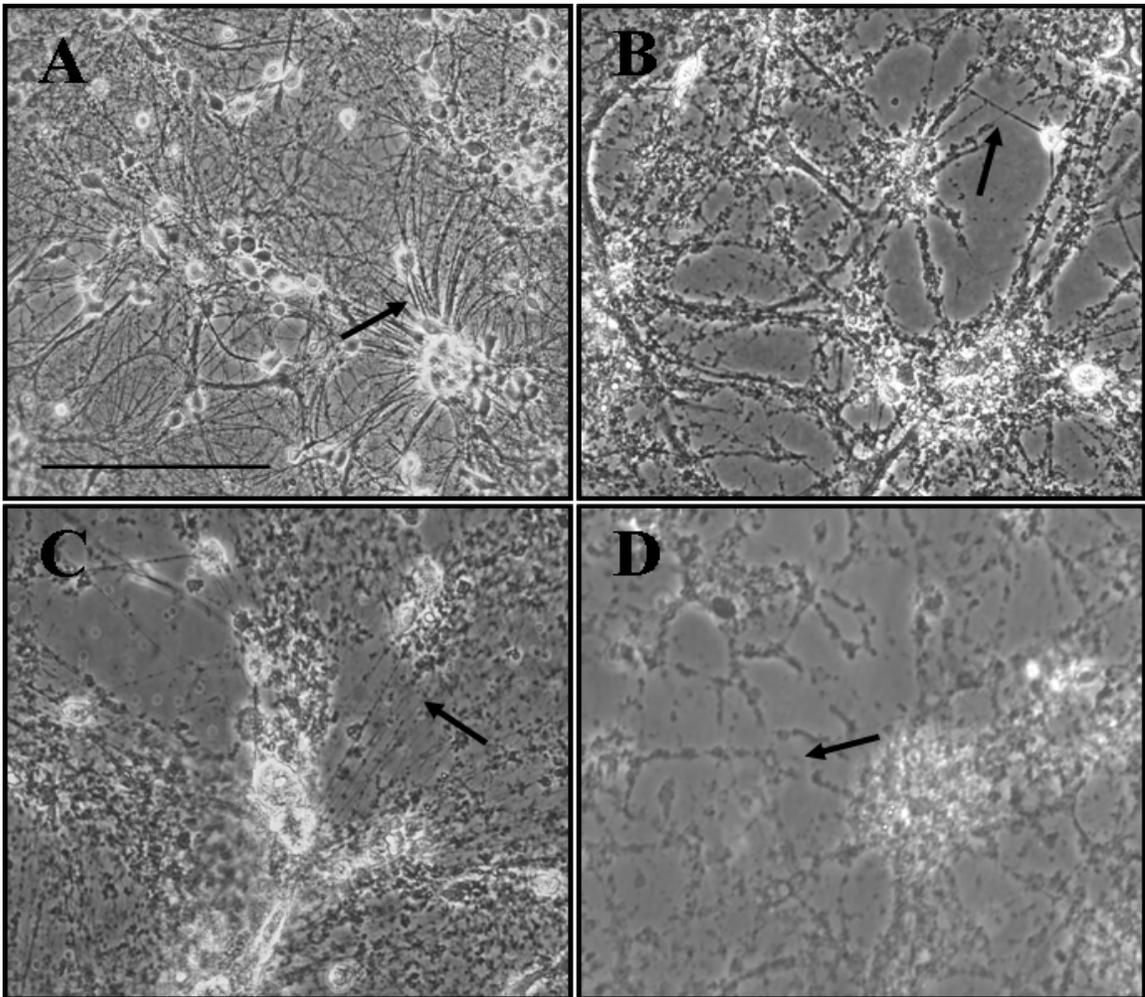


Figure 4-1. Cerebrocortical neurons after 48 h of drug incubation or saline treated control. Over 80% of cells in the cultures were neurons. Control neurons (A) had healthy cell bodies and a well-defined neurite network (arrow). In contrast, after MDMA (2mM) (C) or Meth (2mM) (D) drug treatment, significant axonal degeneration was observed (arrows). Positive control, NMDA 300 μ M showed similar axonal degeneration (arrow) as the drug treated cultures (B). Similar results were seen after 24 h and also for the 1 mM concentrations of MDMA and Meth (data not shown). Scale bar 200 μ m.

The phase contrast observations after 48 h of the control cultures showed healthy cell bodies and well-defined neurite networks including axons and dendrites (Figure 4-1A). Challenge with glutamate analogue N-methyl-D-aspartic acid (NMDA) (300 μ M), used as a positive control, resulted in excitotoxicity and significant degeneration in the cell body and neurites, including abnormal nodes along the neurite coupled with blebbing (Figure 4-1B). Challenge with MDMA (2 mM) induced apparent cell injury shown as deformed neuronal dendrites and the presence of shrunken cells and blebbing with undefined neuronal cell bodies (Figure 4-1C). A similar profile of degenerating neuronal neuritis was observed with the 2 mM Meth challenge (Figure 4-1D).

LDH release

Lactate dehydrogenase (LDH), normally a cytosolic enzyme has been shown to be released into cell culture medium when cell plasma membrane integrity is compromised due to injury (Koh and Choi 1987). We previously reported that LDH release occurs both in primary necrosis (oncosis) as well as secondary necrosis as a result of apoptosis (Nath et al. 1996; Posmantur et al. 1998). The magnitude of LDH release after drug challenges, presented as the mean absorbance \pm standard error of measurement (SEM), for the NMDA, MDMA and Meth treatment groups were increased over saline controls and were significantly higher after 48 h versus 24 h and each treatment group (Figure 4-2). The NMDA and all drug treatment groups were significantly increased over saline treated controls for both time points and both levels of drug treatment ($\#p < 0.001$ Student's T-test for NMDA versus saline control, $*p < 0.001$ One-way analysis of variance (ANOVA) with Dunnett post-hoc pairwise comparison test for drug treatment versus saline control). Also, LDH release was significantly increased for the 48 h groups versus the 24 h groups for all treatments ($**p < 0.01$ Student's T-test).

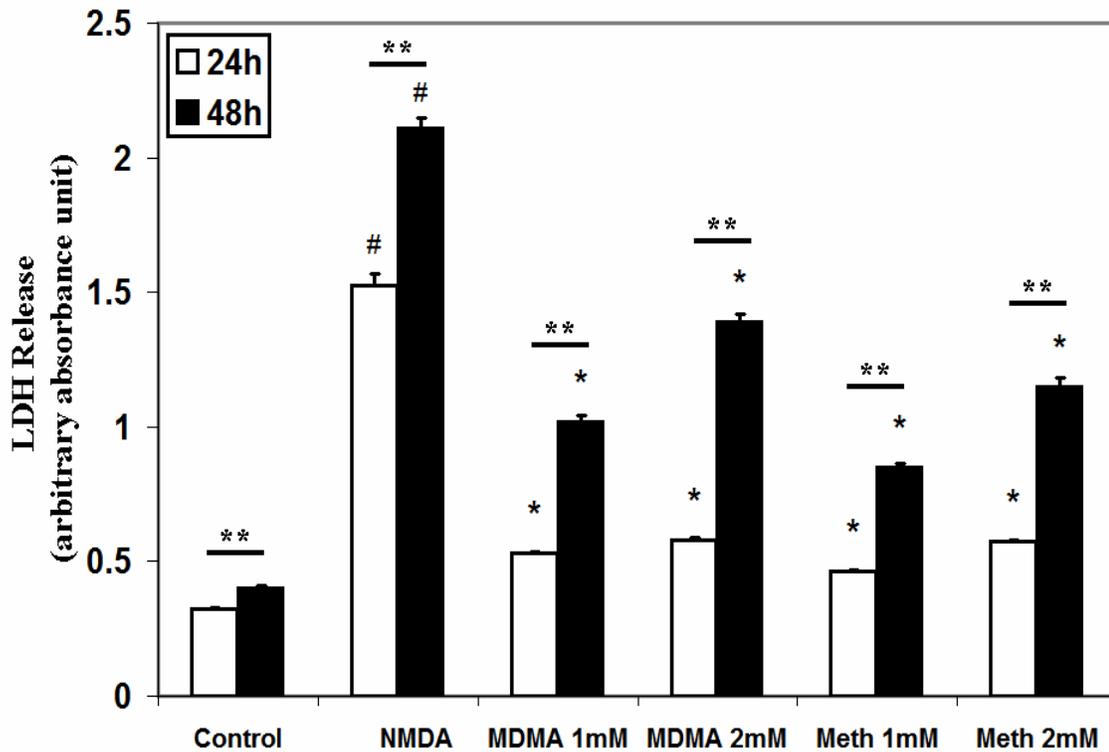


Figure 4-2. Lactate dehydrogenase (LDH) released into cell-conditioned media following insult. Each column represents the mean \pm SEM value ($n = 4$ cultures). # $p < 0.001$ Student's *T*-test for NMDA versus saline control, * $p < 0.001$ One-way ANOVA with Dunnett post-hoc pairwise comparison test for drug treatment versus saline control, ** $p < 0.01$ Student's *T*-test.

Cortical Cultures with Club Drugs

Analyses of western blots for axonally enriched cytoskeletal proteins α II-spectrin and tau and their breakdown products in the rat cerebrocortical neuron cultures 24 h and 48 h after challenge showed robust increase in breakdown products after treatment (Figure 4-3).

The total α II-spectrin antibody readily identified the parent protein at 280 kDa and the modest increases in its breakdown products (SBDPs) at 150, 145 and 120 kDa after 24 hours of MDMA and Meth (1 and 2 mM) treatments (Figure 4-3A). This contrasts with 24 h of NMDA exposure, used as a positive control, which resulted in substantial increases of each SBDP. For tau, after 24 h of MDMA and Meth exposure, the parent

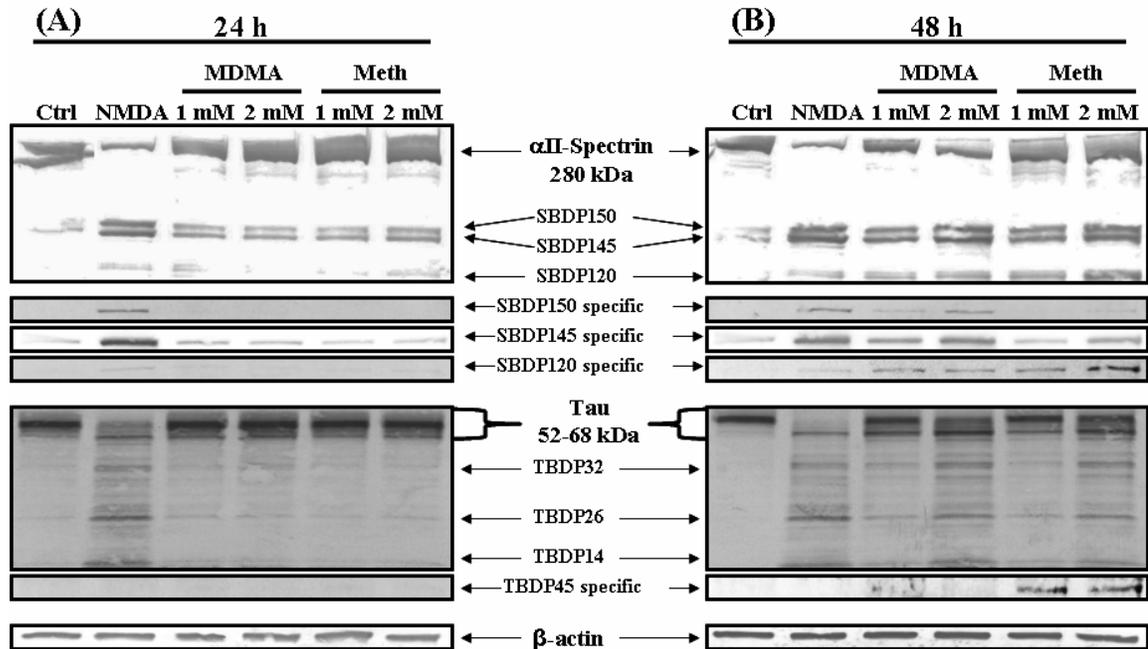


Figure 4-3. Proteolysis of α II-spectrin and tau in rat cortical neuron cell cultures following club drug treatment. A) 24 h. B) 48 h. The α II-spectrin and tau parent proteins as well as breakdown products at 150 kDa, 145 kDa, and 120 kDa for α II-spectrin and 32 kDa, 26 kDa, and 12 kDa for tau showed increases following drug treatment. In general, breakdown products increased globally after 48 h of drug exposure compared to 24 h. Additionally, the use of the fragment specific antibody to the TBDF45 allowed for the visualization of the 45 kDa tau breakdown product, normally not distinguishable from the parent band, and to show that activated caspases cleave tau.

protein isoforms in the range of 52 to 68 kDa were detected using the total tau antibody as were the calpain specific breakdown products (TBDFs) at 32, 26, and 14 kDa (Figure 4-3A). After 48 h for both MDMA and Meth, the resulting SBDP bands appear more robust, results that are comparable to NMDA (Figure 4-3B). Similar to spectrin, for tau breakdown products a more robust signal was observed after 48 h of treatment and with the higher drug concentrations. Also, interestingly, the antibody specific for caspase cleaved 45 kDa tau fragment detected TBDF45 after 48 h of treatment with the higher drug dosages.

Quantification of the breakdown products was performed (Figure 4-4).

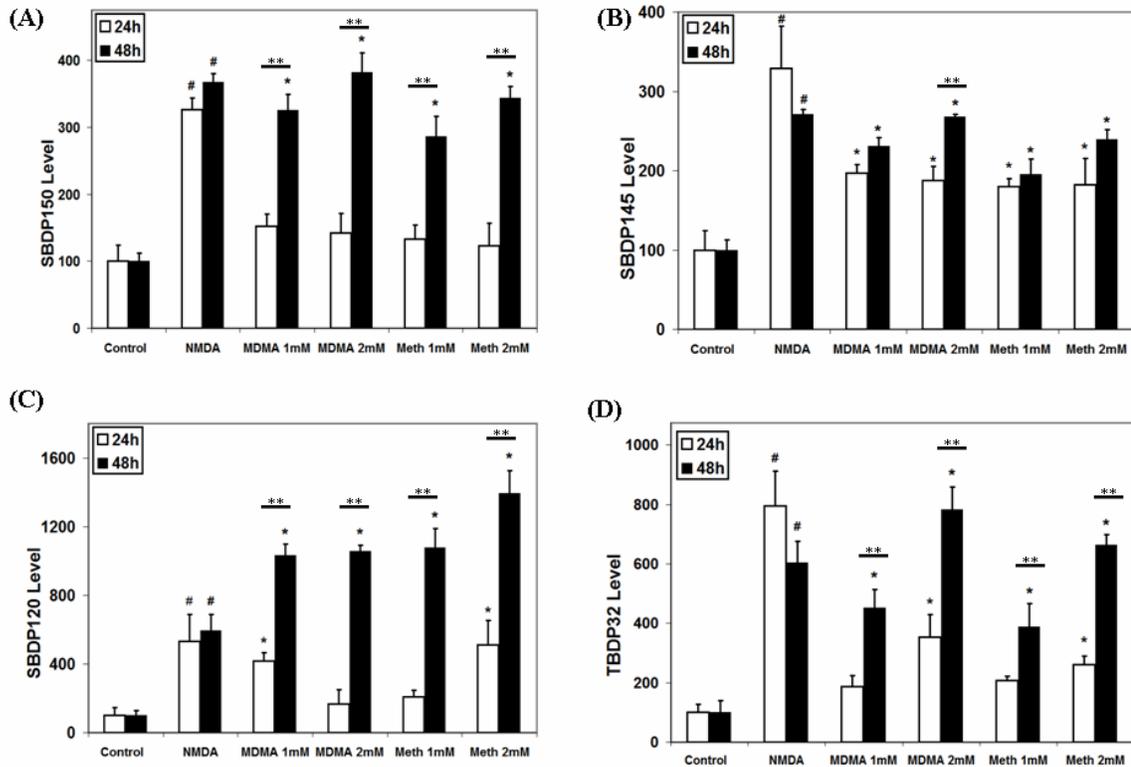


Figure 4-4. Accumulation of α II-spectrin and tau breakdown products in rat cortical neuron cell cultures following club drug treatment. 150 kDa (A), 145 kDa (B) and 32 kDa (D) were due to calpain activation and 120 kDa (C) was due to caspase activation in cortical neuron cultures 24 h and 48 h following drug administration. Each column represents the mean \pm SEM value ($n = 4$ cultures). # $p < 0.05$ Student's *T*-test, * $p < 0.05$ Dunnett post-hoc pairwise comparison test, ** $p < 0.05$ Student's *T*-test.

One-way ANOVA tests revealed differences among accumulation of breakdown products for drug treatment groups compared to saline controls for each band and time point except SBDP150 after 24 h. Accumulation of breakdown products for specific drug treatment groups were significantly increased over saline controls for all conditions after 48 h (* $p < 0.05$ Dunnett post-hoc pairwise comparison test). After just 24 h, significant increases were seen only for the SBDP145 band (all conditions), the 1 mM MDMA and 2 mM Meth conditions for SBDP120 and the 2 mM drug doses for TBDP32. For drug treatment groups, significant increases in accumulation of breakdown products were

detected after 48 h compared to 24 h for all conditions other than SBDP145 (** $p < 0.05$ Student's T-test). Similar results were seen for TBDP26 and TBDP14 (data not shown).

Calpain specific breakdown products showed significant increases over saline controls following drug challenges by both MDMA and Meth and for both dosage levels. The only statistically significant differences were seen after 48 h (Figure 4-4A) as determined by the breakdown product antibody specific for calpain SBDP150. In contrast, specific antibodies for the calpain SBDP145 (Wang, 2000) showed an increase in SBDP145 over saline controls for both drugs at both time points (Figure 4-4B). The antibody for the caspase SBDP120 (Wang, 2000) was only able to discern a statistically significant increase in SBDP120 after 24 h for the 1 mM MDMA and 2 mM Meth doses, but was able to discern a significant increases for both drugs after 48 h, with the more robust signal seen for Meth (Figure 4-4C). Furthermore, these data suggest that MDMA and Meth challenges after 48 h induced more caspase-mediated apoptosis than the positive control excitotoxic NMDA challenge.

Tau proteolysis can be quantified by measuring the calpain mediated TBDP32 (Figure 4-4D). Breakdown products of TBD32 appeared to increase over saline controls following MDMA and Meth challenges (1-2 mM) after both 24 and 48 h although they were not statistically significant for the 1 mM drug doses after 24 h. Similar results were seen for TBDP26 and TBDP14, both the result of calpain mediated proteolysis (data not shown).

Cortical Cultures with Club Drugs and Protease Inhibitors

Western blots analyses for α II-spectrin and tau and their breakdown products were also performed on cell lysate from rat cerebrocortical neuron cultures 48 h after a 1 h

protease inhibitor incubation with z-VAD-fmk (ZVAD), Z-D-DCB (ZD) or SJA6017 (SJA) and subsequent drug challenge by MDMA and Meth (Figure 4-5).

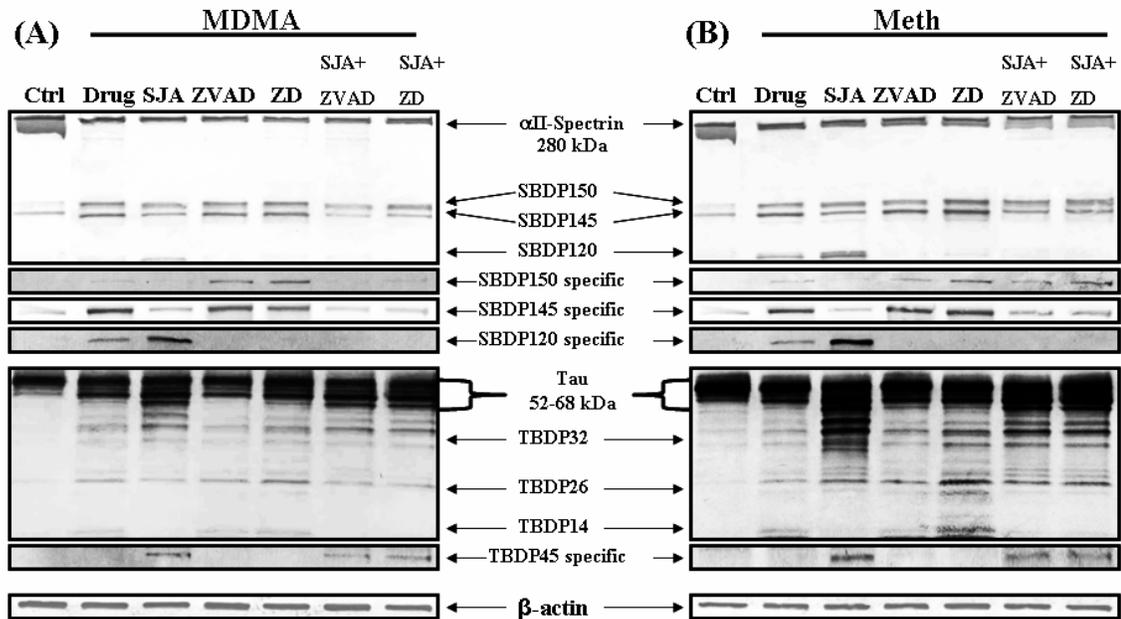


Figure 4-5. Proteolysis of α II-Spectrin and tau in rat cortical neuron cell cultures following preincubation with inhibitors prior to drug treatment (2 mM, 48 h). A) MDMA. B) Meth. α II-Spectrin and tau parent proteins as well as their breakdown products at the 150 kDa, 145 kDa, and 120 kDa for α II-spectrin and 32 kDa, 26 kDa, and 12 kDa for tau showed effects of the protease inhibitors. The calpain (SJA) and caspase (ZVAD, ZD) inhibitors appeared to decrease accumulation of calpain and caspase mediated accumulation of breakdown products, respectively. The use of fragment specific antibody allowed for the visualization of the 45 kDa tau breakdown product that is normally obscured by the parent protein.

With the total α II-spectrin antibody, the parent protein at 280 kDa and the breakdown products (SBDPs) at 150, 145 and 120 kDa were observed for MDMA and Meth. With the total tau antibody, for both MDMA and Meth challenges, beside the parent protein bands in the range of 52 to 68 kDa, a number of putative calpain specific tau breakdown products of 32, 26, and 14 kDa were observed. The signals appeared to be more robust for the Meth treatments than MDMA. The calpain and caspase inhibitors

appeared to decrease accumulation of calpain and caspase mediated breakdown products, respectively. Quantification was performed (Figure 4-6).

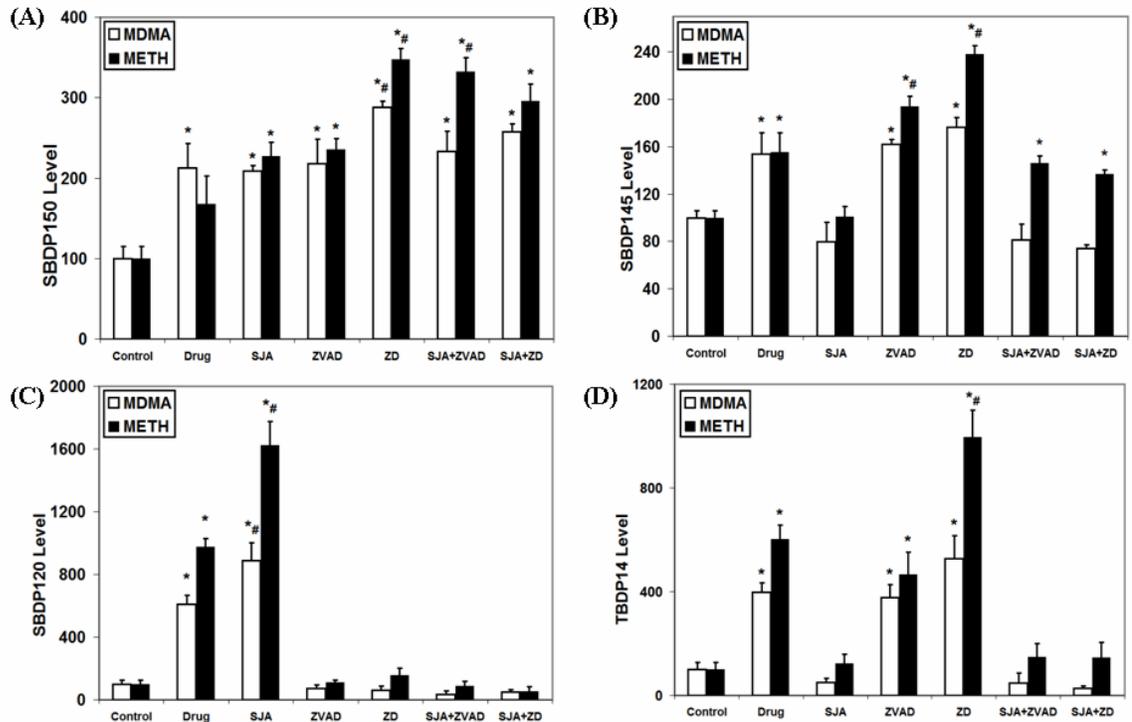


Figure 4-6. Accumulation of α II-Spectrin and tau breakdown products in rat cortical neuron cell cultures following preincubation with inhibitors prior to drug treatment. 150 kDa (A), 145 kDa(B) and 14 kDa (D) were due to calpain activation and 120 kDa (C) was due to caspase activation in cortical neuron cultures 48 h following treatment with various inhibitors (SJA for calpains; ZVAD or ZD for caspases) and drug administration (2 mM). Each column represents the mean \pm SEM value (n = 4 cultures). *One-way ANOVA*, $p < 0.001$ for all, * $p < 0.05$ Dunnett post-hoc pairwise comparison test, # $p < 0.05$ Dunnett post-hoc pairwise comparison test.

For MDMA and Meth, a significant difference in accumulation of breakdown products among treatment groups was detected for each band (One-way ANOVA, $p < 0.001$ for all). For the SBDP150, accumulation of breakdown products was increased over saline controls for all groups except Meth alone (* $p < 0.05$ Dunnett post-hoc pairwise comparison test). Breakdown products were enhanced over the levels of drug alone for the ZD treatment for both drugs and the SJA+ZVAD treatment for Meth (# $p < 0.05$

Dunnett post-hoc pairwise comparison test). For the SBDP145, SJA had the effect of significantly decreasing accumulation of breakdown products to the level of saline controls. The same was true for the mixtures of SJA with either ZVAD or ZD for the MDMA treatment group. Breakdown products were enhanced over the levels of drug alone for Meth cultures treated with either ZVAD or ZD. For the SBDP120, any drug group treated with ZVAD or ZD alone or in mixture with SJA had its accumulation of breakdown products reduced to that of saline controls. Breakdown products were enhanced over the levels of drug alone for the SJA treatments in either drug. For TBDP14, each drug group treated with SJA alone or in combination with ZVAD or ZD had the breakdown product accumulation reduced to that of saline controls. Breakdown products were enhanced over the levels of drug alone for the Meth cultures treated with ZD. Similar results were seen for TBDP32 and TBDP26 (data not shown).

For both drug treatments, prior to pretreatment with the protease inhibitors, the SBDP150/SBDP145 doublet was visible, although the calpain specific SBDP150 was not detectable (Figures 4-5A, 4-5B). Our results demonstrated that the calpain specific antibody to SBDP150 was enhanced in the presence of the caspase inhibitors (ZVAD or ZD). Quantification of data based on the SBDP150, including both calpain-mediated SBDP150 and caspase-mediated SBDP150i (Wang 2000), from the non-specific total α II-spectrin antibody did not show an overall decrease in breakdown products after inhibitor treatment suggesting a mixture of caspase and calpain activity (Figure 4-6A). In fact, breakdown products appeared to have increased after treatment with the caspase inhibitors. Although some calpain-specific SBDP145 background signal was detected in control cells, there were significant increases in the SBDP145 signals for MDMA and

Meth treatments. Meth showed a small but statistically significant increase of SBDP145 after preincubation with the caspase inhibitors ZVAD and ZD. In contrast, calpain inhibitor SJA suppressed both the Meth and MDMA-induced SBDP145 signals (Figure 4-5). Quantification data of SBDP145 confirmed that SBDP145 was suppressed by calpain inhibition (Figure 4-6B). Caspase specific SBDP120 was significantly elevated for both Meth and MDMA, and was suppressed to control levels by the presence of the caspase inhibitors ZVAD or ZD. Interestingly, the presence of calpain inhibitor SJA enhanced the level of SBDP120 dramatically (Figure 4-6C). Taken together, these data suggest α II-spectrin, in Meth and MDMA treated cortical cells, is a target of both calpain and caspase proteases. By suppressing one protease, proteolysis of spectrin by the other protease becomes more dominant.

Quantification data of the 14 kDa tau breakdown product indicated calpain degradation is occurring (Figure 4-6D) (Park and Ferreira, 2005). TBDP14 increased following drug challenge by MDMA and Meth but when treated with the calpain inhibitor SJA, the breakdown products decreased to near control levels. To ascertain whether caspases also contribute to tau breakdown, we employed the caspase-3 specific TBDP45 antibody (Chung et al., 2001), but observed no signal with MDMA or Meth treatment alone (Figure 4-5D). However, as with the case of SBDP120, the presence of calpain inhibitor SJA has allowed the TBDP45 signal to be visualized (Figure 4-5). This is believed to have occurred because SJA strongly suppresses the calpain-mediated TBDP14 preventing further cleavage of the higher molecular weight TBDP45 by caspase. Since the caspase-3 tau breakdown fragments are of higher molecular weight, they were likely subjected to further proteolysis by calpain into BDPs of 36, 32 and 14 kDa.

Conversely, ZD and ZVAD enhanced the calpain-mediated TBDP14 (Figure 4-6) while suppressing the SJA-enhanced TBDP45 (Figure 4-5). Applying both calpain and caspase inhibitors also resulted in complete reduction of TBDP14 levels and a partial reduction of TBDP45 levels (Figure 4-5B). Taken together, these tau data suggest there is a dual proteolysis of tau by calpains and caspases. Thus, pharmacological suppression of only calpains would still allow for caspase activity as observed by the increase in TBDP45. The caspase inhibitors had no significant inhibition effect on the TBDP14 and, actually, the breakdown products appeared enhanced for the Meth group following treatment with ZD. Similar results were seen for TBDP32 and TBDP26 which are also the result of calpain cleavage activity (not shown).

Discussion

The results of the LDH assays, immunoblot quantification and visual observation demonstrate that when primary cerebrocortical cell cultures are challenged by MDMA and Meth they appear to show all the signs of having undergone a neurotoxic challenge. While the 1 mM and 2 mM concentrations of Meth and MDMA used in this treatment paradigm are higher than what is considered to be physiologically relevant and may not reflect the toxic doses utilized in *in vivo* rat and mouse models, these concentrations were selected since they would achieve a minimal neurotoxic effect on primary cortical cells both for cell survival and protein proteolysis. It is worth noting that in most *in vivo* drug abuse studies drug concentrations needed to be from 3 and 5 mM to achieve similar neurotoxicity (Cadet et al. 1997; Stumm et al. 1999; Deng et al. 2002b; Genc et al. 2003; Jiminez et al. 2004). Generally, *in vitro* drug abuse studies require higher concentrations of these drugs when compared to *in vivo* studies reflecting the complexity of the CNS where neurotoxic effects can be exacerbated by the synergistic effects of other factors

that may lead to neuronal cell death. These factors include excitotoxic amino acids, hyperthermia, monoamine oxidation, cytokine production and hyperthermia. These have been shown to be major contributors in mediating cell death (Deng et al. 2002b; Cadet et al. 2003).

Neurotoxicity, as measured by the concentration of BDPs was more apparent after 48 h than 24 h and the results show they were dose dependent. The increase in both calpain-mediated SBDPs at 150 and 145 kDa and TBDPs at 14 kDa are evident after 48 h, as is the increase in caspase-mediated SBDP of 120 kDa. Protease inhibitors do not appear to be effective at least when examining the total SBDP at 150 kDa, but this may be due to combination of the calpain-induced and caspase-induced cleavage products, or it may be due the activity of other proteases such as interleukin 1 beta-converting-enzyme (ICE)-like (Nath et al. 1996). The fragment specific spectrin 150 kDa antibody revealed that there was a downregulation of the breakdown product when the cells were pretreated with the calpain inhibitor SJA. In addition, SJA reduced the levels of the calpain-induced 145 kDa band to that of controls. The mixture of SJA with calpain inhibitors appeared to lessen the effect of caspase inhibition after 48 h. A similar profile is seen with the calpain-induced 14 kDa band from total tau. The caspase-induced 120 kDa spectrin fragment, when examined with the total spectrin antibody, was reduced to control level with the addition of either caspase inhibitor ZVAD or ZD, but there was no change when the cells were pretreated with SJA. Similar results were noted with the fragment specific antibody for SBDP120. The fragment specific antibody to the 45 kDa tau breakdown product, a caspase-induced fragment, was suppressed by the caspase inhibitors ZVAD and ZD. This was partially offset by the addition of the calpain inhibitor SJA, for reasons

determined in future studies. These results essentially agree with those found using rat cerebellar granule cell cultures in which MDMA and Meth toxicity were seen to increase from 24 h to 48 h and in which caspase inhibition by ZVAD was also displayed (Jimenez et al. 2004). Also, ZVAD was found to suppress apoptosis in neuron-like PC12 cells treated with Meth (Uemura et al. 2003). In general when one pathway of proteolysis is inhibited, it seems that an alternative pathway appears to be enhanced as reflected by the spectrin and tau breakdown products (see Figure 1-3).

The results of these studies imply that MDMA and Meth serve to activate both the pro-apoptotic caspase system as well as the pro-necrotic calpain system (Wang 2000) in rat cerebrocortical neuron cultures. The degradation of spectrin and tau breakdown products appears to be dose and time dependent. The protease inhibitors were effective in attenuating the neurotoxicity brought on by the drugs as measured by decreases in protein breakdown products. Future studies need to be conducted to see if a combination of these inhibitors would be effective in preventing α II-spectrin and tau degeneration observed in MDMA and Meth exposure in vivo (Warren et al. 2005a). Such experiments could point to clinical tools useful in treating acute drug overdose or in preventing chronic structural brain damage of addicted patients.

CHAPTER 5 NEURONDOCRINE TOXICITY

Introduction

The hazardous effects of ecstasy (MDMA) and methamphetamine (Meth) occur partly from the release of large amounts of serotonin and 5-HT nerve terminal degeneration, which hinders normal cellular function and repair (Battaglia et al. 1987; Gianwi et al. 2005). The monoamines released following drug use are normally involved in appetite regulation, therefore, increased concentrations caused by drug use may also result in the observed negative effects on these systems (Jaehne et al. 2005; Malpass et al. 1999), including pharmacologically-induced anorexia. Thus, this study focuses on examining of the effects of acute MDMA and Meth administration on the serum concentrations of leptin, ghrelin, growth hormone and neuropeptide-Y (NPY) (Chapter 1) to determine if changes in these hormones occur. This has the potential to explain anorexia resulting from drug use.

Results

Methamphetamine

Weight

Changes in weight from baseline occurred after Meth administration (Figure 5-1). For all drug treatment groups, rat weight decreased immediately in a dose-dependent manner. Maximum weight loss was achieved after 6 hours, beyond which weight gain was experienced for all groups except the 40 mg/kg Meth group which experienced

further weight loss between 24 and 48 h. A one-way analysis of variance (ANOVA) test revealed that treatment groups were different for each time point ($p < 0.001$ for each).

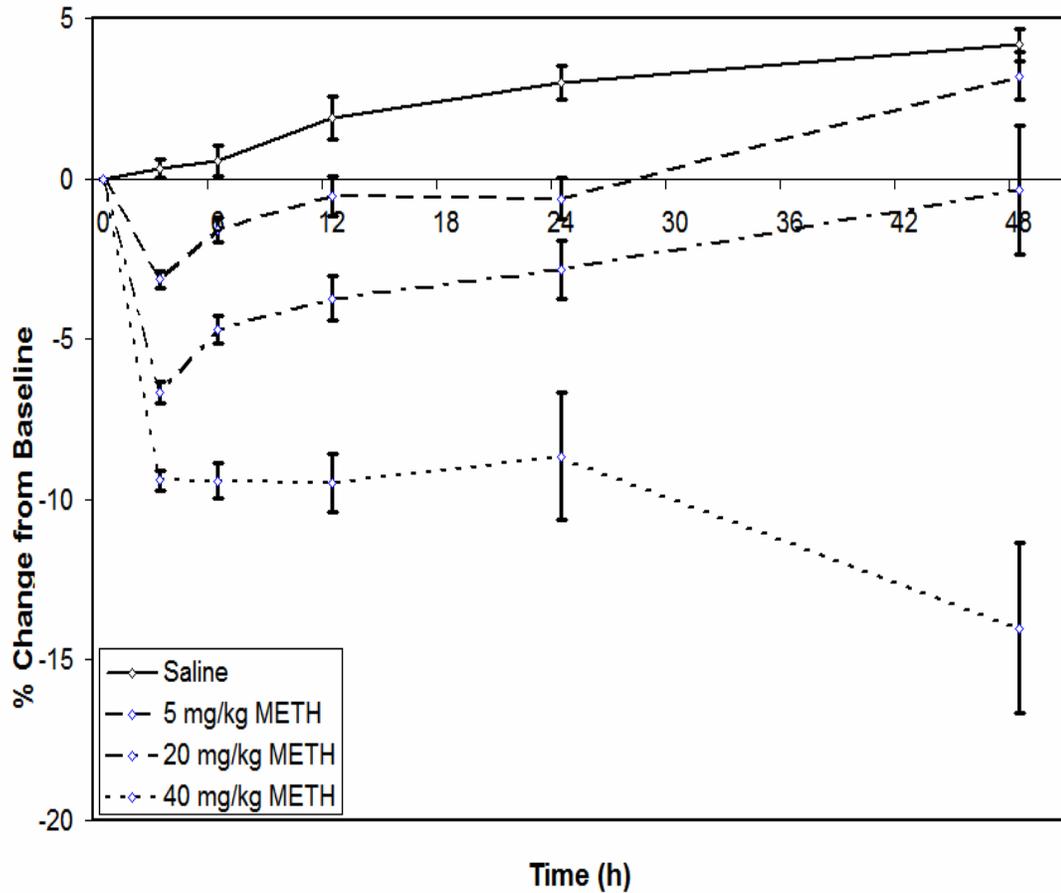


Figure 5-1. Changes in rat weight after Meth administration. For saline treatments, $n=6$, for Meth treatments, $n=5$. Rat weight decreased immediately following Meth administration and began to recover after 6 h, except with the highest doses of Meth. A dose response was indicated.

Leptin

Analysis of the serum leptin levels of rats administered Meth was performed (Figure 5-2). A trend, similar to what is seen with MDMA, was suggested via a transient decrease in leptin followed by recovery, but did not reach statistical significance except for the 6 h 20 mg/kg sample. A one-way ANOVA test revealed no significant difference.

The closest to significance was that of the 6 h group ($p=0.11$) and is most noticeable with the 20 mg/kg group.

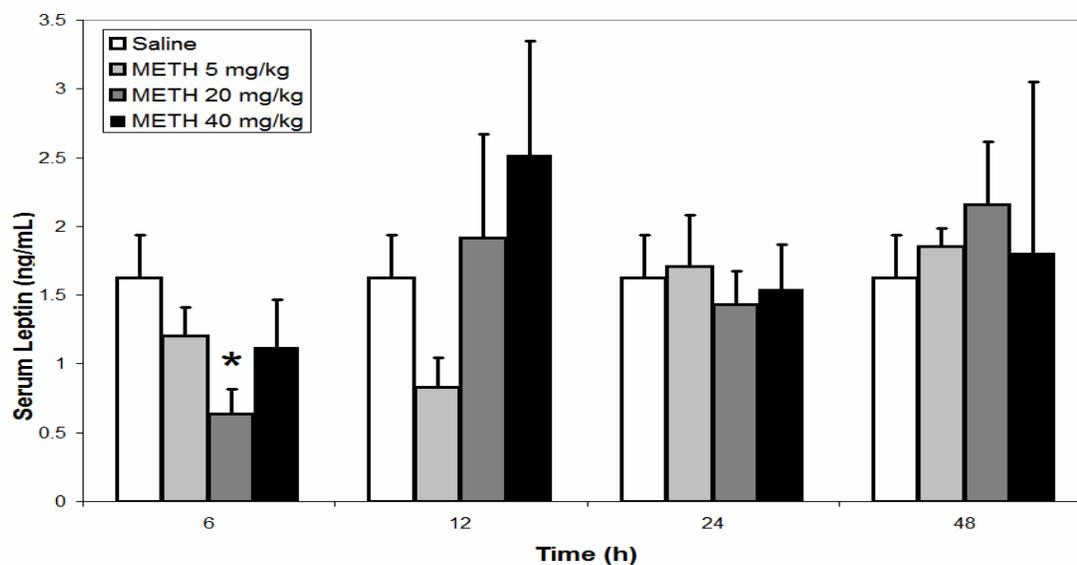


Figure 5-2. Changes in serum leptin following Meth administration. For saline treatments $n=6$, and for Meth treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

Ghrelin

Analysis of the serum ghrelin in rats administered Meth was performed (Figure 5-3). A trend, again similar to MDMA, was suggested via a transient increase in ghrelin followed by normalization, but does not reach statistical significance except with the 20 mg/kg dose at 6 h. A one-way ANOVA test revealed no significant difference. The closest to significance was that of the 6 h group ($p=0.10$).

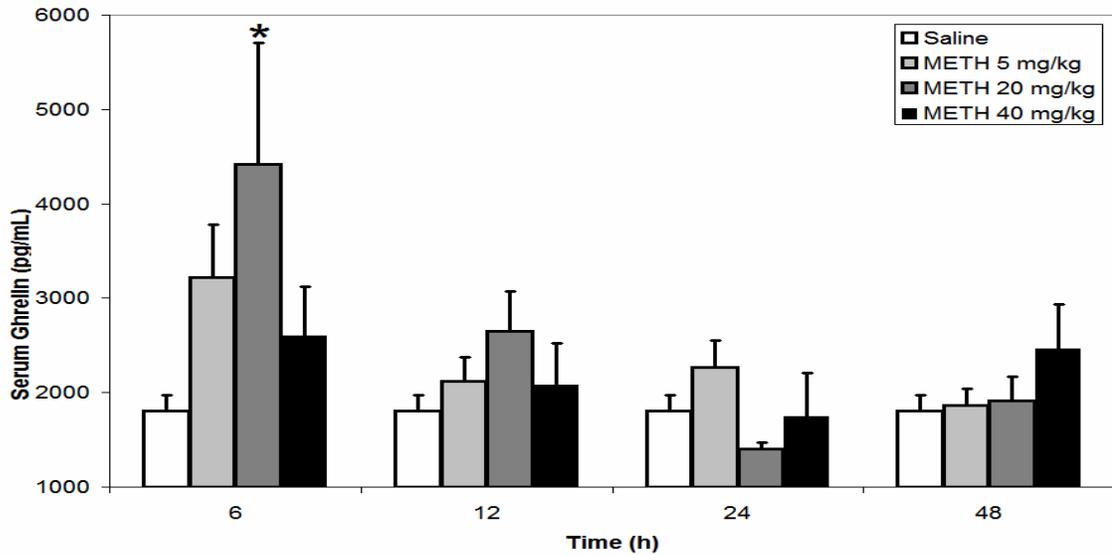


Figure 5-3. Changes in serum ghrelin following Meth administration. For saline treatments $n=6$, and for Meth treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

Growth hormone

Analysis of the serum growth hormone levels of rats administered Meth was performed (Figure 5-4).

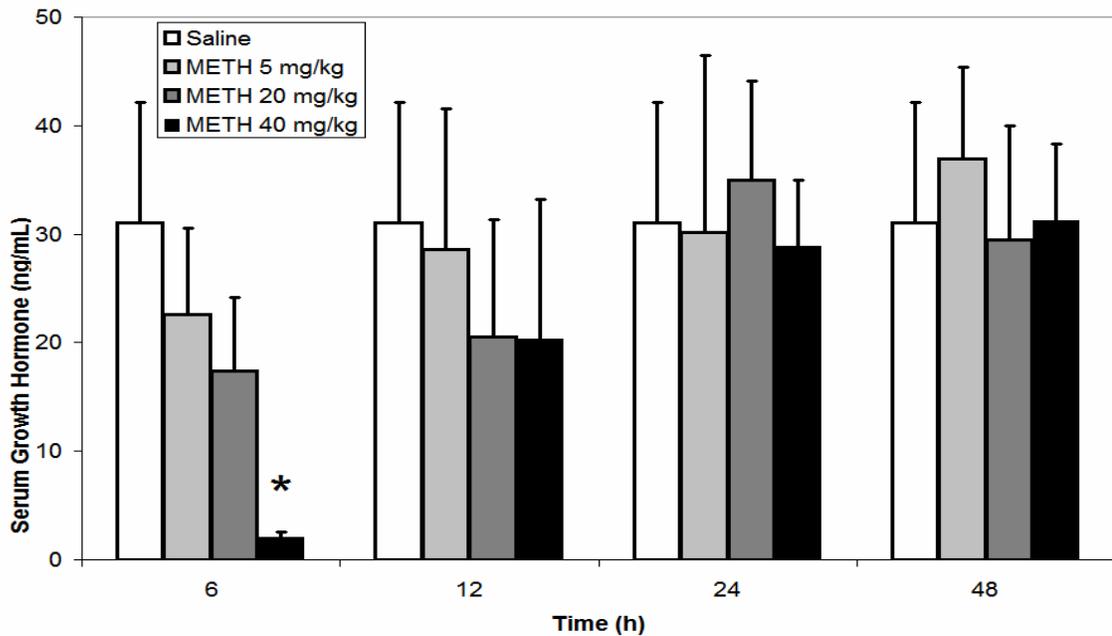


Figure 5-4. Changes in serum growth hormone following Meth administration. For saline treatments $n=6$, and for Meth treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

A trend, also similar to MDMA, was suggested at 6 h via a transient decrease in growth hormone followed by recovery, but only reached statistical significance for the 40 mg/kg dose. A one-way ANOVA test revealed no significant difference. The closest to significance was that of the 6 h group ($p=0.12$).

Neuropeptide-Y

Analysis of the serum NPY levels of rats administered Meth was performed (Figure 5-5).

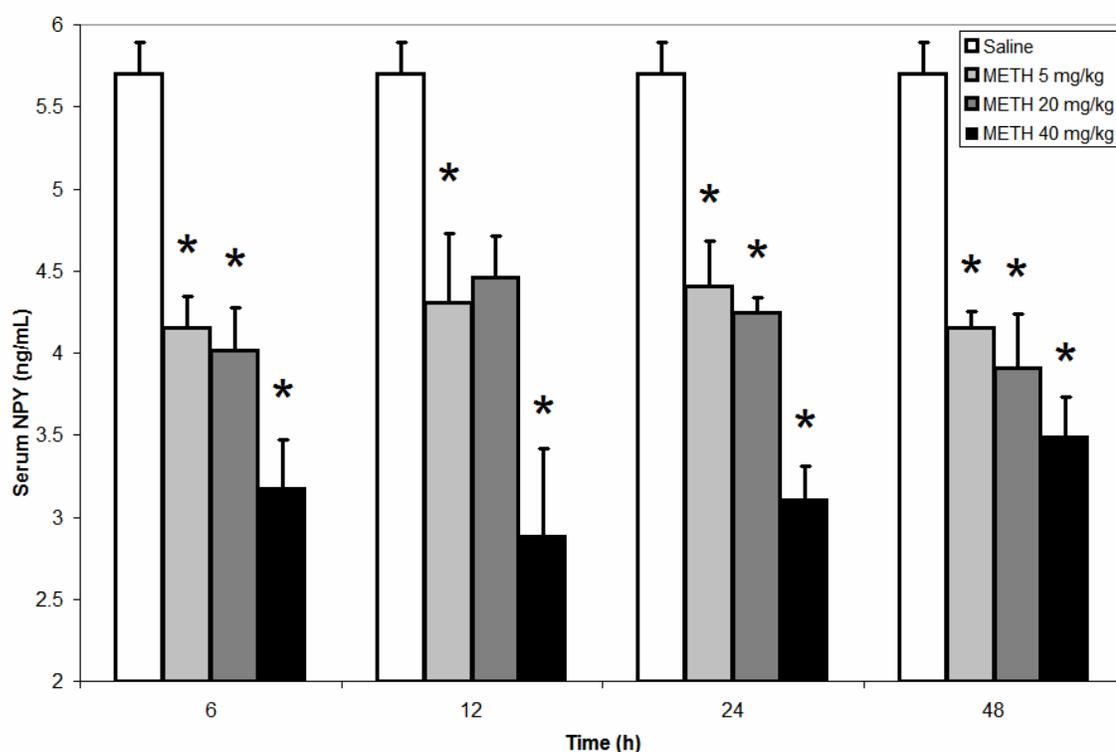


Figure 5-5. Changes in serum neuropeptide-Y following Meth administration. For saline treatments $n=6$, and for Meth treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

NPY levels appeared to decrease over time in a dose-dependent manner while no recovery was seen within the first 48 h. A one-way ANOVA test revealed serum neuropeptide-Y levels were different among treatment groups at all time points ($p<0.001$).

for all). Dunnett's post hoc pairwise comparison tests revealed statistically significant decreases virtually across the board.

Ecstasy

Weight

Changes in weight from baseline after MDMA administration was performed (Figure 5-6).

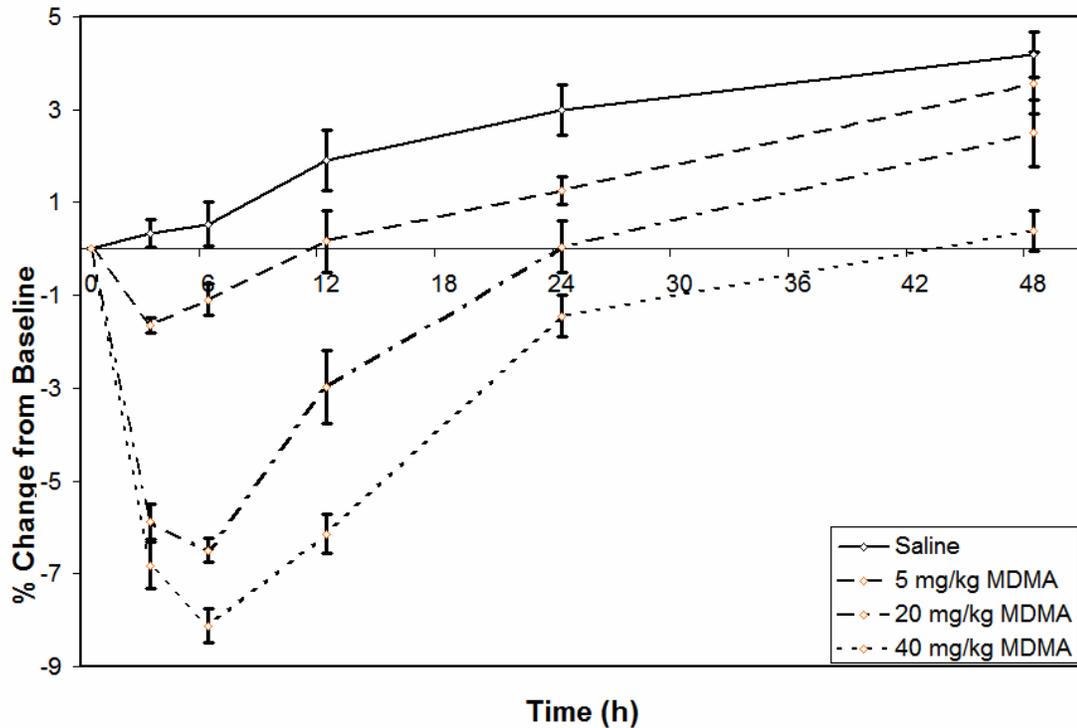


Figure 5-6. Changes in rat weight after MDMA administration. For saline treatments, n=6, for MDMA treatments, n=5. Rat weight decreased immediately following MDMA administration and began to recover after 6 h. A dose response was indicated.

For all drug treatment groups, rat weight decreased immediately in a dose-dependent manner. Maximum weight loss was achieved after 6 hours, beyond which weight gain was experienced. A one-way ANOVA test revealed that treatment groups are different for each time point ($p < 0.001$ for each).

Leptin

Analysis of the serum leptin levels of rats administered MDMA was performed (Figure 5-7).

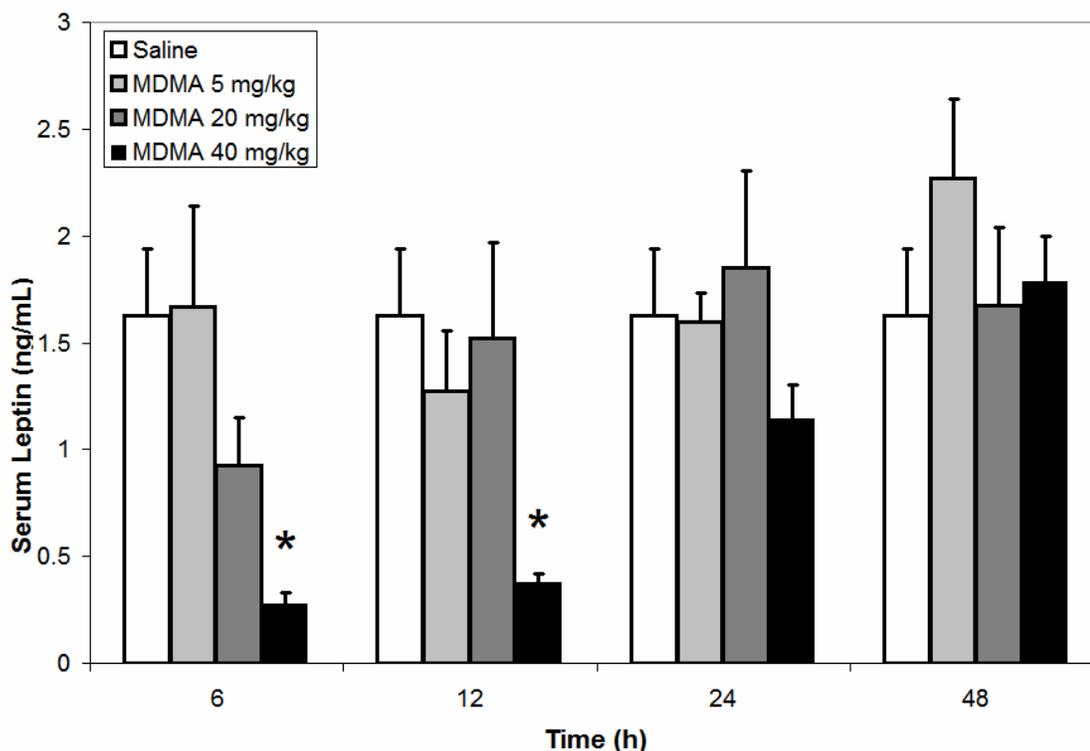


Figure 5-7. Changes in serum leptin following MDMA administration. For saline treatments $n=6$, and for MDMA treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

At the highest dose, a transient decrease in leptin was observed which recovers after 24 h.

A one-way ANOVA test revealed serum leptin levels were different among treatment groups at 6 and 12 h ($p=0.02, 0.04$). A Dunnett's post hoc pairwise comparison test revealed that the 40 mg/kg MDMA group had significantly decreased serum leptin values as compared to control values at 6 and 12 h.

Ghrelin

Analysis of the serum ghrelin levels of rats administered MDMA was performed (Figure 5-8). Similar but opposite to the leptin, a transient increase in serum ghrelin is

observed which normalizes after 24 h. A one-way ANOVA test revealed serum ghrelin levels were different among treatment groups at 6 and 12 h ($p < 0.01$ for both). A Dunnett's post hoc pairwise comparison test revealed that the 40 mg/kg MDMA group

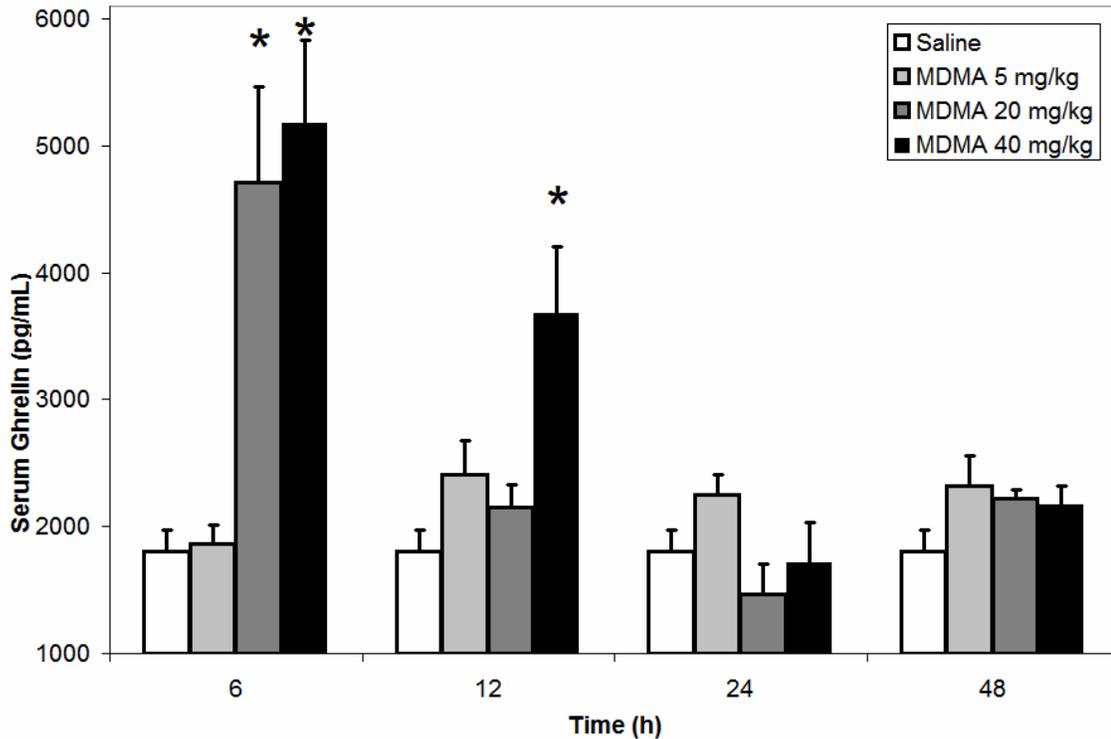


Figure 5-8. Changes in serum ghrelin following MDMA administration. For saline treatments $n=6$, and for MDMA treatments $n=5$. $*p < 0.05$ Dunnett post-hoc pairwise comparison test.

had significantly increased serum ghrelin values as compared to control values at 6 and 12 h while the 20 mg/kg MDMA group also had increased ghrelin at 6 h.

Growth hormone

Analysis of the serum growth hormone levels of rats administered MDMA was performed (Figure 5-9). Though no direct statistical significance was achieved, trends suggested a transient decrease in growth hormone, recovering after 24 h. A one-way ANOVA test revealed serum growth hormone levels were different among treatment groups at 12 h ($p=0.02$) and were trending towards significance at 6 h ($p=0.06$).

Dunnett's post hoc pairwise comparison tests were insignificant but revealed trends for decreases in growth hormone for the 20 mg/kg MDMA group at 6 h and 12 h and also the 40 mg/kg group at 12 h.

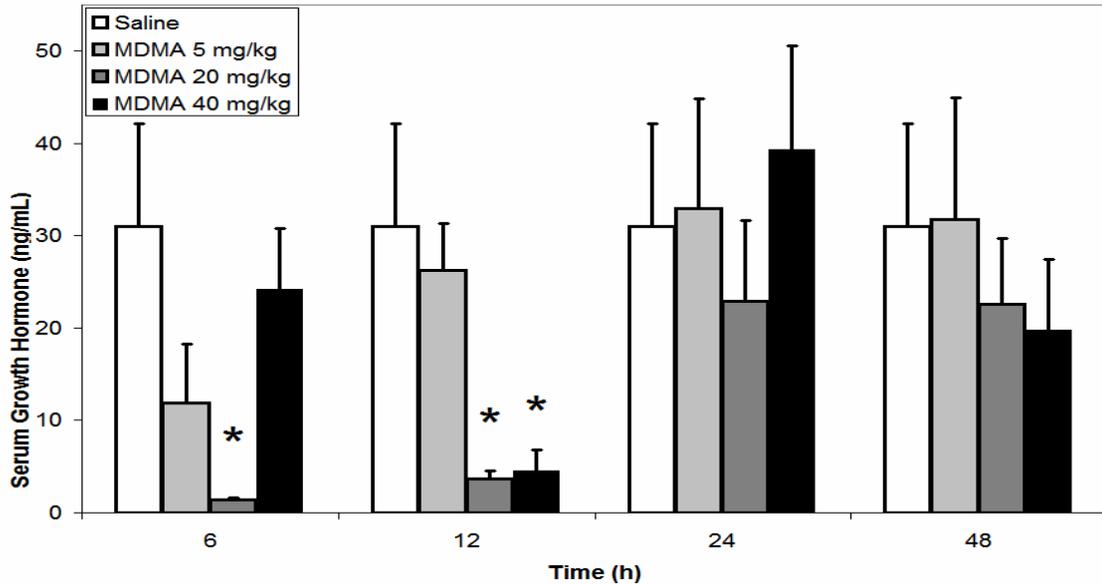


Figure 5-9. Changes in serum growth hormone following MDMA administration. For saline treatments $n=6$, and for MDMA treatments $n=5$. $*p<0.05$ Dunnett post-hoc pairwise comparison test.

Neuropeptide-Y

Analysis of the serum NPY levels of rats administered MDMA was performed (Figure 5-10). NPY levels appeared to decrease over time in a dose-dependent manner with no recovery seen within the first 48 h. A one-way ANOVA test revealed serum neuropeptide-Y levels were different among treatment groups at all time points ($p<0.01$ for all). Dunnett's post hoc pairwise comparison tests revealed a significant decrease when compared to saline controls for the 20 and 40 mg/kg doses of MDMA.

Comparison to Traumatic Brain Injury

Analyses of the serum hormone levels of rats administered traumatic brain injury (TBI) versus naïve controls were performed (Figure 5-11). Although a slight decrease in

serum leptin and ghrelin was detect in the TBI animals versus the naïve controls, they did not achieve statistical significance. There was virtually no difference in measured growth hormone, however, a significant increase in NPY was detected in the TBI group.

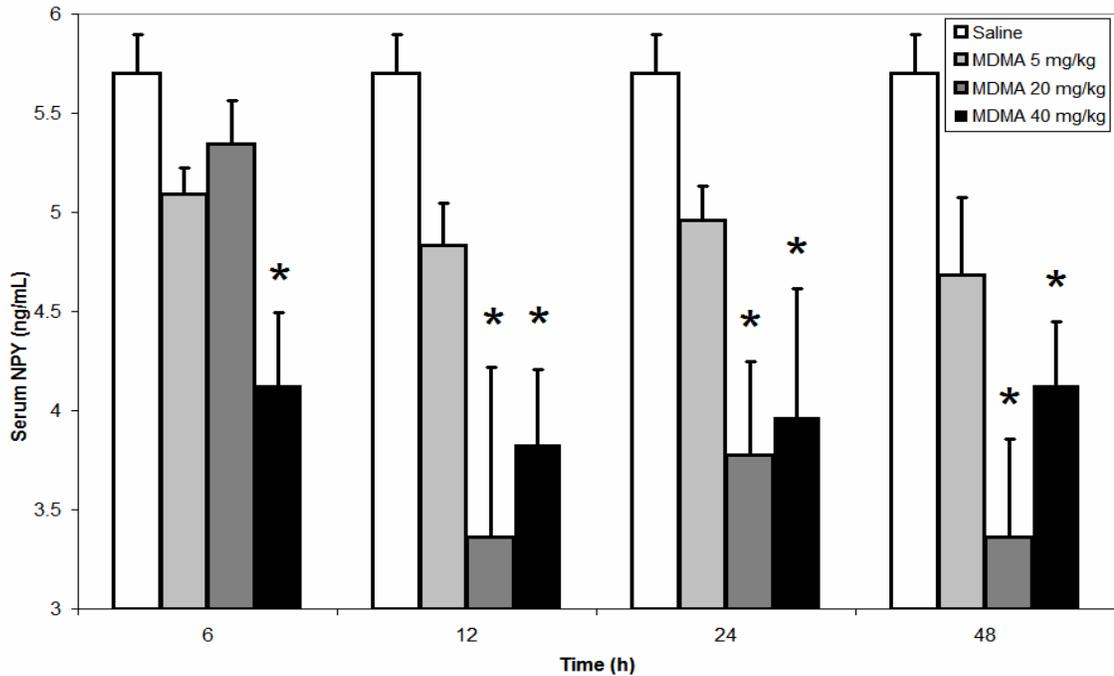


Figure 5-10. Changes in serum neuropeptide-Y following MDMA administration. For saline treatments $n=6$, and for MDMA treatments $n=5$. * $p<0.05$ Dunnett post-hoc pairwise comparison test.

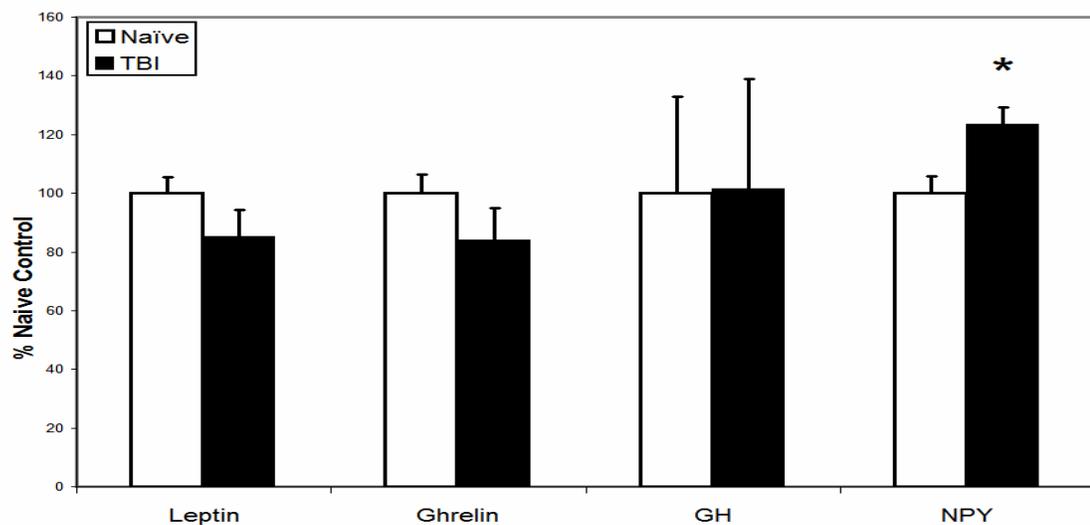


Figure 5-11. Changes in serum hormones after TBI versus naïve. For all treatments $n=5$. * $p<0.05$ Student's *T*-test.

Discussion

In this study, acute administration of MDMA and, to a lesser extent, Meth had significant effects on serum hormone levels compared in rats to controls. A dose response was indicated, in that a single 5 mg/kg MDMA dose did not have an observable effect on the serum leptin or ghrelin levels over the different time intervals, but the higher dosages (20 mg/kg and 40 mg/kg) demonstrated an initial leptin decrease and ghrelin increase when compared to controls. For the higher dosage, 40 mg/kg MDMA, both a significant reduction in serum leptin and a significant increase in serum ghrelin were observed. At the 24 and 48 h time points, the leptin and ghrelin levels of the 20 mg/kg and 40 mg/kg groups began to normalize to the control values, suggesting a transient effect.

Rats lost weight in the first 24 h after MDMA administration in previous studies (De Souza et al. 1996). All rats administered MDMA at all dose levels had shown significant weight loss, with the greatest weight loss at the highest MDMA doses. This may indicate that rats were initially in a state of negative energy balance following drug exposure, possibly due to hyperactivity and water loss but not chronic effects on satiety hormones. The fasting state would result in a rise in ghrelin levels, and because their intake is significantly reduced (De Souza et al. 1996), normal leptin secretion that is stimulated by feeding will not occur (Sanchez et al. 2004). Because the observed changes in leptin and ghrelin would be expected to increase appetite, if the animals are prevented from eating by central effects of the MDMA, then the observed changes would be expected as they respond to an empty stomach (Nogueiras et al., 2004). Thus, it may not be accurate to say that the drugs have an effect, *per se*, on the leptin and ghrelin hormones, but rather another effect on food intake which in turn affects these hormones.

A transient decrease in growth hormone was suggested by the data, but did not reach statistical significance. NPY was significantly decreased, with the 20 mg/kg and 40 mg/kg groups having the most significant reductions, but did not recover within the 48 h window of the study. These results are similar to the results found in experiments where animals given Meth and fed *ad libitum* had reduced serum leptin and increased serum ghrelin concentrations (Crowley et al. 2005). However, in that study, it was observed that the NPY expression was increased in *ad libitum*-fed animals, but inhibited in schedule-fed animals. In another study, NPY expression has been shown to be significantly decreased 24 h following amphetamine administration (Hseigh et al. 2005). NPY is a strong appetite stimulator (Kalra and Kalra 2004), and a decrease in its expression has been suggested as an underlying mechanism for psychostimulant-induced anorexia (Crowley et al. 2005). In this study, it was observed that leptin and NPY serum levels were both decreased. Leptin inhibits NPY and ghrelin stimulates NPY production; therefore, it would seem that NPY levels should be increased due to ghrelin stimulation. However, it could be possible that the increase of 5-HT released due to drug administration (Battaglia et al. 1987) may have a stronger effect on inhibiting NPY expression than ghrelin has on stimulating NPY production. Ecstasy could also be affecting the hormones individually and disrupting the feedback mechanisms that they have on one another, so that NPY and growth hormone could not be responding to ghrelin or leptin influence.

The serum hormone levels for the experimental groups administered Meth followed the same trend as the values for the groups administered MDMA, however, all the experimental values were not yet considered significant except the values for serum NPY

concentrations. A sample size larger than $n=5$ may prove to be more significant. Similar to what was observed following MDMA administration, most of the serum NPY levels for all the Meth dosage groups at all time intervals were seen to be significantly less than the control serum NPY levels.

With regards to the TBI model, differences were more difficult to detect. The increase in serum NPY should serve to stimulate appetite, but animals tend to have decreased appetite and lose weight following trauma. This could be evidence of a protective reaction not having sufficient countering effects.

The doses of Meth and MDMA administered to the rats were chosen to simulate acute usage that would be considered ‘normal’ or ‘binge’ for humans. The normal estimated dose that human MDMA users administer is between 1-4 mg/kg. Because of the interspecies scaling and administration route, the dosages given to the rats in this study would be representative of about a normal human dose (5 mg/kg) up to human “binge” doses (20 mg/kg to 40 mg/kg) (Morley et al. 2001). The duration of Meth exposure in rats and human is very different, with the half-life of Meth in rats lasting 70 min vs. 12 h in humans. Therefore, an acute Meth “binge” may be simulated in rats by administering multiple injections separated by intervals of time less than the half-life of the drug (20 mg/kg and 40 mg/kg groups) (Davidson et al. 2005; Cho et al. 2001).

These experiments observed the changes in serum hormones that occurred with an acute dose of MDMA and Meth. It was shown that leptin, and growth hormone levels initially decreased while ghrelin increased and then all three normalized towards control values after 24 h. NPY proved to be the exception; it decreased and did not normalize within the 48 h window of the study. This study indicates that drug use affects serum

appetite regulating hormones. However, since it is only an acute dose study, it cannot explain long term anorexia induced by chronic drug use. The acute Meth or MDMA dosing schedule used would be similar to an overdose in a naïve user.

Therefore, a model where chronic administration of a daily low dose of MDMA or Meth is needed for a more accurate determination of long term changes in serum hormones levels resulting from drug use. Future studies need to be performed to see how chronic administration will affect these hormones and solve questions concerning the physiological mechanisms involved in causing hypophagia that results from drug use. A chronic drug administration model, using experiments involving administration of a low dose of MDMA or Meth (2 mg/kg) to rats daily for a month, and measurements of daily food and water intake and weight change could yield more accurate and informative results. An alternative explanation for the observed chronic effects of weight loss in drug abusers may reside in the competition between food and drug for reward in the brain. Studies have shown that, at high body mass index, human patients have a tendency for lower drug use (Kleiner et al. 2004; Warren et al. 2005b). This may be due to the “hijacking” of the reward center nucleus accumbens (Hernandez and Hoebel 1988) or alteration of dopamine receptors (Wang et al. 2001).

This study indicates that acute administration of MDMA and Meth affects the serum levels of leptin, ghrelin, growth hormone and NPY in rats. MDMA administration (40 mg/kg) caused transiently reduced leptin levels and increased ghrelin levels compared to control groups. A transient decrease in growth hormone is also suggested. Both MDMA and Meth administration resulted in significantly reduced NPY levels. The impact of Meth and MDMA on serum appetite hormone levels has important societal

implications for studying the mechanisms of drug-induced anorexia. Further studies need to be performed to examine the effects of chronic drug administration on appetite regulation to potentially understand why hypophagia results from drug use.

CHAPTER 6 CONCLUSIONS

Summary and Application of Findings

The findings incorporated in this dissertation represent the first attempt to study drug abuse from the proteomic approach currently employed in traumatic brain injury. This approach has the potential to present to clinicians and scientists powerful tools for understanding the mechanism of pathology and the diagnosis and treatment of drug-related diseases. The development of simple, rapid and accurate blood or tissue tests based on proteomic markers, similar to those being developed for traumatic brain injury (TBI), will assist in the establishment of clinical endpoints for determining the existence and extent of injury as well as the progress of treatment. Proteomic tools elucidating the cascade of steps involved in toxicity at a molecular level will further our understanding of biological changes and will help to develop and target new pharmacologic agents for the treatment of related diseases. Spectrin and tau breakdown products were identified in brain tissue following drug exposure, allowing for mechanistic study. Results from these studies imply that a single dose of methamphetamine may be as neurotoxic as a TBI. Neuroendocrine hormones were also found to respond to drug use.

The *in vivo* rat models showed that the level of neurotoxicity in the cortex and hippocampus was at least equivalent to the model of TBI. The impact of drug abuse on cell degeneration has important prevention and clinical implications. Given the effects of the drug on the cortex and hippocampus, cortical- and memory-related clinical outcomes should be considered when treating a patient with methamphetamine toxicity. Calpain-

associated necrosis was evident in both brain regions. Caspase-associated apoptosis was clear in the hippocampus after 24 h and appeared later in the cortex. The evidence of damage was more striking for methamphetamine (Meth) than ecstasy (MDMA), which does not come as a surprise as it correlates with clinical data. On the other hand, MDMA was shown to induce apoptosis in the liver. Immunohistochemistry revealed the co-localization of either upregulated calpain-1 or caspase-3 in cortical neurons, but not glial cells, after drug use. Specific breakdown products for spectrin were also co-localized with neuronal marker NeuN and were particularly noticeable in the axons.

The *in vitro* studies showed a dose and time-dependent vulnerability of cultured neurons to club drug insult. It is worth noting that our drug abuse studies were able to elucidate toxicity at concentrations lower than that of other experiments (1 to 2 mM compared to 3 and 5 mM). The use of protease inhibitors was successful in reducing specific pathways of neurotoxicity. Furthermore, it was apparent that when one pathway of proteolysis is inhibited, an alternative pathway is enhanced. The results imply that MDMA and Meth serve to activate both the pro-apoptotic caspase system and the pro-necrotic calpain system concurrently.

Neuroendocrine studies showed that changes in serum hormones occurred with use of MDMA and Meth in a dose dependent manner. Leptin and growth hormone levels initially decreased while ghrelin increased with all hormones normalizing towards control values after 24 h. Neuropeptide-Y decreased for both drugs and did not normalize within the 48 h window of the study. This experiment indicates that drug use affects serum appetite regulation hormones, however, since it is only an acute dose study, it cannot explain the long term anorexia induced by chronic drug use.

Future Directions

This work serves a dual function. First, it lays the foundation a method of drug toxicity investigation from the perspective of traumatic brain injury, a technique never before used. Second, it calls for further studies which need to be conducted to complete our understanding of the effects that club drugs have physiologically. For example, other brain regions such as the cerebellum, striatum and thalamus need to be examined. A low-dose, multiple administration chronic model examination to discover if similar neurotoxicity is seen when compared to this acute, single-use model needs to be performed. The combination of club drug usage with TBI, as opposed to in comparison, would provide a model for the clinical scenario of a motor vehicle accident while under the influence of drugs. In addition to rat studies, post-mortem studies could be performed in human brain tissues following deaths involving club drugs after they have been identified in the blood. Of particular interest would be to study the use of the calpain and caspase inhibitors in clinical trials to treat drug induced proteolysis. Similar work has already begun with some success in TBI models (Buki et al. 2003; Knoblach et al. 2004). Finally, the basic principles applied here could be used to study other drugs of abuse such as stimulants or hallucinogens.

APPENDIX HEPATOTOXICITY

Ecstasy (MDMA) has been shown to be hepatotoxic in humans, with some evidence of apoptosis in cultured hepatocytes. Illustrating the toxicity and understanding its mechanism is important to developing preventions and treatments for MDMA abuse patients. Here, we analyzed the liver from club drug exposed rats using Western blot analysis to evaluate hepatotoxicity as evidenced by α II-spectrin breakdown products (SBDPs). Degradation of α II-spectrin proteins as a result of MDMA or methamphetamine (Meth) exposure after 72 h is evident (Figure A-1).

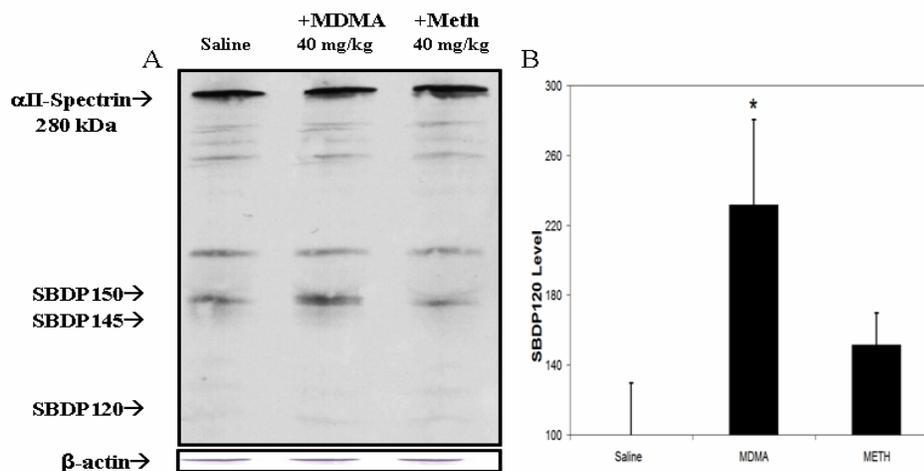


Figure A-1. α II-Spectrin proteolysis and accumulation in rat liver 72 h after MDMA or Meth exposure.⁴ A) Breakdown products of spectrin was seen in the liver tissue lysates after drug exposure at 150, 145 and 120 kDa. β -actin was shown as an internal standard. B) Accumulation of α II-spectrin breakdown products. * $p < 0.05$ One-way ANOVA with Bonferroni post-hoc pairwise comparison test for treatment versus negative control.

⁴ Figure A-1 reprinted from Journal of Addictive Diseases, In press, Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KKW, Ecstasy toxicity: A comparison to methamphetamine and traumatic brain injury, 2006, with permission from The Haworth Press, Binghamton NY. Article copies available from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: docdelivery@haworthpress.com ; <http://www.HaworthPress.com> .

Breakdown product signal is weaker for the liver than for previous cortex studies, but appear to be increased over saline treated liver controls (Fig. A-1A). Quantitative analysis of the pro-apoptotic SBPD120 (Fig. A-1B) suggests that the 40 mg/kg dose of MDMA produces a significant increase over saline controls after 72 h ($p=0.05$). The evidence of liver apoptosis after MDMA exposure, which was not seen with Meth exposure, is concerning. Post-mortem studies should be performed in human liver tissues following deaths involving MDMA presence to see if the same biomarkers are found.

LIST OF REFERENCES

- Ali S, Chang L, Slikker W. (1991) Biogenic amines as biomarkers for neurotoxicity. *Biomedical Environmental Science* 4(1-2):207–216.
- Alonso A, Del C, Zaidi T, Novak M, Grundke-Iqbal I, Iqbal K. (2001) Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. *Proceedings of the National Academy of Sciences* 98:6923–6928.
- Ameisen J. C. (2002) On the origin, evolution and nature of programmed cell death: a timeline of four billion years. *Cell Death and Differentiation* 9:367–393.
- Azam M. (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Molecular and Cellular Biology* 21:2213–2220.
- Baggott M, Heifets B, Jones RT, Mendelson J, Sferios E, Zehnder BS. (2000) Chemical Analysis of Ecstasy Pills. *Journal of the American Medical Association*. 284:2190.
- Bahr B A, Tiriveedhi S, Park GY, Lynch G. (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *Journal of Pharmacology and Experimental Therapeutics* 273:902–908.
- Bakhit C, Morgan MA, Peat MA, Gibb JW. (1981) Long-term effects of methamphetamine on the synthesis and metabolism of 5-hydroxytryptamine in various regions of the rat brain. *Neuropharmacology* 20:1135–1140.
- Banik NL, Matzelle DC, Gantt-Wilford G, Osborne, Hogan EL. (1997) Increased calpain content and progressive degradation of neurofilament protein in spinal cord injury. *Brain Research* 752:301–306.
- Battaglia G, Yeh S, O’Hearn E, Molliver M, Kuhar M, De Souza E. (1987) 3,4 Methylenedioxymethamphetamine and 3,4 methylenedioxyamphetamine destroy serotonin terminals in rat brain: Quantification of neurodegeneration by measurement of [3H] paroxetine-labeled serotonin uptake sites. *Journal of Pharmacology and Experimental Therapeutics* 242(3):911–91.
- Baucum AJ, Rau KS, Riddle EL, Hanson GR, Fleckenstein AE. (2004) Methamphetamine increases dopamine transporter higher molecular weight complex formation via a dopamine- and hyperthermia-associated mechanism. *Journal of Neuroscience* 24:3436–3443.

- Bernath E, Kupina N, Liu MC, Hayes RL, Meegan C, Wang KKW. (2006) Elevation of cytoskeletal protein breakdown in aged Wistar rat brain. *Neurobiology of Aging* 27(4):624–632.
- Buki A, Farkas O, Doczi T, Povlishock J. (2003) Preinjury administration of calpain inhibitor MDL-28170 attenuates traumatically induced axonal injury. *Journal of Neurotrauma* 20(3):261–268.
- Bullock R, Chestnut R, Clifton G. (1995) Guidelines for the management of severe brain injury. New York: Brain Trauma Foundation/American Association of Neurological Surgeons.
- Cadet JL, Ordonez SV, Ordonez JV. (1997) Methamphetamine induces apoptosis in immortalized neural cells: Protection by the proto-oncogene, bcl-2. *Synapse* 25:176–184.
- Cadet JL, Thiriet N. (2001) Acute transcriptional effects of MDMA on the rat cortex: Evidence from cDNA analysis. Poster presented at MDMA/Ecstasy Research: Advances, Challenges and Future Directions, Bethesda, MD.
- Cadet JL, Javanthi S, Deng X. (2003) Speed kills: Cellular and molecular basis of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. *Federation of American Societies for Experimental Biology Journal* 13:1775–1788.
- Canu N, Dus L, Barbato C, Ciotti M, Brancolini C, Rinaldi A, Novak M, Cattaneo A, Bradbury A, Calissano P. (1998) Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. *Journal of Neuroscience* 18(18):7061–7074.
- Carafoli E, Molinari M. (1998) Calpain: a protease in search of a function? *Biochemistry and Biophysics Research Communications* 247:193–203.
- Centers for Disease Control. (1997) Sports-related recurrent brain injuries – United States. *MMWR* 46(10):224–227.
- Centers for Disease Control. (2001) Annual data submission standards: Guidelines for surveillance of central nervous system injury. Atlanta, GA.
- Centers for Disease Control. (2006) Methamphetamine use and HIV risk behaviors among heterosexual men—preliminary results from five northern California counties, December 2001–November 2004. *Morbidity and Mortality Weekly Report* 55(10):273–277.
- Chan Y, Steiner R, Clifton D. (1996) Regulation of hypothalamic neuro peptide-Y neurons by growth hormone in the rat. *Endocrinology* 137(4):1319–1325.
- Chan SL, Mattson MP. (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. *Journal of Neuroscience Research* 58:167–190.

- Chen J, Li Y, Wang L, Lu M, Chopp M. (2002) Caspase inhibition by z-VAD increases the survival of grafted bone marrow cells and improves functional outcome after MCAo in rats. *Journal of the Neurological Sciences* 199:17–24.
- Cho A, Melega W, Kuczenski R, Segal D. (2001) Relevance of pharmacokinetic parameters in animal models of methamphetamine abuse. *Synapse* 39:161–166.
- Choi DW, Rothman SM. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annual Review of Neuroscience* 13:171–182.
- Choi H, Yoo T, Chung S, Yang J, Kim J, Ha E, Hwang O. (2002) Methamphetamine-induced apoptosis in a CNS-derived catecholaminergic cell line. *Molecules and Cells* 13(2):221–227.
- Chua BT. (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *Journal of Biological Chemistry* 275:5131–5135.
- Chung CW, Song YH, Kim IK, Yoon WJ, Ryu BR, Jo DG, Woo HN, Kwon YK, Kim HH, Gwag BJ, Jung IHM, Jung YK. (2001) Proapoptotic effects of tau cleavage product generate by caspase-3. *Neurobiology of Disease* 8:162–172.
- Clark RS, Kochanek PM, Watkins SC, Chen M, Dixon CE, Seidberg NA, Melick J, Loeffert JE, Nathaniel PD, Jin KL, Graham SH. (2000) Caspase-3 mediated neuronal death after traumatic brain injury in rats. *Journal of Neurochemistry* 74:740–753.
- Commins DL, Axt KJ, Vosmer G, Seiden LS. (1987) 5,6-Dihydroxytryptamine, a serotonergic neurotoxin is formed endogenously in the rat brain. *Brain Research* 403:7–14.
- Cohen GM. (1997) Caspases: the executioners of apoptosis. *Biochemical Journal* 326:1–16.
- Conti AC, Raghupathi R, Trojanowski JQ, McIntosh TK. (1998) Experimental brain injury induces regionally distinct apoptosis during the acute and delayed posttraumatic period. *Journal of Neuroscience* 18:5663–5672.
- Croall DE, DeMartino GN. (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiological Reviews* 81:813–847.
- Crowley W, Ramoz G, Keefe K, Torto R, Kalra S, Hanson G. (2005) Differential effects of methamphetamine on expression of neuropeptide Y mRNA in hypothalamus and on serum leptin and ghrelin concentrations in ad libitum-fed and schedule-fed rats. *Neuroscience* 132:167–173.
- Curzon G, Gibson E, Oluyomi A. (1997) Appetite suppression by commonly used drugs depends on 5-HT receptors but not on 5-HT availability. *Trends in Pharmacological Sciences* 18(1):21–25.

- Dafters RI, Lynch E. (1998) Persistent loss of thermoregulation in the rat induced by 3,4-methylenedioxymethamphetamine (MDMA or "Ecstasy") but not by fenfluramine. *Psychopharmacology* 138:207–212.
- Darvesh AS, Shankaran M, Gudelsky GA. (2001) MDMA produces glycogenolysis and increases the extracellular concentration of glucose in the rat brain. Poster presented at MDMA/Ecstasy Research: Advances, Challenges and Future Directions, Bethesda, MD.
- Davidson C, Lee T, Ellinwood E. (2005) Acute and chronic continuous methamphetamine have different long-term behavioral and neurochemical consequences. *Neurochemistry International* 46:189–203.
- Drug Abuse Warning Network. (2003) Series D-24, DHHS Publication No. (SMA) 03-3780, Rockville, MD. Published August, 2003.
- Denecker G, Vercammen D, Steemans M, Vanden Berghe T, Brouckaert G, Van Loo G, Zhivotovsky B, Fiers W, Grooten J, Declercq W, Vandenabeele P. (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. *Cell Death and Differentiation* 8:829–840.
- Deng X, Jayanthi S, Ladenheim B, Krasnova I, Cadet JL. (2002a) Mice with partial deficiency of c-Jun show attenuation of methamphetamine-induced neuronal apoptosis. *Molecular Pharmacology* 62(5):993–1000.
- Deng X, Cai N, McCoy M, Chen W, Trush M, Cadet JL. (2002b) Methamphetamine induces apoptosis in an immortalized rat striatal cell line by activating the mitochondrial cell death pathway. *Neuropharmacology* 42(6):837–845.
- De Souza I, Kelly J, Harkin A, Leonard B. (1996) An appraisal of the pharmacological and toxicological effects of a single oral administration of 3,4-methylenedioxymethamphetamine (MDMA) in the rat. *Pharmacological and Toxicology* 80:207–210.
- De Vito M, Wagner G. (1989) Methamphetamine-induced neuronal damage: a possible role for free radicals. *Neuropharmacology* 28(10):1145–1150.
- Dietrich WD. (1994) Morphological manifestations of reperfusion injury in brain. *Annals of the New York Academy of Sciences* 723:15–24.
- Dixon CE, Clifton GL, Lighthall JW, Yahhmai AA, Hayes RL. (1991) A controlled cortical impact model of traumatic brain injury in the rat. *Journal of Neuroscience Methods* 39:253–262.
- Dutta S, Chiu Y, Probert A, Wang, KKW. (2002) Selective release of calpain produced alphaII-spectrin (alpha-fodrin) breakdown products from acute neuronal cell death. *Biological Chemistry* 383(5):785–791.

- Earnshaw WC, Martins LM, Kaufmann SH. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual Review of Biochemistry* 68:383–424.
- Egecioglu E, Bjursell M, Ljungberg A, Dickson S, Kopchick J, Bergstrom G, Svensson L, Oscarsson J, Tornell J, Bohlooly M. (2005) Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice. *American Journal of Physiology – Endocrinology and Metabolism* 290(2):317–325.
- Ellis RC, O’Steen WA, Hayes RL, Nick HS, Wang KKW, Anderson DK. (2005) Cellular localization and enzymatic activity of cathepsin B after spinal cord injury in the rat. *Experimental Neurology* 193(1):19–28.
- Fischer U, Janicke RU, Schulze-Osthoff K. (2003) Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death and Differentiation* 10:76–100.
- Fukiage C, Azuma M, Nakamura Y, Tamada Y, Makamura M, Shearer TR. (1997) SJA6017, a newly synthesized peptide aldehyde inhibitor of calpain: Amelioration of cataract in cultured rat lenses. *Biochimica et Biophysica Acta* 1361:304–312.
- Genc K, Genc S, Kizildag S, Sonmez U, Yilmaz O, Tugyan K, Ergur B, Sonmez A, Buldan Z. (2003) Methamphetamine induces oligodendroglial cell death in vitro. *Brain Research* 982(1):125–130.
- Gennarelli TA. (1993) Mechanisms of brain injury. *Journal of Emergency Medicine* 11 Suppl 1:5–11.
- Ginawi O, Al-Majed A, Al-Suwilem A. (2005) Ondansetron, a selective 5-HT₃ antagonist, antagonizes methamphetamine-induced anorexia in mice. *Pharmacological Research* 51:255–259.
- Gluck M, Moy L, Jayatilleke E, Hogan K, Manzano L, Sonsalla P. (2001) Parallel increases in lipid and protein oxidative markers in several mouse brain regions after methamphetamine treatment. *Journal of Neurochemistry* 79(1):152–160.
- Gonzalez R, Rippeth JD, Carey CL, Heaton RK, Moore DJ, Schweinsburg B. (2004) Neurocognitive performance of methamphetamine users discordant for history of marijuana exposure. *Drug and Alcohol Dependence* 72(2):181–190.
- Goodman JC, Simpson RK. (1996) Biochemical monitoring in head injury. In: R. K. Narayan, J. E. Wilberger and J. T. Povlishock, eds. *Neurotrauma*, pp. 577–591. McGraw-Hill, New York.
- Goodman SR, Zagon IS, Kulikowski RR. (1981) Identification of a spectrin-like protein in nonerythroid cells. *Proceedings of the National Academy of Science* 78:7570–7574.

- Graham DI, Adams JH, Nicoll JA, Maxwell WL, Gennarelli TA. (1995) The nature, distribution and causes of traumatic brain injury. *Brain Pathology* 5:397–406.
- Graham DI, McIntosh TK, Maxwell WL, Nicoll JA. (2000) Recent advances in neurotrauma. *Journal of Neuropathology and Experimental Neurology* 59:641–651.
- Guroff G. (1964) A neutral calcium-activated proteinase from the soluble fraction of rat brain. *Journal of Biological Chemistry* 239:149–155.
- Halford J, Blundell J. (2000) Pharmacology of appetite suppression. *Progress in Drug Research* 54:25–58.
- Harris AS, Croall DE, Morrow JS. (1989) The calmodulin-binding site in α -fodrin is near the calcium dependant protease-I cleavage site. *Journal of Biological Chemistry* 263:15754–15761.
- Hatzidimitriou G, McCann UD, Ricaurte GA. (1999) Altered serotonin innervation patterns in the forebrain of monkeys treated with (+/-) 3,4 methylenedioxymethamphetamine seven years previously: factors influencing abnormal recovery. *Journal of Neuroscience* 19:5096–5107.
- Hengartner MO. (2000) The biochemistry of apoptosis. *Nature* 407:770–776.
- Herndon DN, Hart DW, Wolf SE, Chinkes DL, Wolfe RR. (2001) Reversal of catabolism by beta-blockade after severe burns. *New England Journal of Medicine* 345:1223–1229.
- Hosoda H, Kojima M, Kangawa K. (2002) Ghrelin and the regulation of food intake and energy balance. *Molecular Interventions* 2:494–503.
- Hsieh Y, Yang S, Chiou H, Kuo D. (2005) Activations of c-fos/c-jun signaling are involved in the modulation of hypothalamic superoxide dismutase (SOD) and neuropeptide-Y (NPY) gene expression in amphetamine-mediated appetite suppression. *Toxicology and Applied Pharmacology*. August 3 [Epub ahead of print].
- Huber A, Ling W, Shoptaw W, Gulati V, Brethen P. (1997) Integrating treatments for methamphetamine abuse: A psychosocial perspective. *Journal of Addictive Disease* 16(4):41–50.
- Ishigami A, Tokunaga I, Gotohda T, Kubo S. (2003) Immunohistochemical study of myoglobin and oxidative injury-related markers in the kidney of methamphetamine abusers. *Legal Medicine (Tokyo)* 5(1):42–48.
- Islam S, Hossain K, Ahmed A, Ahsan M. (2002) Nutritional status of drug addicts undergoing detoxification: prevalence of malnutrition and influence of illicit drugs and lifestyle. *British Journal of Nutrition* 88(5):507–513.

- Jaehne E, Salem A, Irvine R. (2005) Effects of 3,4-methylenedioxymethamphetamine and related amphetamines on autonomic and behavioral thermoregulation. *Pharmacology Biochemistry and Behavior* 81:485–496.
- Jiminez A, Jorda EG, Verdaguer E, Pubill D, Sureda FX, Canudas AM, Escubedo E, Camarasa J, Camins A, Pallas M. (2004) Neurotoxicity of amphetamine derivatives is mediated by caspase pathway activation in rat cerebellar granule cells. *Toxicology and Applied Pharmacology* 196(2):223–234.
- Lockshin RA, Zakeri, Z. (2001) Programmed cell death and apoptosis: origins of the theory. *Nature Reviews - Molecular Cell Biology* 2:545–550.
- Jacobson MD, Weil M, Raff MC. (1997) Programmed cell death in animal development. *Cell* 88: 347–354.
- Jonas J, Gold MS. (1986) Cocaine abuse and eating disorders. *Lancet* 1:390–391.
- Kalra S, Kalra P. (2004) Overlapping and interactive pathways regulation appetite and craving. *Journal of Addictive Diseases* 23(3):5–21.
- Kaufmann SH, Hengartner MO. (2001) Programmed cell death: alive and well in the new millennium. *Trends in Cell Biology* 11:526–534.
- Kerr JF, Wyllie AH, Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26:239–257.
- Kesler SR, Adams HF, Bigler ED. (2000) SPECT, MR and quantitative MR imaging: correlates with neuropsychological and psychological outcome in traumatic brain injury. *Brain Injury* 14:851–857.
- Kish SJ, Furukawa Y, Ang L, Vorce SP, Kalasinsky KS. (2000) Striatal serotonin is depleted in brain of a human MDMA (Ecstasy) user. *Neurology* 55: 294–296.
- Kleiner K, Gold MS, Frost-Pineda K, Lenz-Brunsmann B, Perri. (2004) Body Mass Index and Alcohol Use. *Journal of Addictive Disease* 23(3):105–118.
- Komoriya A, Packard BZ, Brown MJ, Wu ML, Henkart PA. (2000) Assessment of caspase activities in intact apoptotic thymocytes using cell-permeated fluorogenic caspase substrates. *The Journal of Experimental Medicine* 191(11):1819–1828.
- Kontos HA. (1989) Oxygen radicals in CNS damage. *Chemico-Biological Interactions* 72:229–255.
- Knoblach S, Alroy D, Nikolaeva M, Cernak I, Stoica B, Faden A. (2004) Caspase inhibitor z-DEVD-fmk attenuates calpain and necrotic cell death in vitro and after traumatic brain injury. *Journal of Cerebral Blood Flow and Metabolism* 24(10):1119–1132.

- Koda LY, Gibb JW. (1973) Adrenal and striatal tyrosine hydroxylase activity after methamphetamine. *Journal of Pharmacology and Experimental Therapeutics* 185:42-48.
- Koh JY, Choi DW. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *Journal of Neuroscience Methods* 20(1):83-90.
- Kupina NC, Nath R, Bernath EE, Inoue J, Misuyoshi A, Yuen PW, Wang KKW, Hall ED. (2001) The novel calpain inhibitor SJA6017 improves functional outcome after delayed administration in a mouse model of diffuse brain injury. *Journal of Neurotrauma* 18(11):1229-1240.
- Larner SF, Hayes RL, McKinsey DM, Pike BR, Wang KKW. (2004) Increased expression and processing of caspase-12 after traumatic brain injury in rats. *Journal of Neurochemistry* 88(1):78-90.
- Larner SF, McKinsey DM, Hayes RL, Wang KKW. (2005) Caspase 7: Increased expression and activation after traumatic brain injury in rats. *Journal of Neurochemistry* 94(1):97-108.
- Larsen K, Fon E, Hastings T, Edwards R, Sulzer D. (2002) Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. *Journal of Neuroscience* 22(20):8951-8960.
- Launay S, Hermine O, Fontenay M, Kroemer G, Solary E, Garrido C. (2005) Vital functions for lethal caspases. *Oncogene* 24:5137-5148.
- Levi L, Guilburd JN, Lemberger A, Soustiel JF, Feinsod M. (1990) Diffuse axonal injury: analysis of 100 patients with radiological signs. *Neurosurgery* 27:429-432.
- Makino Y, Tanaka S, Kurobane S, Nakauchi M, Terasaki T, Ohta S. (2003) Profiling of illegal amphetamine type stimulant tablets in Japan. *Journal of Health Science* 49:129-137.
- Malpass A, White J, Irvine R, Somogyi A, Bochner F. (1999) Acute toxicity of 3,4-methylenedioxymethamphetamine (MDMA) in Sprague-dawley and Dark Agouti rats. *Pharmacology, Biochemistry and Behavior* 64(1):29-34.
- Mansoor O, Beaufreere B, Boirie Y, Ralliere C, Taillandier D, Aourousseau E, Schoeffler P, Arnal M, Attaix D. (1996) Increased mRNA levels for components of the lysosomal, Ca²⁺-activated, and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients. *Proceedings of the National Academy of Sciences* 93:2714-2718.
- Marchesi VT, Steers E. (1968) Selective solubilization of a protein component of the red cell membrane. *Science* 159:203-204.

- Marion DW. (1996) Outcome from severe head injury. In: R. K. Narayan, J. E. Wilberger and J. T. Povlishock, eds. *Neurotrauma*, pp. 767–777. McGraw-Hill, New York.
- Martin C, Warren MW, Gold MS. (2003) Methamphetamine. *Law Enforcement Executive Forum* 3(5):159–170.
- Mashima T, Naito M, Kataoka S, Kawai H, Tsuruo T. (1995) Aspartate-based inhibitor of interleukin-1 beta-converting enzyme prevents antitumor agent-induced apoptosis in human myeloid leukemia U937 cells. *Biochemical and Biophysical Research Communications* 209:907–915.
- McCann UD, Szabo Z, Scheffel U, Dannals RF, Ricaurte GA. (1998) Positron emission tomographic evidence of toxic effect of MDMA ('Ecstasy') on brain serotonin neurons in human beings. *Lancet* 352:1433–1437.
- Metzstein MM, Stanfield GM, Horvitz HR. (1998) Genetics of programmed cell death in *C. elegans*: past, present and future. *Trends in Genetics* 14:410–416.
- Meyer JS, Grande M, Johnson K, Ali SF. (2004) Neurotoxic effects of MDMA ("ecstasy") administration to neonatal rats. *International Journal of Developmental Neuroscience* 22(5-6):261–271.
- Montiel-Duarte C, Varela-Rey M, Oses-Prieto JA, Lopez-Zabalza MJ, Beitia G, Cenarruzabeitia E, Iraburu MJ. (2002) 3,4-Methylenedioxymethamphetamine ("Ecstasy") induces apoptosis of cultured rat liver cells. *Biochimica et Biophysica Acta* 1588(1):26–32.
- Morgan MJ. (1999) Memory deficits associated with recreational use of "ecstasy" (MDMA). *Psychopharmacology* 141: 30–36.
- Morley K, Gallate J, Hunt G, Mallet R, McGregor I. (2001) Increased anxiety and impaired memory in rats 3 months after administration of 3,4-Methylenedioxymethamphetamine ("Ecstasy"). *European Journal of Pharmacology* 433:91–99.
- Nakagawa T, Yuan J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *Journal of Cellular Biology* 150:887–894.
- Nash JF, Nichols DE. (1991) Microdialysis studies of 3,4-methylenedioxymethamphetamine and structurally related analogues. *European Journal of Pharmacology* 200:53–58.
- Nath R, Raser K, McGinnis K, Madimpalli R, Stafford D, Wang KKW. (1996) Effects of calpain and ICE-like protease inhibitors on neuronal apoptosis. *Neuroreport* 8:249–256.

- Nath R, Probert A, McGinnis KM, Wang KKW. (1998) Evidence for activation of caspase-3-like protease in excitotoxic- and hypoxia/hypoglycemia-injured neurons. *Journal of Neurochemistry* 71(1):186–195.
- Nath R, Huggins M, McGinnis K, Nadimpalli D, Wang KKW. (2000) Development and characterization of antibodies specific to caspase-3-produced alpha II-spectrin 120 kDa breakdown product: Marker for neuronal apoptosis. *Neurochemistry International* 36:351–361.
- National Association of Counties. (2005) The meth epidemic in America. National Association of Counties, July 5, 2005.
- National Institute on Drug Abuse. (1997) Methamphetamine Abuse (NIDA Capsules).
- National Institute on Drug Abuse. (2002) NIDA research report – Methamphetamine abuse and addiction. NIH Publication No. 02-4210.
- National Institute on Drug Abuse. (2005a) National Survey Results on Drug Use from Monitoring the Future Study, 1975–2004.
- National Institute on Drug Abuse. (2005b) NIDA research report – MDMA abuse (Ecstasy). NIH Publication No. 05-4728.
- Nicholson W, Thornberry N. (1997) Caspases, killer proteases. *Trends in Biochemical Sciences* 22:299–306.
- National Institute on Drug Abuse. (2006) Community epidemiology work group: A retrospective analysis of drug abuse data/information reported by the CEWG.
- Newcomb JK, Zhao X, Pike BR, Hayes RL. (1999) Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat. *Experimental Neurology* 158:76–88.
- Nogueiras R, Tovar S, Mitchell S, Rayner D, Archer Z, Dieguez C, Williams L. (2004) Regulation of growth hormone secretagogue receptor gene expression in the arcuate nuclei of the rat by leptin and ghrelin. *Diabetes* 53:2552–2558.
- Nordahl TE, Salo R, Leamon M. (2003) Neuropsychological effects of chronic methamphetamine use on neurotransmitters and cognition: A review. *Journal of Neuropsychiatry and Clinical Neuroscience* 15(3):317–325.
- Park SY, Ferreira A. (2005) The generation of a 17 kDa neurotoxic fragment: An alternative mechanism by which tau mediates β -amyloid-induced neurodegeneration. *The Journal of Neuroscience* 25(22):5365–5375.
- Parrott AC. (2001) Neuropsychopathology associated with recreational Ecstasy/MDMA. Presented at MDMA/Ecstasy Research: Advances, Challenges and Future Directions, Bethesda, MD.

- Paul S, Hulihan-Giblin B, Skolnick P. (1982) (+/-)Amphetamine binding to rat hypothalamus: Relation to anorexic potency for phenylethylamines. *Science* 218(4571):487–490.
- Pettigrew L, Holtz M, Craddock S, Minger S, Hall N, Geddes J. (1996) Microtubular proteolysis in focal cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism* 16(6):1189–1202.
- Pike BR, Zhao X, Newcomb JK, Posmantur RM, Wang KKW, Hayes RL. (1998) Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury. *Neuroreport* 9:2437–2442.
- Pike B, Flint J, Dutta S, Johnson E, Wang KKW, Hayes RL. (2001) Accumulation of non-erythroid α II-spectrin and calpain-cleaved α II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. *Journal of Neurobiochemistry* 78:1297–1306.
- Pike B, Flint J, Jitendra D, Lu X, Wang KKW, Tortella F, Hayes RL. (2003) Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived α II-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats, *Journal of Cerebral Blood Flow and Metabolism* 24:98–106.
- Pineda J, Wang KKW, Hayes RL. (2004) Biomarkers of proteolytic damage following traumatic brain injury. *Brain Pathology* 14(2):202–209.
- Postmantur R, Wang KKW, Gilbersten RB. (1998) Caspase-3-like activity is necessary for IL-2 release in activated Jurkat T-cells. *Experimental Cell Research* 244(1):302–309.
- Raghupathi R, Graham D, McIntosh T. (2000) Apoptosis after traumatic brain injury. *Journal of Neurotrauma* 17(10):927–938.
- Rao RV, Hermel E, Castro-Obregon S, del Rio G, Ellerby LM, Ellerby HM, Bredesen DE. (2001) Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *Journal of Biological Chemistry* 276:33869–33874.
- Reneman L, Booij J, Schmand B, Vandenbrink W, Gunning B. (2000) Memory disturbances in "Ecstasy" users are correlated with an altered brain serotonin neurotransmission. *Psychopharmacology* 148:322–324.
- Ricaurte GA, Guillery RW, Seiden LS, Schuster CR. (1980) Selective neurodegenerative changes in the somatosensory cortex following treatment with methylamphetamine, p-chloroamphetamine but not 6-HDA. *Society of Neuroscience* 6(259.7):764.

- Ricaurte GA, Bryan G, Strauss L, Seiden L, and Schuster C. (1985) Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. *Science* 229:986–988.
- Ricaurte, GA. (1989) Studies of MDMA-induced neurotoxicity in nonhuman primates: a basis for evaluating long-term effects in humans. *NIDA Research Monogram* 94:306–322.
- Ricaurte GA, Yuan J, Hatzidimitriou G, Cord BJ, McCann UD. (2003) Retraction of “Severe dopaminergic neurotoxicity in primates after a common recreational dose regimen of MDMA (“ecstasy”)”. *Science* 301(5639):1479.
- Robbins TW, Everitt BJ. (1999) Drug addiction: Bad habits add up. *Nature* 398(6728):567–570.
- Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *Journal of Neuroscience* 14:3934–3944.
- Rohn T, Rissman R, Davis M, Kim Y, Cotman C, Head E. (2002) Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain. *Neurobiology of Disease* 11(2):341–354.
- Sakas DE, Bullock R, Teasdale G. (1995) One-year outcome following craniotomy for traumatic brain hematoma in patients with fixed dilated pupils. *Journal of Neurosurgery* 82:961–965.
- Sanchez J, Oliver P, Pico C, Palou A. (2004) Diurnal rhythms of leptin and ghrelin in the systemic circulation and in the gastric mucosa are related to food intake in rats. *European Journal of Physiology* 448:500–506.
- Santolaria F, Perez-Cejas A, Aleman M, Gonzalez-Reimers E, Milena A, Jose De la Vega M, Gomez-Rodriguez M. (2003) Low Serum leptin levels and malnutrition in chronic alcohol misusers hospitalized by somatic complications. *Alcohol and Alcoholism* 38(1):60–66.
- Schifano F, Corkery J, Deluca P, Oyefeso A, Ghodse AH. (2006) Ecstasy (MDMA, MDA, MDEA, MBDB) consumption, seizures, related offences, prices, dosage levels and deaths in the UK (1994-2003). *Journal of Psychopharmacology* 20(3):456–463.
- Schmidt CJ, Taylor VL. (1987) Depression of rat brain tryptophan hydroxylase activity following the acute administration of methylenedioxymethamphetamine. *Biochemical Pharmacology* 36:4095–4102.
- Schroder N, O'Dell SJ, Marhsall JF. (2003) Neurotoxic methamphetamine regimen severely impairs recognition memory in rats. *Synapse* 49(2):89–96.

- Seiden LS, Fischman MW, Schuster CR. (1976) Long-term methamphetamine-induced changes in brain catecholamines in tolerant rhesus monkeys. *Drug and Alcohol Dependence* 3:215–219.
- Seiden LS, Ricaurte GA. (1987) Neurotoxicity of methamphetamine and related drugs. In: Meltzer, H., ed. *Psychopharmacology: The Third Generation of Progress*. New York: Raven Press. pp. 359–366.
- Shankaran M, Yamamoto BK, Gudelsky, GA. (1999) Involvement of the serotonin transporter in the formation of hydroxyl radicals induced by 3,4-methylenedioxymethamphetamine. *European Journal of Pharmacology* 385:103–110.
- Shiraha H, Glading A, Chou J, Jia Z, Wells A. (2002) Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Molecular and Cell Biology* 22:2716–2727.
- Shotton D, Burke BE, Branton D. (1979) The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. *Journal of Molecular Biology* 131:303–329.
- Sikorski AF, Terlecki G, Zagon IS, Goodman SR. (1991) Synapsin I-mediated interaction of brain spectrin with synaptic vesicles. *Journal of Cell Biology* 114:313–318.
- Siman R, Baudry M, Lynch G. (1984) Brain fodrin: Substrate for calpain I, an endogenous calcium-activated protease. *Proceedings of the National Academy of Science* 81:3572–3576.
- Simantov R, Tauber M. (1997) The abused drug MDMA (Ecstasy) induces programmed death of human serotonergic cells. *Federation of American Societies for Experimental Biology Journal* 11:141–146.
- Slee EA, Adrain C, Martin SJ. (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry* 276:7320–7326.
- Sonsalla PK, Nicklas WJ, Heikkila RA. (1989) Role for excitatory amino acids in methamphetamine-induced nigrostriatal dopaminergic toxicity. *Science* 243:398–400.
- Springer JE, Azbill RD, Knapp PE. (1999) Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nature Medicine* 5:943–946.
- Stumm G, Schlegel J, Schafer T, Wurz C, Mennel H, Krieg J, Vedder H. (1999) Amphetamines induce apoptosis and regulation of bcl-x splice variants in neocortical neurons. *Federation of American Societies for Experimental Biology Journal* 13(9):1065–1072.

- Substance Abuse and Mental Health Services Administration. (2004) Overview of Findings from the 2003 National Survey on Drug Use and Health (Office of Applied Statistics, NSDUH Series H-24, DHHS: Publication No. SMA 04-3963.
- Sutton RL, Lescaudron L, Stein DG. (1993) Unilateral cortical contusion injury in the rat: vascular disruption and temporal development of cortical necrosis. *Journal of Neurotrauma* 10:135–149.
- Suzuki K, Hata S, Kawabata Y, Sorimachi H. (2004) Structure, activation, and biology of calpain. *Diabetes* 53:12–18.
- Taffe MA, Davis S, Yuan J, Schroeder R, Hatzidimitriou G, Parsons LH, Ricaurte GA, Gold LH. (2001) Behavioral Sensitivity to Serotonergic Challenge in MDMA-Treated Rhesus Monkeys. Poster presented at MDMA/Ecstasy Research: Advances, Challenges and Future Directions, Bethesda, MD.
- Taniguchi T, Kawamata T, Mukai H, Hasegawa H, Isagawa T, Yasuda M, Hashimoto T, Terashima A, Nakai M, Mori H, Ono Y, Tanaka C. (2001) Phosphorylation of tau is regulated by PKN. *Journal of Biological Chemistry* 276:10025–10031.
- Teasdale G, Jennett B. (1974) Assessment of coma and impaired consciousness, a practical scale. *Lancet* 2:81–84.
- Thiriet N, Jayanthi S, McCoy M, Ladenheim B, Cadet JL. (2001). Methamphetamine increases expression of the apoptotic c-myc and L-myc genes in the mouse brain. *Brain Research* 90(2):202–204.
- Thomas DM, Dowgiert J, Geddes TJ, Francescutti-Berbeem D, Liu X, Kuhn DM. (2004) Microglial activation is a pharmacologically specific marker for the neurotoxic amphetamines. *Neuroscience Letters* 367:349–354.
- Thornberry NA, Lazebnik Y. (1998) Caspases: enemies within. *Science* 281:1312–1316.
- Thurman D, Alverson C, Dunn K, Guerrero J, Sniezek J. (1999) Traumatic brain injury in the United States: A public health perspective. *Journal of Head Trauma and Rehabilitation* 14(6):602–615.
- Thurman D. (2001) The epidemiology and economics of head trauma. In: Miller L, Hayes R, editors. *Head Trauma: Basic, Preclinical, and Clinical Directions*. New York (NY): Wiley and Sons.
- Uemura K, Aki T, Yamaguchi K, Yoshida K. (2003) Protein kinase C-epsilon protects PC12 cells against methamphetamine-induced death: possible involvement of suppression of glutamate receptor. *Life Sciences* 72(14):1595–1607.
- Verkes RJ, Gijsman HJ, Pieters MSM. (2001) Cognitive performance and serotonergic function in users of ecstasy. *Psychopharmacology* 153:196–202.

- Wagner GC, Lucot JB, Schuster CR, Seiden LS. (1983) Alphanethyltyrosine attenuates and reserpine increases methamphetamine-induced neuronal changes. *Brain Research* 270:285–288.
- Wallace T, Vorhees C, Zemlan F, Gudelsky G. (2003) Methamphetamine enhances the cleavage of the cytoskeletal protein tau in the rat brain. *Neuroscience* 116:1063–1068.
- Wang GJ, Volkow ND, Logan J. (2001) Brain dopamine and obesity. *Lancet* 357:354–357.
- Wang KKW, Nath R, Raser K, Hajimohammadreza I. (1996) Maitotoxin induces calpain activation in SH-SY5Y neuroblastoma cells and cerebrocortical cultures. *Archives of Biochemistry and Biophysics* 331(2):208–214.
- Wang KKW. (1998) Simultaneous degradation of alpha-II and beta-II spectrin by caspase 3 (CPP32) in apoptotic cells. *Journal of Biological Chemistry* 273:22490–22497.
- Wang KKW. (2000) Calpain and caspase: Can you tell the difference? *Trends in Neuroscience* 23(1):20–26.
- Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KKW. (2005a) Concurrent calpain and caspase-3 mediated proteolysis of α II-spectrin and tau in the rat brain after methamphetamine exposure: A similar profile to traumatic brain injury. *Life Sciences* 78:301–309.
- Warren MW, Frost-Pineda K, Gold MS. (2005b) Body mass index and marijuana use. *Journal of Addictive Disease* 24(3):95–100.
- Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KKW. (2006) Ecstasy toxicity: A comparison to methamphetamine and traumatic brain injury. *Journal of Addictive Disease* (accepted for publication).
- White SR, Duffy P, Kalivas PW. (1994) Methylendioxyamphetamine depresses glutamate-evoked neuronal firing and increases extracellular levels of dopamine and serotonin in the nucleus accumbens in vivo. *Neuroscience* 62:41–50.
- Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes and Development* 12:806–819.
- Wyllie AH, Kerr JF, Currie AR. (1980) Cell death: The significance of apoptosis. *International Review of Cytology* 68:251–306.

- Xie H, Johnson G. (1997) Ceramide selectively decreases tau levels in differentiated PC12 cells through modulation of calpain I. *Journal Neurochemistry* 69(3):1020–1030.
- Xu W, Zhu JPQ, Angulo JA. (2005) Induction of striatal and pre- and postsynaptic damage by methamphetamine requires the dopamine receptors. *Synapse* 58:110–121.
- Yakovlev AG, Knobloch SM, Fan L, Fox GB, Goodnight R, Faden AI. (1997) Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience* 17:7415–7424.
- Yakovlev AG, Faden AI. (2001) Caspase-dependent apoptotic pathways in CNS injury. *Molecular Neurobiology* 24:131–144.
- Yamamoto BK, Nash JF, Gudelsky GA. (1995) Modulation of methylenedioxymethamphetamine-induced striatal dopamine release by the interaction between serotonin and gamma-aminobutyric acid in the substantia nigra. *Journal of Pharmacology and Experimental Therapeutics*. 273:1063–1070.
- Yamamoto BK. (2001) Neurochemical mediators and neurophysiological consequences of acute MDMA exposure in rats. Presented at MDMA/Ecstasy Research: Advances, Challenges and Future Directions, Bethesda, MD.
- Yamashima R. (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Progress in Neurobiology* 62:273–295.
- Yang L, Ksiezak-Reding H. (1995) Calpain-induced proteolysis of normal human tau and tau associated with paired helical filaments. *European Journal of Biochemistry* 233(1):9–17.
- Yen S, Easson C, Nacharaju P, Hutton M, Yen S. (1999) FTDP-17 tau mutations decrease the susceptibility of tau to calpain I digestion. *Federation of European Biochemical Societies* 461(1-2):91–95.
- Yuen PW, Wang KKW. (1998) Calpain inhibitors, novel neuroprotectants and potential anticataractic agents. *Drugs Future* 23:741–749.
- Zhu JPQ, Xu W, Angulo JA. (2005) Disparity in the temporal appearance of methamphetamine-induced apoptosis and depletion of dopamine terminal markers in the striatum of mice. *Brain Research* 1049:171–181.

BIOGRAPHICAL SKETCH

Matthew Wesley Warren was born in 1978 and originally hails from Raleigh, NC. He graduated from the University of North Carolina at Chapel Hill in 2001 with Highest Honors in Research and Highest Distinction, earning a Bachelor of Science degree in chemistry (biochemistry track) with a minor in mathematical science (statistics option). During his undergraduate studies Matthew was inducted into Phi Beta Kappa and earned research awards from the Emmitt-Rand and National Starch and Chemical Company scholarship funds. Matthew then began the combined MD/PhD program at the University of Florida (Gainesville, FL) College of Medicine, was named a University Alumni Fellow and completed two years of medical school, passing Step 1 of the United States Medical Licensing Exam. In 2006, he earned a Doctor of Philosophy in medical sciences (neuroscience concentration) while studying under the guidance of Mark S. Gold and Kevin K.W. Wang in the Departments of Psychiatry and Neuroscience. During his graduate work, Matthew earned research honors including the Washington University Guze Award, the American Society of Addiction Medicine Young Investigator Award and the American Academy of Addiction Psychiatry Medical Student Award. Matthew was also given recognition as an Outstanding Graduate Student Teacher. Upon completion of this dissertation, he returns to medical school to complete the final two years of his Doctor of Medicine degree.