THE EFFECTS OF A NEUROTOXIC DOSE OF METHAMPHETAMINE ON PRESYNAPTIC MORPHOLOGY AND GLUTAMATE IMMUNOCYTOCHEMISTRY WITHIN THE STRIATUM AND MOTOR CORTEX OF THE RAT

by

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LIST OF ABBREVIATIONS

AMPH	Amphetamine
ANOVA	Analysis of Variance
CD/P	Caudate/Putamen
CNS	Central Nervous System
CTX	Cortex
D1R	D1 Type Dopamine Receptor
D2R	D2 Type Dopamine Receptor
DA	Dopamine
DHBA	Dihydrobenzylamine
DL	Dorsolateral
DM	Dorsomedial
DMSO	Dimethyl Sulfoxide
DOPAC	Dihydroxyphenyl Acetic Acid
EXP	Experiment
GABA	γ-Aminobutyric acid
GFAP	Glial Fibrillary Acidic Protein
GLU	Glutamate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HPLC	High Performance Liquid Chromatography
HVA	Homovanillic Acid
MAO	Monoamine Oxidase
MCTX	Motor Cortex
METH	Methamphetamine
MP	Mobile Phase
NAC	Nucleus Accumbens
NE	Norepinephrine
NMDA	N-Methyl-D-aspartate
NMDAR	NMDA Type Glutamate Receptor

6-OHDA	6-Hydroxydopamine
PBS	Phosphate Buffered Saline
SAL	Saline
SN	Substantia Nigra
TBST	Tris Buffered Saline with Triton X-100
TH	Tyrosine Hydroxylase
VL	Ventrolateral
VM	Ventromedial
VTA	Ventral Tegmental Area

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ABSTRACT

Methamphetamine (METH) is a potent drug of abuse that is known to induce presynaptic terminal damage within the striatal dopamine (DA) system. It has been suggested that METH-induced toxicity is due to excess DA overflow which could lead to the endogenous formation of oxidative neurotoxins. In addition, a delayed glutamate (GLU) overflow, of unknown origin, is believed to be important in the neurotoxic effects of METH (Nash and Yamamoto, 1992). These studies were designed to test the hypothesis that the depletion of DA, produced by moderate doses of METH, is due to the degeneration of DA containing terminals within the caudate/putamen (CD/P). In addition, these experiments were designed to determine if a decrease in presynaptic GLU levels is temporally correlated with the delayed overflow of GLU.

Sprague Dawley rats were treated with METH (5 mg/kg every 2 hrs, 4 times) and killed at various time points in order to look for evidence of terminal degeneration and changes in presynaptic GLU immunoreactivity within the motor cortex (MCTX) and three different regions of the striatum. Animals treated with METH did show pronounced hyperthermia in the range found to correlate with DA depletion (Bowyer *et al.*, 1992), indicating that a loss of DA likely occurred. However, no ultrastructural evidence of terminal degeneration (i.e., darkened nerve terminal cytoplasm) was seen in any region at any time following METH administration. Examination of the anatomical distribution of changes in GLU immunoreactivity revealed that significant decreases in the density of nerve terminal

GLU immunolabeling occurred 12 hrs after METH administration within the MCTX and the ventrolateral (VL) CD/P. Although decreases within the dorsolateral (DL) CD/P were not significant, the time course of the changes in labeling closely resembled those found within the VL CD/P and MCTX.

In order to demonstrate that METH administration in these studies results in DA toxicity as previously reported by others, changes in catecholamines and their metabolites were measured in a separate group of animals treated with the same dose of METH. The results replicate previous experiments demonstrating a significant loss of approximately 50% of striatal DA, without reduction of norepinephrine, 1 week following METH treatment. Although DA metabolites were not significantly decreased following METH administration, there was a consistent 25% depletion of both DOPAC and HVA. Small decreases in the level of striatal DA and DA metabolites continued to be found 4 weeks following METH administration suggesting that low doses of METH may have small but long-lasting effects on the basal ganglia. Although it has been reported that the effect of METH is heterogeneous within the striatum, the present study found similar DA depleting effects in all three striatal regions. One possible explanation for this difference may be ascribed to how the different regions were defined in the current study.

The lack of degeneration, coupled with the recovery from DA depletion in comparably treated animals, suggests that moderate doses of METH may inhibit DA biosynthesis without widespread

terminal loss. Furthermore, the results indicate that METH administration results in a decrease in presynaptic GLU that correlates temporally with the delayed GLU overflow, suggesting that neuronally derived GLU may play a role in METH-induced neurotoxicity. This METH-induced decrease in GLU immunoreactivity does not appear to be a generalized effect, but is specific to the known circuitry of the dorsal striatum. This evidence is consistent with the hypothesis that METH administration may lead to the delayed activation of the thalamocorticostriatal loop.

INTRODUCTION

The amphetamine (AMPH) derivative methamphetamine (METH), is a potent psychostimulant that has recently reemerged as a popular drug of abuse (Derlet and Heischober, 1990; Sato *et al.*, 1992). METH overdose can be fatal, and is associated with seizures, cerebrovascular or cardiovascular accidents, and severe hyperthermia (Callaway and Clark, 1994). Chronic use of METH can lead to weight loss, lack of sleep, and the deterioration of both mental and physical function known as psychostimulant-psychosis. This syndrome is virtually indistinguishable from schizophrenia and is characterized by compulsive behavior, paranoia and auditory and visual hallucinations (Sato *et al.*, 1992).

Psychosis is not solely attributed to the acute psychogenic action of METH. Symptoms can last up to a month after drug use has ended, and patients who have experienced an episode of psychosis are more vulnerable to relapses. These episodes occur within 1 week of drug use, even in patients who have abstained from METH usage for up to 5 years (Sato *et al.*, 1983). In addition, relapse without substance abuse has been reported (Sato *et al.*, 1983; Sato, 1992). Undefined neuronal damage produced gradually during chronic METH abuse is assumed to be the cause of psychostimulant-psychosis and the apparent long-lasting sensitization to the actions of the drug (Sato, 1992).

Animal research has found that administration of psychostimulants, within the dose range abused by humans, can produce long lasting effects on the central nervous system. These

changes have been found in rats, guinea pigs, mice, cats, and rhesus monkeys (Kleven and Seiden, 1992). Of particular interest is the finding that METH induces long lasting depletions of the catecholamine dopamine (DA) in the striatum. This neurotransmitter system has been implicated in several human disorders, including schizophrenia, Huntington's, and Parkinson disease (Seiden *et al.*, 1975/76; Lucot *et al.*, 1980; Ricaurte *et al.*, 1980).

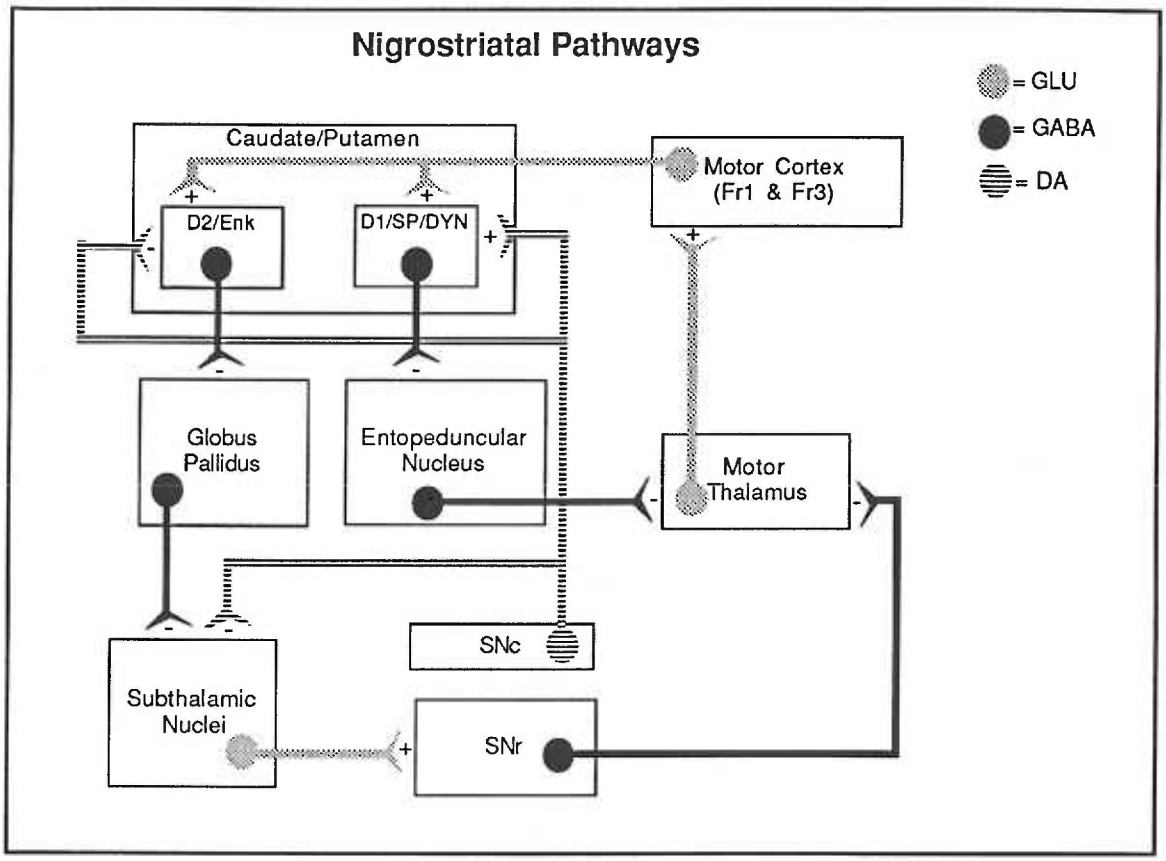
Organization of the Striatum

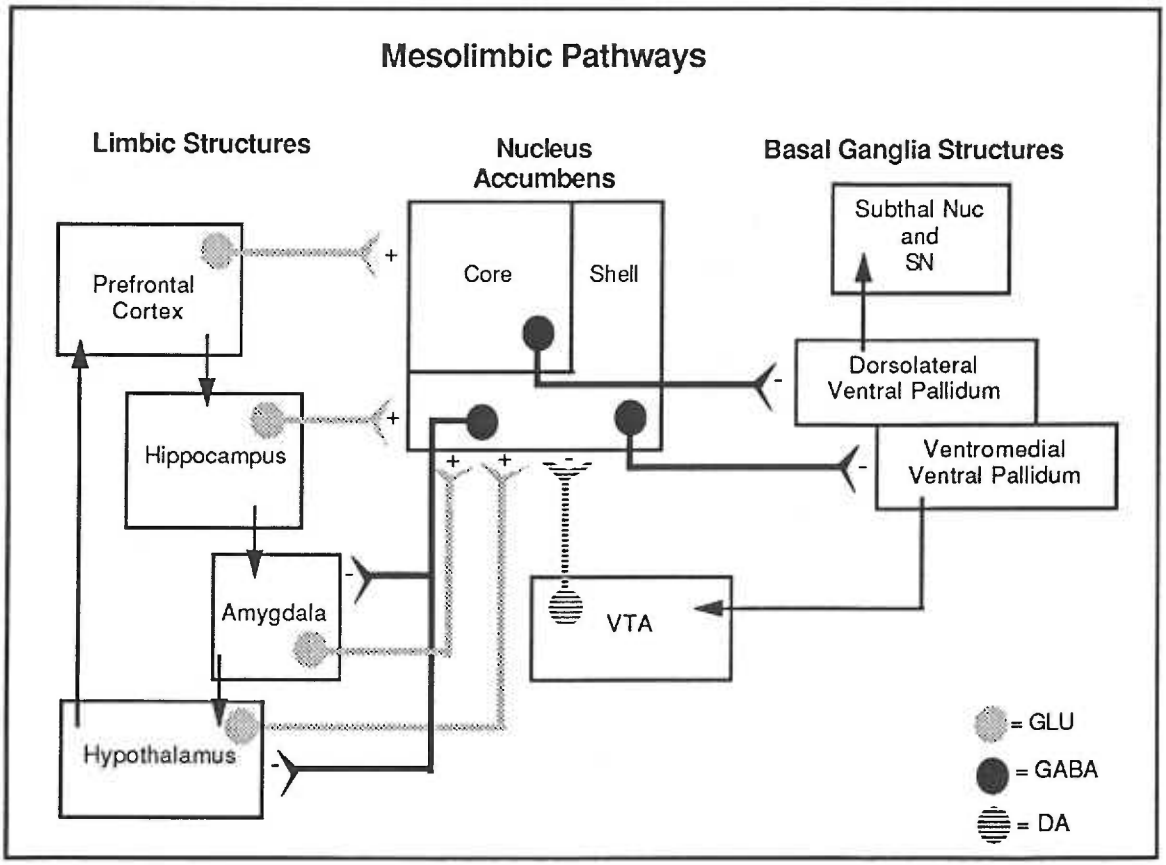
The neurotoxic effects of METH on the striatum are heterogeneous. In order to define the anatomical terms referenced in this paper, the basic organization of the striatum will be briefly reviewed. The nigrostriatal and mesolimbic pathways are illustrated in Figure 1.

The Dorsal Striatum

The dorsal striatum of the rat consists of the dorsolateral (DL), dorsomedial (DM) and ventrolateral (VL) portions of the caudate-putamen (CD/P) (Heimer and Alheid, 1991). This region of the striatum receives excitatory glutamatergic input primarily from the neocortex. In particular, the primary motor cortex (MCTX) projects to the DL and VL CD/P, whereas the DM CD/P receives cortical input primarily from auditory and visual regions of the neocortex (McGeorge and Faull, 1989). Dopaminergic projections to the CD/P arise from the substantia nigra (SN) pars compacta in the midbrain (Fallon and Moore, 1978).

Figure 1. Diagram of the nigrostriatal and mesolimbic pathways (+ indicates excitatory input, - indicates inhibitory input). This figure was adapted from several sources (McGeorge and Faull, 1989; Alexander and Crutcher, 1990; Zahm and Heimer, 1990; Heimer and Alheid, 1991; Heimer *et al.*, 1991; Zahm and Heimer, 1993; McGinty, 1995).


Nigrostriatal Pathways
= GLU
= GABA
= DA
Caudate/Putamen
D2/Enk
D1/SP/DYN
Motor Cortex
(Fr1 & Fr3)
Globus
Pallidus
Entopeduncular
Nucleus
Motor
Thalamus
SNc
Subthalamic
Nuclei
SNr


Mesolimbic Pathways
Limbic Structures
Nucleus
Accumbens
Basal Ganglia Structures
Prefrontal
Cortex
Core
Shell
Subthal Nuc
and
SN
Dorsolateral
Ventral Pallidum
Hippocampus
Ventromedial
Ventral Pallidum
Amygdala
VTA
Hypothalamus
= GLU
= GABA
= DA

The MCTX of the rat consists of regions Fr1 and Fr3 of the frontal neocortex. This region receives excitatory afferents from motor thalamus (VL and VM regions) and sends excitatory efferents to the CD/P (Beckstead, 1976). The thalamic inputs innervate primarily the superficial layers of MCTX (layers I-III). In accordance with these findings, immunohistochemistry has revealed that the excitatory amino acid neurotransmitter glutamate (GLU) and its corresponding binding sites are found in layers I-III (Ottersen and Storm-Mathisen, 1984).

Afferents arising from the centromedial and parafascicular thalamic nuclei also provide a large glutamatergic input to the dorsal striatum (Dubé *et al.*, 1988; Berendse and Groenewegen, 1990). This thalamostriatal pathway can be morphologically distinguished from the corticostriatal input. In primates, only approximately 20% of thalamostriatal terminals synapse upon dendritic spines (Sadikot *et al.*, 1992). In the rat, thalamostriatal terminals primarily form asymmetrical synapses with dendritic shafts, while afferents arising from the neocortex synapse exclusively onto the spines of what appear to be morphologically distinct neurons (Dubé *et al.*, 1988).

The Ventral Striatum

The ventral striatum of the rat is composed of the ventromedial (VM) portions of the CD/P, the nucleus accumbens (NAC), and the olfactory tubercle. The NAC and VM CD/P receive excitatory cortical input from the limbic portions of the mesocortex and allocortex (including the hippocampus and amygdala) (McGeorge

and Faull, 1989). The NAC receives DA afferents from the ventral tegmental area (VTA) through the mesolimbic pathway (Hillarp *et al.*, 1966; Fallon and Moore, 1978).

The caudal NAC can be divided into two basic subregions, the shell (VM NAC) and core (DL NAC). The partitioning of the NAC is based on both morphological and pharmacological differences (Heimer *et al.*, 1991; Deutch and Cameron, 1992). Although both regions share common afferent input from the VTA and limbic structures, they differ in their efferent projections. The core and shell project to different regions within the ventral pallidum, the subcommissural portion of the pallidal complex that is a continuation of the more dorsal globus pallidus (Zahm and Heimer, 1988). The core projects to the DL ventral pallidum which, in turn, sends efferents to the subthalamic nucleus and SN. These regions are a part of the thalamocorticostriatal motor loop. In contrast, the shell projects to the VM ventral pallidum which feeds back onto the VTA. In addition, the shell sends projections to the lateral hypothalamus and amygdala of the limbic system (Zahm, 1989; Heimer and Alheid, 1991; Heimer *et al.*, 1991). The shell of the NAC is therefore considered to be part of the limbic system and is believed to play a role in emotional and motivational functions. In contrast, the core of the NAC is considered to be a part of the striatopallidal motor complex and is believed to be involved in somatomotor functions (Alheid and Heimer, 1988).

Pharmacology of Methamphetamine's Effects

The physiological effects of amphetamines on central DA pathways have been well characterized. AMPH and its derivative METH have similar effects on neurochemistry and behavior, although METH has greater efficacy in the central nervous system (CNS) and fewer peripheral actions (Lake and Quirk, 1984; Hoffman and Lefkowitz, 1990). In addition, METH has a longer half-life (Ellison and Switzer, 1993). METH increases the amount of extracellular DA by blocking its reuptake and inducing the release of newly synthesized DA from the cytosolic pool (McMillen, 1983). This DA release is mediated by METH's ability to reverse the energy-dependent high-affinity DA transporter (Raiteri *et al.*, 1979; Liang and Rutledge, 1982). It has been suggested that amphetamines also interact with the vesicular transporter, resulting in a redistribution of DA from the vesicles into the cytosol, thereby increasing the releasable pool (Sulzer *et al.*, 1995). METH also prolongs DA activity by competing with catecholamines for metabolism by monoamine oxidase (MAO) (Suzuki *et al.*, 1980).

Evidence of METH-Induced Neurotoxicity

Seiden *et al.* (1988) defined drug-induced neurotoxicity by the presence of decreased levels of transmitter for at least 2 weeks following drug administration, accompanied by a decreased number of transmitter uptake sites without a change in affinity, decreased activity of the rate limiting enzyme, and morphological evidence of cell damage. In the absence of degeneration, neurotoxicity can be

presumed by fulfillment of the first three criteria. There is substantial evidence, as detailed below, suggesting that amphetamines, including METH, satisfy these requirements.

Depletion of DA and Tyrosine Hydroxylase

METH induces DA and tyrosine hydroxylase (TH) depletion within the CD/P but does not appear to affect the noradrenergic system (Fibiger and McGeer, 1971; Seiden *et al.*, 1975/76; Ellison *et al.*, 1978; Wagner *et al.*, 1979; Lucot *et al.*, 1980; Seiden *et al.*, 1988). Repeated administration of METH, ranging from 4 to 50 mg/kg, results in approximately a 50% decrease in DA content (Table 1). It has been reported that the primary DA metabolites dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) also show a decrease in levels following a neurotoxic regimen of METH (Ricaurte *et al.*, 1982; Schmidt and Gibb, 1985; Sonsalla *et al.*, 1986; Abekawa *et al.*, 1994; Pu *et al.*, 1994). However, reduction of DA metabolites has not been consistently reported (Table 2).

There is evidence that a dose-dependent recovery of DA content and TH activity can occur (Kogan *et al.*, 1976; Wagner *et al.*, 1980; Jonsson and Nwanze, 1982; Fields *et al.*, 1991). As an example, Wagner *et al.* (1980) reported that in rats treated with either 12.5 or 50 mg/kg/day METH for 4 days, DA depletions lasting greater than 2 weeks were found only in animals receiving the higher dose. Others have found that reductions of DA and TH last for 1-4 months with no signs of recovery (Ellison *et al.*, 1978; Hotchkiss and Gibb, 1980; Lucot *et al.*, 1980; Nwanze and Jonsson, 1981). Recovery of striatal DA content suggests that low doses of METH

Table 1. Representative studies and drug doses resulting in METH-induced DA depletion in rats

Study	Dose METH	Change in DA in CD/P	Change in DA in NAC	Time After METH
O'Dell *et al.* (1991)	4.0 mg/kg X 2 hr X 4 inj	-51%	-43%	1 week
Eisch *et al.* (1992)	4.0 mg/kg X 2 hr X 4 inj	-50%	None	1 week
Bowyer *et al.* (1992)	5.0 mg/kg X 2 hr X 4 inj	-60%		3 days
Bowyer *et al.* (1992)	5.0 mg/kg X 2 hr X 4 inj	-30%		14 days
Nash *et al.* (1992)	7.5 mg/kg X 2 hr X 3 inj	-30%		1 week
Stephans *et al.* (1994)	10.0 mg/kg X 2 hr X 3 inj	-45%		1 week
Green *et al.* (1992)	15.0 mg/kg X 3 hr X 4 inj	-75%		3 days
Ricaurte *et al.* (1980)	50.0 mg/kg X 12 hr X 8 inj	-42%	-19%	3 weeks
Ricaurte *et al.* (1982)	50.0 mg/kg X 12 hr X 8 inj	-53%	None	3 weeks
Lucot *et al.* (1980)	50.0 mg/kg X 12 hr X 8 inj	-47%		16 weeks

Table 2. Representative studies and drug doses showing the effects of METH on DA metabolites within the rat CD/P.

Study	Dose METH	Change in DOPAC	Change in HVA	Time After METH
Abekawa *et al.* (1994)	4 mg/kg X 2 hr X 4 inj	-53%*	-52%*	5 days
Bowyer *et al.* (1993)	5 mg/kg X 2 hr X 4 inj	-18%	+1%	3 days
Nash and Yamamoto (1992)	7.5 mg/kg X 2 hr X 3 inj	-15%	-25%	7 days
Schmidt and Gibb (1985)	10 mg/kg X 6 hr X 4 inj	-41%*	-19%*	1 day
Pu *et al.* (1994)	10 mg/kg X 2 hr X 4 inj	-30%*		3 days
Ricaurte *et al.* (1982)	50 mg/kg X 8 hr X 3 inj	-50%*		3 weeks

* reported as a significant decrease

may cause down-regulation of nigrostriatal DA biosynthesis independent of terminal destruction. However, administration of METH resulting in a significant loss of DA lasting at least 3 days after drug treatment is considered to be neurotoxic (Nash and Yamamoto, 1992; Baldwin *et al.*, 1993; Marshall *et al.*, 1993; Bowyer *et al.*, 1994).

Loss of DA Uptake Sites

METH treatment has been found to decrease the number, but not the affinity, of DA uptake sites in the CD/P (Steranka and Sanders-Bush, 1980; Wagner *et al.*, 1980; Muraki *et al.*, 1992). Administration of high doses of METH have been found to decrease [^{3}H]DA uptake in striatal synaptosomal preparations in both rats (Wagner *et al.*, 1980) and monkeys (Finnegan *et al.*, 1982), suggesting that the rate of extracellular DA clearance is compromised in drug treated animals. Eisch *et al.* (1996) recently reported that METH treatment (4 mg/kg every 2 hrs, 4 times) resulted in the loss of DA uptake sites, as measured by decreased [^{3}H]mazindol binding, in all regions of the CD/P. These decreases were present at both 1 week and 1 month following drug administration, and could account for the reduction in DA uptake.

Histochemical and Morphological Changes

Several histochemical techniques have been used to investigate psychostimulant-induced changes within the CD/P. Fluorescent histochemistry for DA localization has revealed swollen, brightly fluorescent striatal axons following AMPH treatment (Ellison *et al.*, 1978; Nwanze and Jonsson, 1981; Jonsson

and Nwanze, 1982). These are indicators of axonal DA accumulation, and suggest that either the terminals have been damaged or axonal transport has been compromised. Patchy TH immunostaining associated with swollen-TH positive processes have also been reported (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; Pu and Vorhees, 1995). Fink-Heimer silver staining, which selectively stains the fine granular argyophyllic debris indicative of nerve fiber degeneration, has been found in the CD/P but not in the SN following METH treatment, indicating a lack of neuronal cell body loss (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; Ellison and Switzer, 1993).

Although histochemical studies have found evidence for psychostimulant-induced damage, there has been little direct morphological evidence of terminal degeneration. Ryan *et al.* (1990) reported that rats chronically administered with low doses of AMPH (under 20 mg/kg/day for 3 days) showed no neuronal degeneration. Infusion with higher doses of AMPH (20-60 mg/kg/day for 3 days) induced degeneration in the CD/P, somatosensory CTX, and MCTX. Electron microscopic evidence included swollen processes (sometimes also labeled for TH immunoreactivity), dark degenerating processes (never stained for TH immunoreactivity), and darkened nerve terminals associated with both symmetrical and asymmetrical synapses. The lack of TH staining of degenerating processes and the darkening of asymmetric terminals indicate that destruction of non-catecholaminergic transmitter systems may have occurred.

In addition to neuronal changes, METH appears to induce astrogliosis in the CD/P (Hess *et al.*, 1989; Miller and O'Callaghan, 1993; Pu *et al.*, 1994; Pu and Vorhees, 1995). This phenomenon is characterized by proliferation and hypertrophy of astrocytes and is considered to be a marker for generalized damage to the CNS (O'Callaghan, 1993). Changes in astrocyte morphology and glial fibrillary acidic protein (GFAP) staining is limited to the CD/P and tends to occur in regions associated with patchy TH immunostaining (Pu *et al.*, 1994; Pu and Vorhees, 1995).

These histochemical and morphological changes have all been found to occur in animals given high doses of psychostimulants (AMPH or METH) either acutely (3-8 injections totaling 40-150 mg/kg) or chronically (pellet releasing 30-150 mg/kg/day for 3-7 days). It is important to note that these doses are known to induce seizures and death (Ansah *et al.*, 1993) and are much higher than those required to induce depletions of DA (Table 1). The fact that high doses of METH appear to be required for terminal degeneration further suggests that lower doses of METH may cause a transient down-regulation of nigrostriatal DA biosynthesis independent of terminal degeneration.

METH-induced Hyperthermia

Temperature may also play a role in METH lethality and neurotoxicity. Hyperthermia has been found to lower the LD_{50}, the dose of drug that is lethal to 50% of animals tested, of AMPH in mice (Craig and Kupferberg, 1972) and may be a contributing factor in fatal METH overdose (Zalis *et al.*, 1967; Callaway and Clark, 1994).

Bowyer *et al.* (1992) have reported that METH administration (5 mg/kg every 2 hrs, 4 times) at an ambient temperature of 23°C resulted in hyperthermia (39.4-42.2°C). Peak body temperature has been found to correlate with DA depletion and administration of METH in a cold environment (4°C) protects against METH toxicity (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994). Hyperthermia alone, caused by warm ambient temperatures, does not produce DA depletion in animals with a peak body temperature of 40.0-41.5°C (Bowyer *et al.*, 1994).

In summary, evidence that psychostimulant administration results in neurotoxicity includes the loss of DA and TH (Seiden *et al.*, 1975/76), decreased DA uptake sites (Steranka and Sanders-Bush, 1980; Wagner *et al.*, 1980), and histochemical and morphological evidence of terminal degeneration in the absence of cell body damage (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; Ellison and Switzer, 1993). These data suggest that presynaptic DA containing terminals in the CD/P are selectively damaged or destroyed following METH treatment. The effects of METH appear to be dose dependent as very high doses of psychostimulants appear to be required for significant terminal degeneration and long-lasting DA depletion without subsequent recovery. Furthermore, there appears to be a correlation between peak body temperature and DA depletion (Bowyer *et al.*, 1992), suggesting that hyperthermia may be a contributing factor in METH toxicity.

Heterogeneity of METH's Effects

Differences Within the Dorsal Striatum

Marshall and Navarrete (1990) have found that METH-induced changes within the CD/P are correlated with the level of endogenous DA content and hypothesized that METH-induced release of DA may be higher in regions with more DA content, thus leading to a greater degree of neurotoxicity. Consistent with this theory, the lateral and ventral portions of the CD/P have been found to have a higher DA content and are more vulnerable to the neurotoxic effects of METH when compared to the DL CD/P (Marshall and Navarrete, 1990). Fluorescent histochemistry has revealed swollen axons in the ventral regions of the CD/P following AMPH treatment (Ellison *et al.*, 1978). In addition, Pu *et al.* (1994) reported that the patchy TH immunostaining and astrogliosis associated with METH administration (10 mg/kg every 2 hrs, 4 times) was primarily confined to the ventral portions of the CD/P. Depletion of DA content and uptake sites following METH administration (4 mg/kg every 2 hrs, 4 times) has also been found to be greatest in the ventral and lateral CD/P (Marshall and Navarrete, 1990; Eisch *et al.*, 1992).

Differences Between the Dorsal and Ventral Striatum

Although there have been reports of METH-induced toxicity within the NAC (Morgan and Gibb, 1980; Ricaurte *et al.*, 1980; Ricaurte *et al.*, 1982; Seiden *et al.*, 1988; O'Dell *et al.*, 1991) others have not been able to repeat this finding (Nwanze and Jonsson, 1981; Jonsson and Nwanze, 1982; Hess *et al.*, 1989; Fields *et al.*, 1991;

Eisch *et al.*, 1992; Ohmori *et al.*, 1993; Eisch *et al.*, 1996). In general, it is believed that the NAC is less vulnerable to the toxic effects of METH. Seiden *et al.* (1988) reported that a single injection of 50 mg/kg METH resulted in approximately a 50% reduction in DA within the CD/P. In contrast, DA depletion (-30%) within the NAC required 100 mg/kg METH. Similarly, Ricaurte *et al.* (1980) found that following high METH administration (100 mg/kg/day for 4 days), DA levels with the CD/P were reduced by 40%, but depletion within the NAC was only 20%. Thus, although toxic effects of METH have been reported to occur with the NAC, a larger dose of METH is usually required to elicit these changes. In addition, degenerating terminals, visualized by silver staining, have been found within the core of the NAC, but not the shell (Ricaurte *et al.*, 1982). This suggests that METH-induced damage within the NAC itself may be heterogeneous.

Regions Outside the Striatum

It has been reported that the DA-rich regions of the frontal CTX can be damaged by high doses of psychostimulants (Ricaurte *et al.*, 1980; Seiden *et al.*, 1988). In addition, Ryan *et al.* (1990) found that chronic infusion of high doses of AMPH produced both neuronal and terminal degeneration in the MCTX. Degenerating terminals in this region often formed asymmetric synaptic contacts and were not always associated with TH staining, suggesting that some of the degenerating terminals may contain an excitatory amino acid transmitter such as GLU (Eccles, 1964).

Outside of the striatum and CTX, METH has little effect on dopaminergic systems. In fact, METH treatment does not induce DA depletion or degenerative changes in the VTA, SN, locus coeruleus, amygdala, dorsal raphe, cerebellum, lateral septum, adrenal medulla, brain stem, hypothalamus, or hippocampus in animals showing striatal damage (Fibiger and McGeer, 1971; Ricaurte *et al.*, 1980; Ricaurte *et al.*, 1982; Ryan *et al.*, 1990).

METH also induces long-term alterations in central serotonin systems including reduced serotonin uptake sites, decreased tryptophan hydroxylase activity (Hotchkiss and Gibb, 1980), and depletion of serotonin in the striatum, frontal CTX, and amygdala (Seiden *et al.*, 1975/76; Ricaurte *et al.*, 1980). Other transmitter systems in the striatum appear to be intact in that glutamate decarboxylase and choline acetyltransferase (GABA and cholinergic terminal markers) and norepinephrine (NE) levels are unchanged following METH treatment (Wagner *et al.*, 1979; Hotchkiss and Gibb, 1980; Lucot *et al.*, 1980; Fields *et al.*, 1991).

In summary, the DA depleting effects of METH are heterogeneous and are concentrated within the striatum and CTX. Within the CD/P, there appears to be a dorsal to ventral increasing gradient of neurotoxic damage (Ellison *et al.*, 1978; Marshall and Navarrete, 1990; Pu *et al.*, 1994). The NAC appears to be less susceptible to the toxic effects of METH in that higher doses of drug are required for DA depletion to occur (Ricaurte *et al.*, 1980; Seiden *et al.*, 1988). There is also evidence that high doses of METH can induce damage within the MCTX. The presence of degenerating

asymmetric terminals suggests that METH may damage excitatory synapses (Eccles, 1964; Ryan *et al.*, 1990).

METH May Induce Oxidative Damage

The Oxidative Damage Hypothesis

Some evidence suggests that METH-induced damage to the nigrostriatal DA system may be mediated by the endogenous formation of reactive oxygen species, including the neurotoxin 6-hydroxydopamine (6-OHDA). According to this hypothesis, prolonged release of DA coupled with the blockade of MAO, could overwhelm the presynaptic terminal's ability to metabolize catecholamines. Under these conditions, autoxidation of DA can lead to the formation of 6-OHDA, which enters DA terminals via uptake by the high-affinity DA transporter (Seiden and Vosmer, 1984; Slivka and Cohen, 1985). 6-OHDA is itself autoxidized, resulting in the formation of quinones, hydrogen peroxide (H_2O_2), and free radicals (O_2^-, and OH) (Cohen and Heikkila, 1974). Quinones can covalently bind to nucleophilic groups on proteins and lead to their denaturation, while free radicals can oxidize enzymes and lipid membranes (Sachs and Jonsson, 1975). Together, this damage can result in the death of the affected cell. In addition, the competitive inhibition of MAO in the presence of METH, could enhance oxidative damage by preventing the degradation of 6-OHDA.

Formation of Endogenous 6-OHDA After METH

In support of this hypothesis, Seiden and Vosmer (1984) reported that endogenous 6-OHDA (0.39 ng/mg of tissue) was formed

in the CD/P two hours after a single large injection of METH (100 mg/kg). This dose of METH-induced a 50% reduction in striatal DA as measured 2 weeks after drug administration. Pretreatment with α-methylparatyrosine, which blocks the synthesis of DA, has been found to attenuate both the formation of 6-OHDA and METH toxicity (Jonsson and Sachs, 1973; Schmidt *et al.*, 1985; Axt *et al.*, 1990). Amfonelic acid, a DA uptake blocker, has been found to prevent METH-induced DA depletion and loss of TH activity but not the formation of 6-OHDA (Marek *et al.*, 1990a). The protective effect occurs even when amfonelic acid is administered 6 hours (Pu *et al.*, 1994) or 8 hours (Marek *et al.*, 1990a) after METH treatment and suggests that 6-OHDA is formed in the synaptic cleft and is transported into the terminal after METH is removed from the synapse. In contrast, pargyline, which inhibits MAO and prevents METH and DA metabolism, enhances both the formation of 6-OHDA and the extent of DA depletion (Marek *et al.*, 1990b).

Several drugs which are known to block the formation of reactive oxygen species also attenuate the toxic effects of METH. METH-induced striatal DA depletion can be significantly attenuated by pretreatment with any of several antioxidants, including ascorbic acid, ethanol, mannitol, vitamin E, and DMSO (Wagner *et al.*, 1985; De Vito and Wagner, 1989). Superoxide dismutase also affords protection against METH toxicity (Cadet *et al.*, 1994) and pretreatment with the superoxide dismutase inhibitor, diethyldithiocarbamate, enhances catecholamine depletion (De Vito and Wagner, 1989).

Similarities Between 6-OHDA and METH Toxicity

The pattern of toxicity following METH treatment resembles that seen following a 6-OHDA lesion. Like METH, administration of 6-OHDA results in a dose dependent reduction of DA (Hedreen and Chalmer, 1972), TH activity (Sorimachi, 1975), and catecholamine uptake sites (Iverson, 1970). As seen with psychostimulant toxicity, intraventricular administration of 6-OHDA has been found to induce dense terminal degeneration, as measured by the presence of silver staining, within the CD/P and sparse degeneration in the NAC. However, unlike METH, 6-OHDA also depletes NE and induces degeneration of dopaminergic cell bodies within the SN pars compacta and VTA when injected intraventricularly (Hedreen and Chalmer, 1972). Bennett *et al.* (1970) noted that a critical concentration of 6-OHDA must be present for degeneration and long-lasting catecholamine depletion to occur. Unless this threshold is reached, only a moderate and transient decrease occurs. This is similar to the finding that low to moderate doses of METH may induce transient DA depletion while high doses produce terminal degeneration.

In summary, administration of METH results in the formation of 6-OHDA (Seiden and Vosmer, 1984), a neurotoxin that is known to selectively damage catecholamine containing terminals within the striatum. It has been suggested that the endogenous formation of 6-OHDA, and other reactive oxygen species, may play a role in METH toxicity. In support of this hypothesis, drugs which block 6-OHDA formation prevent METH-induced DA depletion (De Vito and Wagner,

1989; Axt *et al.*, 1990), and both neurotoxins produce a similar pattern of terminal damage within the nigrostriatal DA system (Iverson, 1970; Hedreen and Chalmer, 1972; Seiden *et al.*, 1988).

Synergistic Effects of Dopamine and Glutamate

METH Induces DA and GLU Overflow

The severity of long term DA depletion is highly correlated with cumulative DA overflow as measured by *in vivo* microdialysis (O'Dell *et al.*, 1991). METH administration (4 mg/kg every 2 hrs, 4 times) increases extracellular DA in both the CD/P and NAC (O'Dell *et al.*, 1991; Abekawa *et al.*, 1994). In contrast, METH treatment induces a delayed overflow of GLU in the CD/P, but not in the NAC. This release of GLU occurs following the final dose of METH and continues to rise after DA levels have returned to baseline (Nash and Yamamoto, 1992; Abekawa *et al.*, 1994).

Blockade of DA or GLU Overflow Confers Protection

The neurotoxic effects of METH can be attenuated by a variety of treatments that selectively prevent either DA or GLU overflow. These include attenuation of DA synthesis (Hotchkiss and Gibb, 1980; Nash and Yamamoto, 1993), and blockade of either DA receptors (Sonsalla *et al.*, 1986; O'Dell *et al.*, 1993; Stephans and Yamamoto, 1994), DA uptake sites (Nwanze and Jonsson, 1981; Stephans and Yamamoto, 1994), or N-methyl-D-aspartate type GLU receptors (NMDAR) (Sonsalla *et al.*, 1989; Weihmuller *et al.*, 1991; Marshall *et al.*, 1993).

Pretreatment with drugs that block DA synthesis or uptake sites, interfere with METH-induced DA release and protects against loss of TH activity, DA depletion, formation of 6-OHDA, and terminal damage sensitive to silver staining (Hotchkiss and Gibb, 1980; Wagner *et al.*, 1983; Ricaurte *et al.*, 1984; Schmidt *et al.*, 1985; Axt *et al.*, 1990). DA uptake inhibitors protect against METH-induced DA depletion and loss of TH activity even when administered 6 hours (Pu *et al.*, 1994) or 8 hours (Marek *et al.*, 1990a) after METH treatment.

Attenuation of DA synthesis by pretreatment with the TH inhibitor α-methylparatyrosine reduces the METH-releasable DA pool and prevents both the DA and GLU overflow (Nash and Yamamoto, 1993). This suggests that the delayed GLU overflow may be dependent upon an initial release of DA. However, DA uptake blockers confer protection against METH toxicity and attenuate the release of DA (from 50-80 times baseline to 15-20 times baseline) without affecting GLU overflow (Stephans and Yamamoto, 1994). Since the severity of METH toxicity is correlated with cumulative DA overflow (O'Dell *et al.*, 1991), perhaps this lower level of extracellular DA is sufficient to induce GLU release but not long-term DA depletion.

Inhibition of the postsynaptic effects of DA transmission by blockade of D1 or D2 type DA receptors (D1R or D2R) significantly attenuates the neurotoxic effects of METH (Buening and Gibb, 1974; Hotchkiss and Gibb, 1980; Sonsalla *et al.*, 1986; Johnson *et al.*, 1989; Marshall *et al.*, 1993). The D2R antagonist haloperidol attenuates METH toxicity and blocks GLU release without reducing DA overflow

(Nash and Yamamoto, 1993; Stephans and Yamamoto, 1994). The D1R antagonist SCH23390 has been found to reduce the DA overflow and prevent DA toxicity (O'Dell *et al.*, 1993).

Competitive and noncompetitive antagonists acting at the NMDAR protect against METH-induced astrogliosis, morphological alterations, and decreases in DA content, TH activity, and DA uptake sites (Sonsalla *et al.*, 1989; Sonsalla *et al.*, 1991; Muraki *et al.*, 1992; Marshall *et al.*, 1993; Miller and O'Callaghan, 1993; Ohmori *et al.*, 1993; Pu and Vorhees, 1995). Pretreatment with the noncompetitive NMDAR antagonist MK-801 attenuates DA overflow and protects against DA depletion (Weihmuller *et al.*, 1991; Marshall *et al.*, 1993). In addition, selective destruction of striatal output neurons by intrastriatal infusion of the excitotoxin quinolinic acid, protects against METH (4 mg/kg every 2 hrs, 4 times) induced DA loss but had no effect on DA overflow (O'Dell *et al.*, 1994). Together, this evidence indicates that METH-induced DA overflow alone is not sufficient to induce toxicity and suggests that the thalamocorticostriatal loop must be intact for DA reduction to occur.

Activation of DA or GLU Receptors Augments Toxicity

Increasing cytosolic DA levels by pretreating with reserpine (results in DA moving from the synaptic vesicles to the cytoplasm), L-dopa (a DA precursor), or iprindole (a MAO inhibitor) enhances METH-induced DA depletion (Wagner *et al.*, 1983; Schmidt, 1992; Weihmuller *et al.*, 1993). METH toxicity can also be exacerbated by

selective activation of NMDARs (Sonsalla *et al.*, 1991; Sonsalla *et al.*, 1992).

Reciprocal Modulation of DA and GLU

As mentioned previously, the CD/P receives glutamatergic input from the MCTX and dopaminergic projections from the SN pars compacta. Although there are few, if any, axoaxonic synapses between corticostriatal and nigrostriatal afferents, there are indications that these systems can functionally modulate each other. Application of DA or D2R agonists have been found to inhibit Ca^{2+} dependent depolarization-evoked release of GLU in the CD/P both *in vitro* (Rowlands and Roberts, 1980; Maura *et al.*, 1988) and *in vivo* (Yamamoto and Davy, 1992). In addition, D2R antagonists have been found to reverse the actions of DA agonists (Maura *et al.*, 1988; Yamamoto and Davy, 1992) and, in some cases, potentiate GLU release (Nieollon *et al.*, 1983). In addition, GLU and NMDAR agonists have been found to enhance the Ca^{2+} dependent, impulse-independent, release of DA within the CD/P (Roberts and Anderson, 1979; Clow and Jhamandas, 1989; Krebs *et al.*, 1991).

This evidence has led to the suggestion that D2Rs are located on corticostriatal terminals, and NMDARs are located on nigrostriatal terminals (Maura *et al.*, 1988; Krebs *et al.*, 1991; Yamamoto and Davy, 1992), although this claim has not been supported by others (Joyce and Marshall, 1987; Hersch *et al.*, 1995). Studies in primates have found that corticostriatal and nigrostriatal afferents terminate on the same striatal neurons (Smith *et al.*, 1994) suggesting that the modulatory effects could be mediated

through postsynaptic mechanisms. In fact, Hersch *et al.* (1995) found that although corticostriatal afferents themselves were not labeled for D1Rs or D2Rs, they often synapsed on dendritic spines that were labeled for either of the DA receptors. This evidence is consistent with the pharmacological studies which indicate that DA inhibits corticostriatal activity and GLU enhances nigrostriatal activity. The means by which these systems influence each other, whether by presynaptic or postsynaptic mechanisms, is unclear at this time.

The interactions between DA and GLU appear to be regionally heterogeneous. Bardgett *et al.* (1993) recently reported that an acute systemic injection of the D2R antagonist haloperidol increased the total amount of GLU in the CD/P but had no effect in the NAC. Although Roberts and Anderson (1979) reported GLU-induced DA release in NAC slices, this accumbal release has not been found by others (Boldry and Uretsky, 1988). As discussed in the previous section, GLU release within the CD/P appears to be a required step in the METH-toxicity cascade. The suggestion that there may be inherent functional differences between the NAC and CD/P, in reference to the modulatory effects between DA and GLU, could help to explain the dichotomy between METH's effects in these regions.

To summarize, in addition to inducing carrier-mediated release of DA, METH administration results in a delayed overflow of GLU selectively within the CD/P (O'Dell *et al.*, 1991; Abekawa *et al.*, 1994). Blockade of DA or GLU receptors, or attenuation of GLU or DA

release, is sufficient to prevent both the neurochemical and morphological changes caused by METH (Hotchkiss and Gibb, 1980; Axt *et al.*, 1990; Stephans and Yamamoto, 1994). The delayed GLU overflow appears to be dependent upon both DA release and activation of D2Rs (Nash and Yamamoto, 1993; Stephans and Yamamoto, 1994). Furthermore, enhancing the cytosolic pool of DA or administration of NMDAR agonists potentiates METH toxicity (Sonsalla *et al.*, 1991; Weihmuller *et al.*, 1993). This evidence suggests that DA and GLU act in a synergistic manner to produce METH toxicity. Indeed, there is substantial evidence to indicate that nigrostriatal and corticostriatal afferents can functionally influence the release of GLU and DA, respectively. Activation of D2Rs inhibits the release of GLU (Yamamoto and Davy, 1992), while stimulation of NMDARs enhances DA release (Krebs *et al.*, 1991).

The Role of GLU in the Oxidative Hypothesis

Glutamate is known to be a potent and rapidly acting neurotoxin in the CNS. Excessive activation of glutamate receptors causes prolonged depolarization and Ca^{2+} influx, ultimately leading to cell death (Choi *et al.*, 1987). However, excitotoxicity alone is unlikely to result in METH-induced DA depletion, in that the pattern of damage differs from what would be expected from an excitotoxic lesion. Presynaptic terminal and axonal damage, the result of METH treatment, is not characteristic of excitotoxic lesions (Robinson and Coyle, 1987). In addition, antagonists of the NMDAR acting at the strychnine-insensitive glycine receptor protect against NMDA mediated excitotoxicity (Patel *et al.*, 1990) but do not protect

against METH-induced toxicity (Layer *et al.*, 1993). Finally, O'Dell *et al.* (1994) have reported that infusion of quinolinic acid into the CD/P induces an excitatory lesion which damages postsynaptic neurons and protects against METH-induced toxicity. This suggests that although GLU may be intricately involved in the neurotoxic action of amphetamines, it is not acting directly to induce the toxicity.

Alternatively, GLU release could lead to a decrease in DA content by furthering the formation of 6-OHDA or other oxidative byproducts. METH- and GLU-induced DA release are additive in that METH administration results in Ca^{2+} independent cytosolic release and GLU induces Ca^{2+} dependent vesicular release (McMillen, 1983; Bowyer *et al.*, 1991; Stephans and Yamamoto, 1994). Blockade of either DA or GLU overflow is sufficient to prevent METH-induced DA depletion (Stephans and Yamamoto, 1994), indicating that both cytoplasmic and vesicular DA release may be necessary for toxicity to occur. In addition, administration of either DA or GLU agonists enhances DA depletion (Sonsalla *et al.*, 1991; Weihmuller *et al.*, 1993), further supporting the idea that both DA and GLU are involved in the toxic effects of psychostimulants. The additive effects of METH- and GLU-induced DA overflow could overwhelm the ability of the presynaptic terminal to metabolize DA and enhance the formation of endogenous neurotoxins.

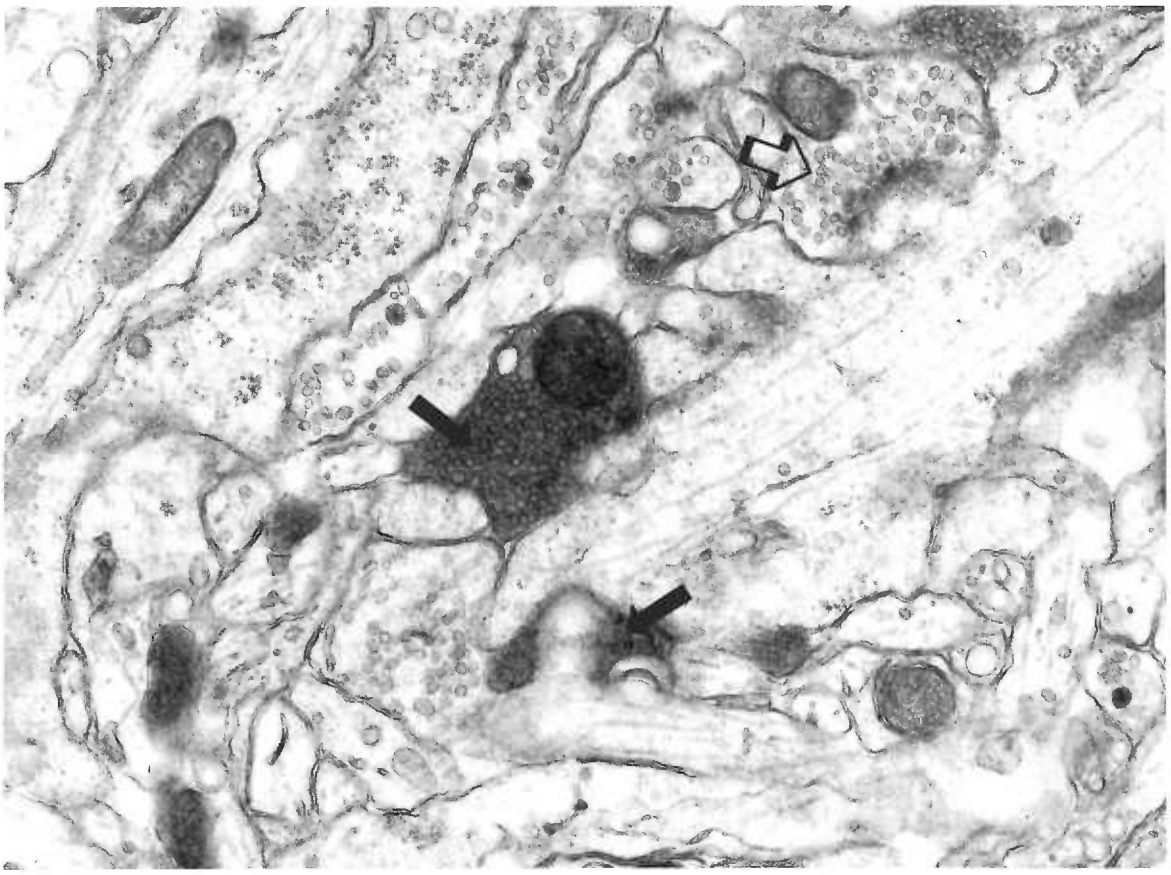
Experiment 1: Examination for Terminal Degeneration

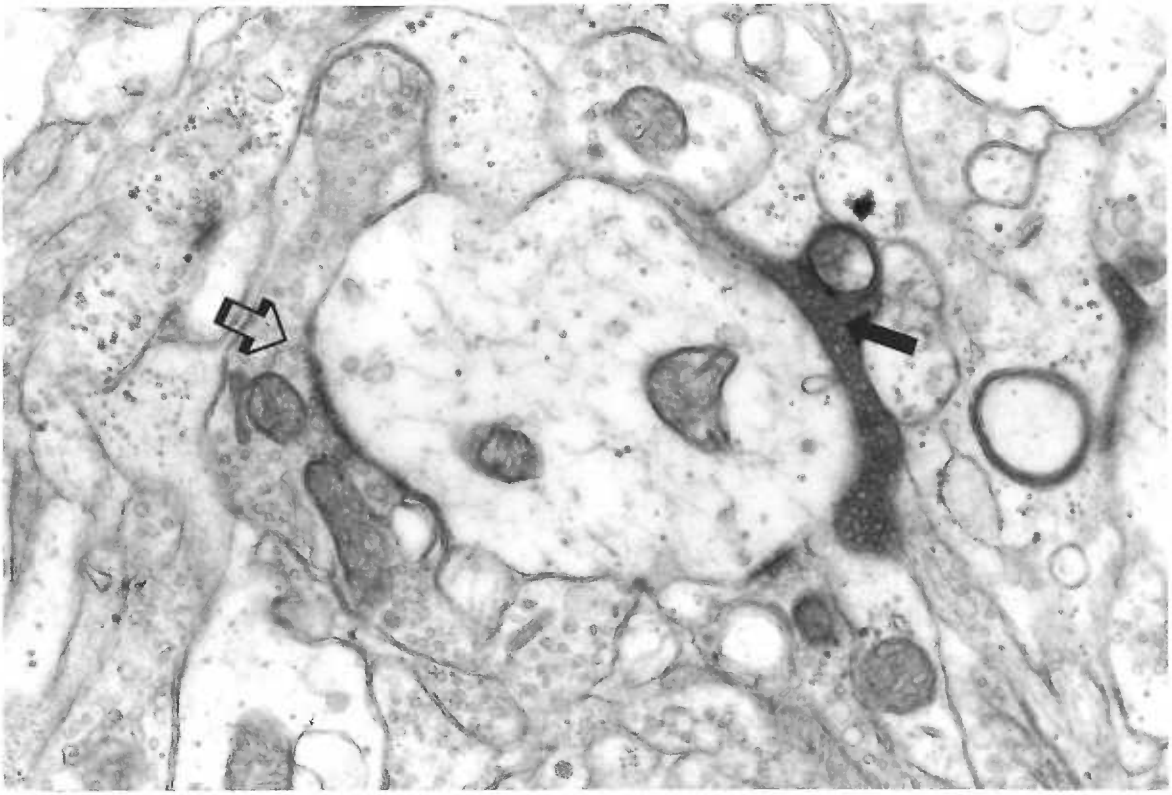
Rationale

There is a substantial amount of indirect evidence suggesting that METH treatment results in a loss of DA containing terminals, but there is little direct ultrastructural evidence supporting this claim. Although Ryan *et al.* (1990) reported electron microscopic evidence of AMPH-induced terminal degeneration, the dosage that resulted in these changes (20-60 mg/kg/day for 3 days) resulted in the death of >50% of the animals and was much higher than the dose of METH required to induce DA depletion (4 mg/kg every 2 hrs, 4 times) (O'Dell *et al.*, 1991). The lack of reported ultrastructural evidence for terminal degeneration following lower doses of METH suggests that METH administration may result in decreased DA synthesis in the absence of terminal degeneration. However, since there are no studies reporting a lack of degeneration following METH, it is not clear if this phenomenon has been examined. This experiment was designed to test the hypothesis that a moderate dose of METH, known to induce DA depletion, results in the degeneration of presynaptic terminals selectively within the CD/P.

Although Ryan *et al.* (1990) reported degeneration following the third day of continuous AMPH treatment, it is not known when METH-induced degeneration is initiated, how long the process of degeneration lasts, or if degeneration continues following removal of the drug. In order to fully understand the nature of METH-induced neurodegeneration, it is important to know the exact time course of degeneration. Tissue from the DL CD/P, VL CD/P, MCTX, and shell of

Figure 2. Degenerating terminals (closed arrows) in spinal cord explants treated with 6-OHDA (initial magnification: X15,000). Note that the degenerating terminals can be easily distinguished from non-degenerating terminals (open arrows). Micrograph courtesy of C. K. Meshul.





Predictions

The introduction described evidence supporting the theory that METH-induced DA depletion within the CD/P is the result of the specific destruction of DA containing terminals arising from the nigrostriatal pathway, and that the damage may be caused by the endogenous formation of oxidative DA metabolites. If this hypothesis is true, then administration of a moderate dose of METH known to induce DA depletion should result in the degeneration of terminals within the CD/P. The severity of METH-induced degeneration should mimic the neurochemical and histochemical damage previously reported. Based on this information, the greatest amount of damage should be found within the VL CD/P, followed by moderate amounts in the DL CD/P and little or no degeneration within the shell of NAC or MCTX.

Detectable levels of endogenous 6-OHDA can be measured 30 min to 2 hrs following a single large injection of METH (100 mg/kg), indicating when maximal concentrations of the neurotoxin are formed (Seiden and Vosmer, 1984; Axt *et al.*, 1990; Marek *et al.*, 1990a). If the oxidative hypothesis is true and METH administration results in the production of significant levels of oxidative neurotoxins, then degeneration of DA containing nerve terminals resulting from METH administration should follow a similar time course. Injection of 6-OHDA intraventricularly induces terminal degeneration in the striatum within 24 hrs. This degeneration peaks 2-3 days following 6-OHDA administration, and declines over the next 7 days (Hedreen and Chalmer, 1972). Based on these

experiments, it is predicted that METH-induced degeneration should peak at 1-3 days, be in the final stages at 7 days, and should be completed by 14 days post drug.

Experiment 2: Analysis of GLU Immunoreactivity

Rationale

There is strong support for the hypothesis that both DA and GLU overflow are required for METH toxicity (Sonsalla *et al.*, 1991; Nash and Yamamoto, 1992; Stephans and Yamamoto, 1994). METH is known to increase the amount of extracellular DA by blocking its reuptake and inducing the release of newly synthesized DA from the cytosolic pool (Raiteri *et al.*, 1979; Liang and Rutledge, 1982; McMillen, 1983). However, the source of the delayed GLU overflow is not known since GLU measured by *in vivo* microdialysis may arise from either glial or neuronal sources (Westerink *et al.*, 1987). There are at least four ways in which an increase in extracellular levels of GLU within the CD/P could occur: (1) GLU could be released from glia, (2) GLU reuptake by glia or nerve terminals could be compromised, (3) GLU could be released locally in an impulse-independent manner, or (4) GLU could be released in an impulse-dependent manner via activation of the thalamocorticostriatal loop (Nieollon *et al.*, 1983; Girault *et al.*, 1986; Paulsen and Fonnum, 1989; Szatkowski *et al.*, 1990).

Application of DA or D2R agonists has been found to inhibit Ca^{2+} dependent depolarization-evoked release of GLU in the CD/P (Rowlands and Roberts, 1980; Yamamoto and Davy, 1992). As

mentioned previously, afferents arising from the MCTX provide substantial GLU input to the CD/P (Beckstead, 1976). Thus, the massive release of DA that follows METH administration could inhibit the impulse-dependent release of GLU from corticostriatal terminals. Once extracellular DA returns to basal levels following the last injection of METH, the excitatory afferents could become disinhibited, leading to the activation of the corticostriatal pathway and the delayed overflow of GLU. Abekawa *et al.* (1994) reported that DA overflow reached similar magnitudes in the CD/P and NAC, but that the GLU overflow occurred only within the CD/P. Such a dichotomy in GLU overflow would be expected if METH led to the delayed disinhibition of the corticostriatal pathway.

These proposed experiments were designed to test the hypothesis that METH administration results in a decrease in presynaptic GLU levels within nerve terminals associated with asymmetric synapses. Decreases in presynaptic GLU levels would be indicative of neuronal release, and analysis of GLU immunoreactivity within presynaptic terminals will provide information about the specific role of neuronally derived GLU in METH toxicity. Because of the limitations associated with *in vivo* microdialysis measurements of extracellular GLU (Nash and Yamamoto, 1992; Stephans and Yamamoto, 1994), it is not known when or if GLU levels return to baseline. For these reasons, tissue from the DL CD/P, VL CD/P, MCTX, and shell of NAC was examined at various time points following METH administration in order to determine (1) if the time course of altered immunolabeling mimics the increased level of

extracellular GLU found in previous *in vivo* microdialysis studies (Abekawa *et al.*, 1994; Stephans and Yamamoto, 1994) and (2) if the change in the density of nerve terminal GLU immunoreactivity is long-lasting. Analysis of GLU immunoreactivity was performed on the same tissue examined for terminal degeneration.

Predictions

There is evidence suggesting that METH-induced damage is partially dependent upon the delayed overflow of GLU. This release of GLU may be the result of activation of the thalamocorticostriatal loop, resulting in the release of neuronal GLU via stimulation of the corticostriatal pathway. If this hypothesis is true, then there should be less GLU, and therefore lower labeling, within asymmetrical presynaptic terminals within the CD/P, but not within the shell of NAC. If the thalamocorticostriatal loop is activated following METH administration, then GLU should be released within the superficial layers of the MCTX where excitatory thalamic inputs terminate. If METH administration results in the release of neuronal GLU within the MCTX, then the density of nerve terminal GLU immunolabeling within this cortical region should also decrease.

If the GLU overflow following METH administration is due to the release of neuronal GLU, then the density of GLU immunolabeling should begin to decrease 2 hrs after the administration of METH and should be maximally decreased 12 hrs after the last dose of METH (Nash and Yamamoto, 1992; Nash and Yamamoto, 1993; Abekawa *et al.*, 1994; Stephans and Yamamoto, 1994). If the effect of METH on excitatory synapses is transient, then the density of nerve terminal

GLU immunolabeling within presynaptic asymmetrical terminals should return to basal levels. If METH has a prolonged effect, then changes in immunolabeling may continue to be seen even 1 or 2 weeks after drug administration.

Experiments 3 and 4: Measures of METH Toxicity

Rationale

There is some evidence that METH-induced toxicity is influenced by environmental factors that may fluctuate between research facilities (John Marshall, personal communication). These studies were designed to test the hypothesis that our schedule of METH administration results in DA toxicity as measured by DA depletion or hyperthermia. Most measures of METH toxicity (i.e., DA levels, TH immunohistochemistry, etc.) cannot be measured in the same animals examined for electron microscopy because of the manner in which the tissue is processed for ultrastructural examination. For this reason, catecholamine content (DA, NE, DOPAC, and HVA) was measured in a separate group of animals exposed to the same METH regimen. Peak body temperature has been found to correlate with DA depletion (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994) and can be easily measured. Therefore, animals used in the immunocytochemical analysis had their core body temperature monitored throughout METH administration.

DA depletions lasting longer than 2 weeks following moderate doses of METH have not been examined; previous studies typically examined DA content 3 to 14 days after drug administration (Table

1). DA content was analyzed in animals allowed to survive for 1 or 4 weeks after METH treatment in order to test the hypothesis that recovery from METH-induced neurotoxicity occurs. Previous studies have suggested that a dose-dependent recovery from METH toxicity can occur. Bowyer *et al.* (1992) reported that METH administration (5 mg/kg every 2 hrs, 4 times) resulted in a 60% reduction of CD/P DA content 3 days following drug treatment. After 14 days, DA content was only depleted by 30%. Thus, it stands to reason that DA content could approach baseline levels after a longer period of time.

Predictions

Peak body temperature has been found to correlate with DA depletion (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994). Therefore, if METH administration results in severe hyperthermia to the degree associated with DA depletion (39.4-42.2°C), it would strongly suggest that METH induced DA depletion in the current studies.

It has been reported that METH administration results in approximately a 50% reduction of DA without affecting NE content (Ellison *et al.*, 1978; Wagner *et al.*, 1979; Lucot *et al.*, 1980; Ricaurte *et al.*, 1982; Seiden *et al.*, 1988). Occasionally, loss of the DA metabolites DOPAC and HVA have also been reported (Table 2). If administration of METH in this study is neurotoxic, then DA, and possibly DOPAC and HVA, should be depleted 1 week following METH treatment. In addition, there may be a dose dependent recovery of DA levels following METH administration (Bowyer *et al.*, 1992). If recovery of DA content does occur, then DA levels should approach

baseline levels in animals allowed to survive 4 weeks after METH treatment.

MATERIALS AND METHODS

General Methods

Subjects

Male Sprague-Dawley rats (Harlan, 200-300 g, age 2-3 months) were housed 2-3 to a cage and maintained on a 12 hr light/dark cycle (lights on at 6:00 am) with food and water available ad lib.

Drug Administration

For all experiments, 5 mg/kg METH (of the salt, s.c., in normal saline) or an equivalent volume of vehicle (SAL) was administered to rats every 2 hours for 4 injections. Experiments were performed on days when cages were normally cleaned. Animals were placed into clean cages 30-45 min before the first injection. At this time, most animals were resting or asleep. Food and water were available to animals at all times throughout the experiments. Room temperature was 22 ± 1°C for all experiments

Drugs

Glutamate antibody (polyclonal rabbit IgG, supplied by Arnel, Brooklyn, NY.) has been previously characterized (Hepler *et al.*, 1988). Secondary antibody (goat anti-rabbit IgG, conjugated to 10 nm gold particles) was supplied by Amersham, Arlington Heights, IL. (+)-Methamphetamine hydrochloride was supplied by Sigma Chemical Co. (St Louis, MO.).

Experiments 1 and 2: Electron Microscopy and Immunocytochemistry

Procedure

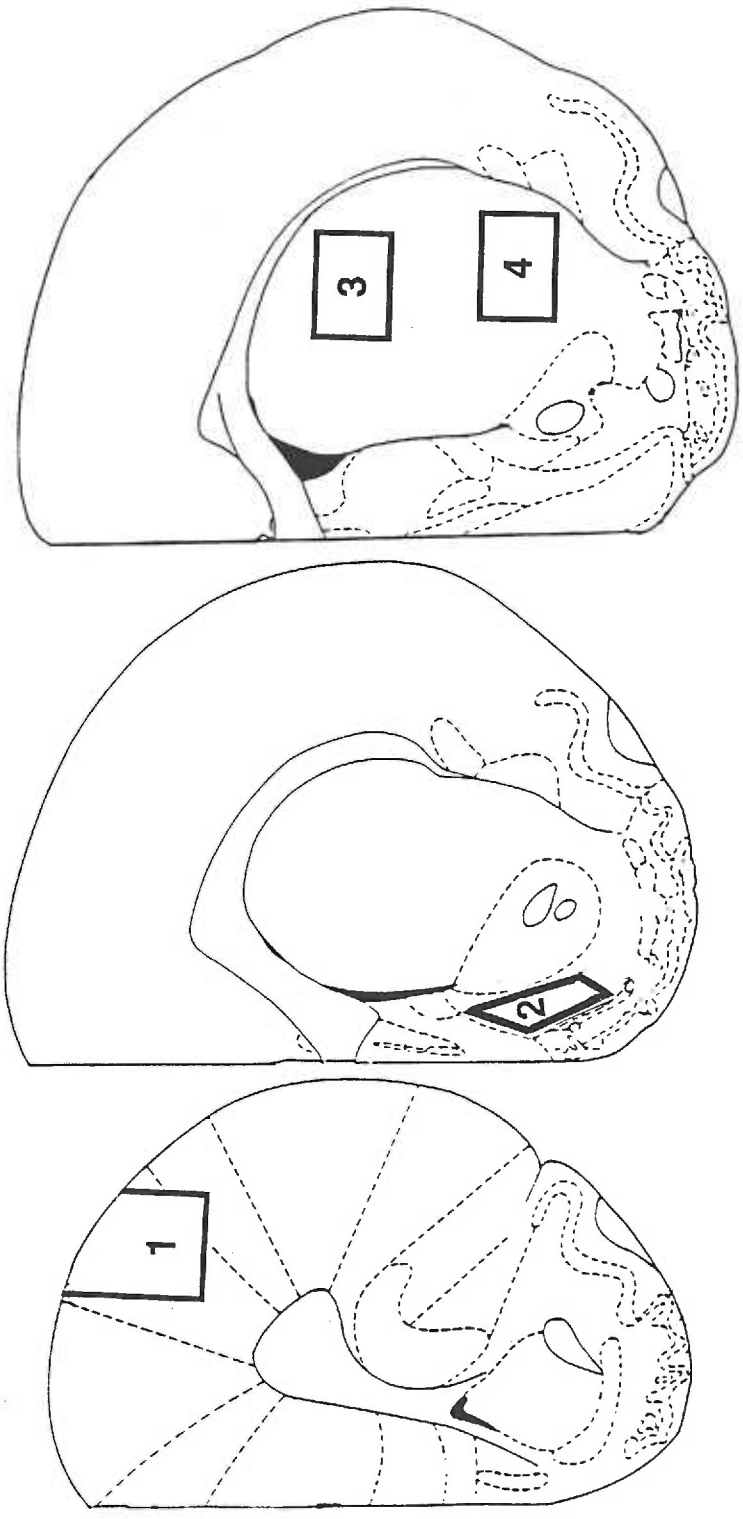
METH or SAL was administered at 4:00 am, 6:00 am, 8:00 am, and 10:00 am. Rats treated with METH were then anesthetized and perfused transcardially with fixative 2 hrs, 12 hrs, 24 hrs, 3 days, 7 days, or 14 days following the last METH injection (n = 4-9 per group, N = 59). SAL treated animals were killed 5 days after treatment.

Preparation of Tissue

Rats were sedated with 1 ml/kg rat cocktail (5% ketamine, 2% xylazine, 1% acepromazine) administered every 20 min for three injections or until animals were deeply anesthetized. The animals were perfused transcardially with fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.1% picric acid in 0.1 M HEPES or 0.1 M phosphate buffered saline (PBS), pH 7.3). Following perfusion, brains were removed and placed in cold fixative overnight (17-20 hrs). Tissue was prepared according to the procedure of Meshul *et al.* (1994).

Brains were washed three times for 30 min each in HEPES or PBS buffer. Brain slices (400 μm) were cut using a vibratome. Pieces of tissue, approximately 1 mm square, from the DL CD/P, VL CD/P, MCTX, and NAC (Figure 3) were dissected and washed in cold HEPES or PBS buffer. Sections were placed into a 1% osmium tetroxide solution with 1.5% potassium ferricyanide for 30-60 min at room temperature. Tissue was washed 4 times in deionized

Figure 3. Diagram illustrating the regions excised for electron microscopic and immunocytochemical analysis. Tissue (approximately 1 mm^2) was taken from the MCTX (**1**), shell of NAC (**2**), DL CD/P (**3**), and VL CD/P (**4**). (Adapted from the atlas by Paxinos and Watson (1986), plates 9, 12, and 16)


3
4
2
1

filtered water and placed into 0.5% aqueous uranyl acetate for 30 min at room temperature. Tissue was dehydrated through an increasing series of ethanols, cleared in propylene oxide, and polymerized in Embed 812/Spurr for no longer than 24 hrs at 60°C.

Electron Microscopy

Processing of tissue for immunocytochemistry involves incubating tissue in solutions containing 0.1% Triton X-100. This etches the surface of the tissue and, as a result, the overall contrast is diminished. Although degenerating profiles would be readily apparent in this tissue, the identification of these profiles could be compromised. For this reason, tissue from 2 animals per group (n = 14) was initially subjected to electron microscopic examination without immunocytochemistry.

Thin sections (approximately 90-100 nm, light gold interference color) were cut and placed on 200 mesh nickel coated grids and allowed to dry overnight at 60°C. Sections were then counterstained with uranyl acetate followed by lead citrate on an Ultrastainer (Leica Co.). Sections were viewed and photographed on a JEOL 1200EX TEM SCAN electron microscope. High magnification photomicrographs of the DL CD/P, VL CD/P, NAC and layers I-III of MCTX (initial magnification: x15,000) were taken randomly throughout the neuropil (10 photos per grid, 2 photos per grid space, photos taken at least 3 grid spaces apart). In addition, low magnification photomicrographs (initial magnification: x2,500) of neuronal cell bodies were taken randomly (5 photos per grid). All photomicrographs were examined under blind conditions (treatment)

for signs of abnormal or degenerating structures. These signs include swelling, darkening, or disruption of axons, dendrites, or terminals in well preserved tissue. An example of terminal degeneration following 6-OHDA treatment is given in Figure 2.

Immunocytochemistry

Post-embedding immuno-gold electron microscopy was performed on tissue from all animals according to a modification of the method by Phend *et al.* (1992). Thin sections (approximately 90-100 nm, light gold interference color) were cut and placed on 200 mesh nickel grids coated with a solution from a Coat-Quik "G" pen (Kiyota International; Elk Grove Village, IL). The sections were allowed to air dry for 3-5 hrs and washed in TBST, pH 7.6 (0.05 M Tris, with 0.9% NaCl and 0.1% Triton X-100). Grids were then transferred to the primary GLU antibody solution. Tissue processed with HEPES as the buffer solution was incubated using the GLU antibody at a dilution of 1:50,000 (EXP 1, see Appendix B) or 1:150,000 (EXP 3), while tissue processed using PBS as the buffer solution was incubated using the GLU antibody at a dilution of 1:200,000 (EXP 2). The GLU antibody was diluted in TBST, pH 7.6 containing 1.0 mM aspartate to prevent the antibody from crossreacting with aspartate in the tissue. Sections were incubated overnight in a moist chamber at room temperature. Grids were washed 3 times (2 X 5 min, then 30 min) in TBST, pH 7.6 and then washed for 5 min in TBST, pH 8.2. Sections were incubated for 1.5 hrs in the secondary antibody (goat anti-rabbit IgG conjugated to 10 nm gold; diluted 1:25 or 1:50 in TBST, pH 8.2). Grids were washed

twice in TBST, pH 7.6, followed by deionized water. Sections were allowed to dry overnight at 60°C. The sections were then counterstained with uranyl acetate followed by lead citrate on an Ultrastainer (Leica Co).

Photomicrographs (initial magnification: X 25,000) were taken randomly throughout the neuropil within the CD/P and NAC, and within layers I-III of MCTX (10 photos per grid, 2 photos per grid space, photos taken at least 3 grid spaces apart in order to account for any unevenness of immunolabeling). The number of gold particles within nerve terminals making asymmetrical synaptic contacts, as determined by the presence of a thickened post synaptic density, were counted and the area of these terminals was determined using a Zeiss Videoplan image analysis system. The GLU antibody is known to crossreact with Krebs cycle intermediates found within mitochondria (Phend *et al.*, 1992). Therefore, any gold-labeling found within mitochondria was excluded from quantitative analysis. The density of labeling of dendritic spines and glial processes in the CD/P has been previously determined for this procedure (Meshul *et al.*, 1994). The specificity of the GLU antibody has also been previously characterized (Hepler *et al.*, 1988; Phend *et al.*, 1992).

Experiment 3: Determination of METH-Induced Hyperthermia

Procedure

Animals receiving SAL (n = 8) or METH (n = 15) had rectal temperatures taken every 30 min beginning at the first injection and ending 2 hours after the final injection. Rectal temperatures were

determined by use of an RET-2 copper-constantan thermocouple rectal probe (Physitemp Instruments, Inc.) and a TH-8 thermalert thermometer (Sensortek, Inc.). Probes were inserted to a depth of 1 inch for 10 sec or until the temperature was steady for approximately 5 sec.

Experiment 4: Determination of Catecholamine levels

Procedure

METH or SAL was administered at 8:00 am, 10:00 am, 12:00 pm, and 2:00 pm. One animal died after receiving the last METH dose. METH treated animals were killed by decapitation 7 days (n = 10) or 28 days (n = 9) following drug administration. SAL (n = 10) treated animals were killed by decapitation 14 days after vehicle administration. All brains were removed and dissected for analysis of DA, NE, HVA and DOPAC content.

HPLC Analysis

Brains were prepared for analysis by high performance liquid chromatography (HPLC) using the method of Feller *et al.* (1993). Briefly, the NAC (+2.2 to +1.0 mm from Bregma), including the core and shell, and CD/P were dissected out (+1.60 to -0.40 mm from bregma). The CD/P was divided into dorsal and ventral halves. Dorsal CD/P, ventral CD/P, and NAC were weighed, quick frozen in liquid nitrogen, and stored at -80°C until assayed. Brains were then thawed at 4 °C and sonicated in 1090LC mobile phase (MP, containing 6.9 g/l sodium phosphate, 250 mg/l heptane sulfonic acid, 80 mg/l EDTA and 7% MeOH v/v, pH=3.7). Samples were rapidly frozen in methanol/dry ice, thawed on ice, and centrifuged at 48,000 x g at 4

°C for 30 min The supernatant (approximately 1.5 mls) was stored at -80 °C until needed. The pellet was stored at -20 °C for later protein analysis.

The supernatant was thawed at 4 °C and centrifuged at 12,000 x g for 15 min prior to HPLC analysis. DA, NE, HVA and DOPAC were separated on an HR-80, C-18 reverse phase column with a flow rate of 0.9 ml/min. Electrochemical detection was carried out using an ESA Coulochem Detector (conditioning cell potential, +0.4 V; detector 1, +0.1 V; detector 2, -0.35 V). Recoveries for each sample were determined by the addition of dihydrobenzylamine (DHBA) as an internal standard. Catecholamine levels, measured as peak height determined by HPLC analysis, were used to calculate the amount in each sample (pg/µl) from standard curves.

Protein Analysis

Pellets were resuspended by sonification in 10 mM TRIS, pH 7.5 with 0.1% Triton X-100. 15 µl of each sample was added to 35 µl of buffer and 5 ml of Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, CA). Samples were allowed to sit for at least 5 minutes. A change in color, detected by a spectrophotometer, was used to calculate the amount of protein in each sample (µg/ml) from standard curves. The amount of catecholamine in each sample (ng/g tissue) was divided by the amount of protein in that sample (mg/g tissue) in order to express the data as the amount of catecholamine per mg protein.

STATISTICAL ANALYSIS

Experiment 2: Analysis of GLU Immunoreactivity

This experiment was designed to examine changes in the density of presynaptic GLU immunoreactivity due to drug treatment. In addition, these changes were assessed in 4 different brain regions. Although all 4 brain regions were taken from some animals, in other cases only 2 or 3 of the regions from a single animal contributed to the analysis. Because brain region is not a purely "between" or "within" subjects factor, data for each region were analyzed separately. Data were analyzed by one-way ANOVA with treatment group (SAL, 2 hrs, 12 hrs, 24 hrs, 72 hrs, 7 days, and 14 days post-METH) as a between-subjects factor. The significance level was set at $\alpha = 0.05$. Significant main effects were further characterized by post-hoc analysis using Peritz' F-test (Einot and Gabriel, 1975; Harper, 1984). Presynaptic terminal area was analyzed similarly.

Experiment 3: METH-Induced Hyperthermia

This experiment was designed to examine changes in body temperature across time and between animals treated with SAL or METH. Data was analyzed by mixed two-factor ANOVA with time as the within-subjects factor (17 time-points, from 0-480 min after the first injection), and drug treatment (SAL or METH) as the between-subjects factor. The significance level was set at $\alpha = 0.05$. In addition, the difference in peak body temperature between SAL and METH treated rats was examined by Student's t-test.

Experiment 4: Determination of Catecholamine levels

This experiment was designed to test for differences in catecholamine content across treatment groups (SAL, 1 week post-METH, and 4 weeks post-METH). To this end, catecholamine content (DA, NE, DOPAC, and HVA) within each brain region (dorsal CD/P, ventral CD/P, and NAC) was analyzed by one-way ANOVA with treatment group as the between-subjects factor. In addition, the ratio of DOPAC/DA was examined in each brain region by one-way ANOVA with treatment group as the between-subjects factor. To control for the possibility of inflated Type I error due to repeated testing, the alpha level was preset at a more stringent level (α = 0.017). This alpha level was determined as follows. Examination of each individual catecholamine (DA, NE, DOPAC, HVA) was treated as a separate experiment containing 3 "sets" of analyses representing catecholamine content at each of the 3 brain regions (i.e., HVA in the dorsal CD/P, HVA in the ventral CD/P, and HVA in the NAC). In order to set the familywise alpha level at α = 0.05 for each of these "sets", the alpha level for each individual comparison was set at α = 0.05/3 = 0.017. Significant main effects were further analyzed by Newman-Keul's test.

RESULTS

Experiment 1: Examination for Terminal Degeneration

No evidence of terminal, axonal, or dendritic degeneration was found in any area at any time following METH administration (**Figures** 4 and 5). Darkened profiles, including glial processes and cell bodies, were occasionally seen in both control and METH treated animals. These profiles were almost always associated with poor tissue preservation and no consistent differences between METH and SAL treated animals were found.

Experiment 2: Analysis of GLU Immunoreactivity

A total of 4710 presynaptic asymmetric terminals were examined (DL CD/P, n = 1433; VL CD/P, n = 1213; MCTX, n = 1238; NAC, n = 826). Approximately 98.4% of these terminals formed asymmetrical synapses with dendritic spines, indicating that the vast majority of synapses examined were of corticostriatal origin (Dubé *et al.*, 1988). Roughly 20 terminals from each of the four different brain regions were photographed and analyzed per animal (Figure 6). Presynaptic asymmetrical terminals were accepted as labeled if the particle density fell within two standard deviations of the subject mean. A total of 10 terminals did not meet this criterion and were excluded from the analysis. For individual subjects, the area of each of these terminals, and the number of gold particles within them, were determined for all 4 brain regions. An average of these terminal measurements was used to represent the subject in all data analyses.

Figure 4. Electron micrograph (initial magnification: X2000) of cell bodies in the DL CD/P 3 days following METH administration. Note the lack of degeneration in the surrounding neuropil (AX = myelinated axon, SYN = synapse, D = dendrite, CB = cell body).

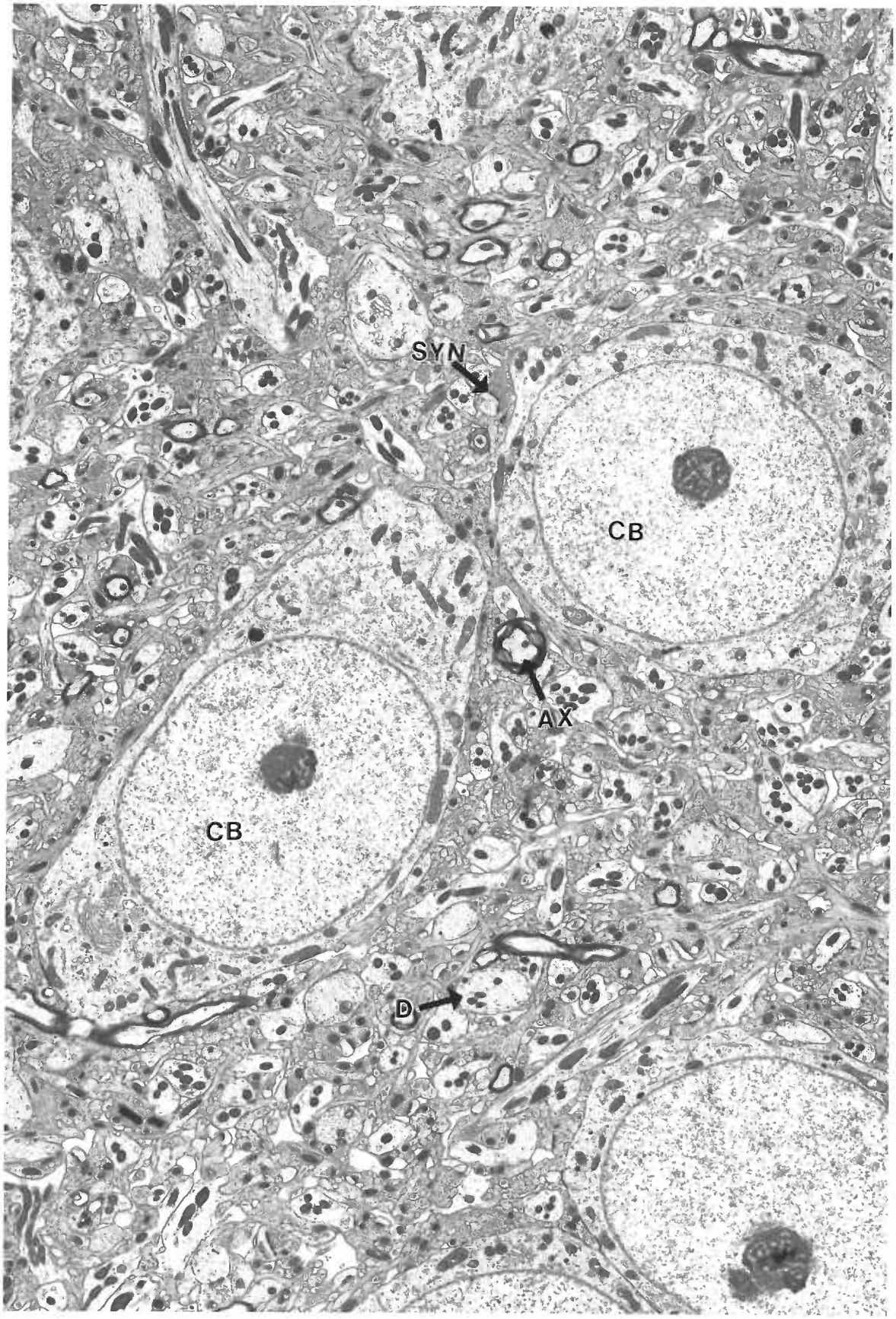

SYN
CB
AX
CB
D

Figure 5. Electron micrograph (initial magnification: X15,000) of synapses in the DL CD/P 3 days following METH administration. Note the lack of degeneration in both symmetrical and asymmetrical synapses (D = dendrite, SP = dendritic spine, NT = presynaptic terminal, closed arrow = asymmetrical contact, open arrow = symmetrical contact).

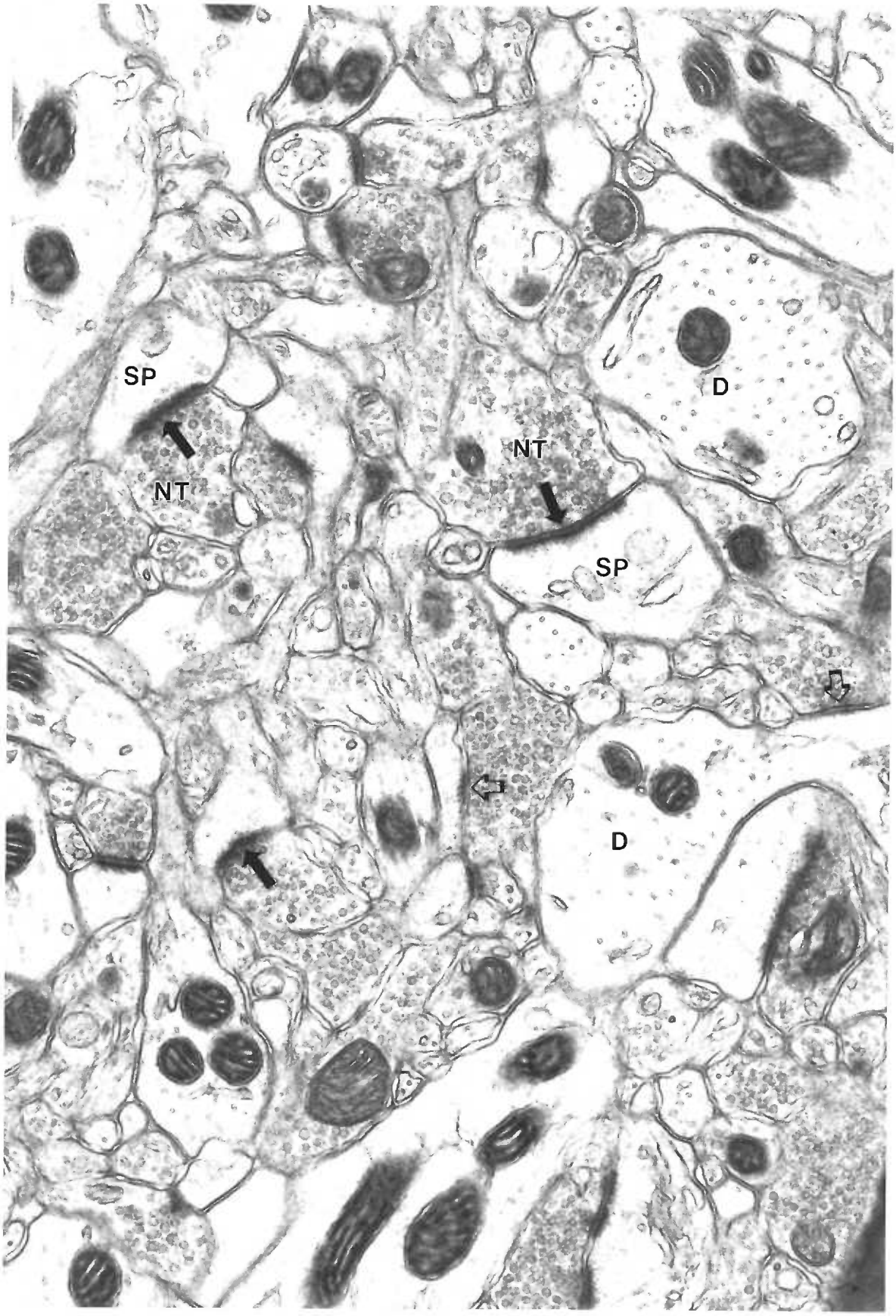
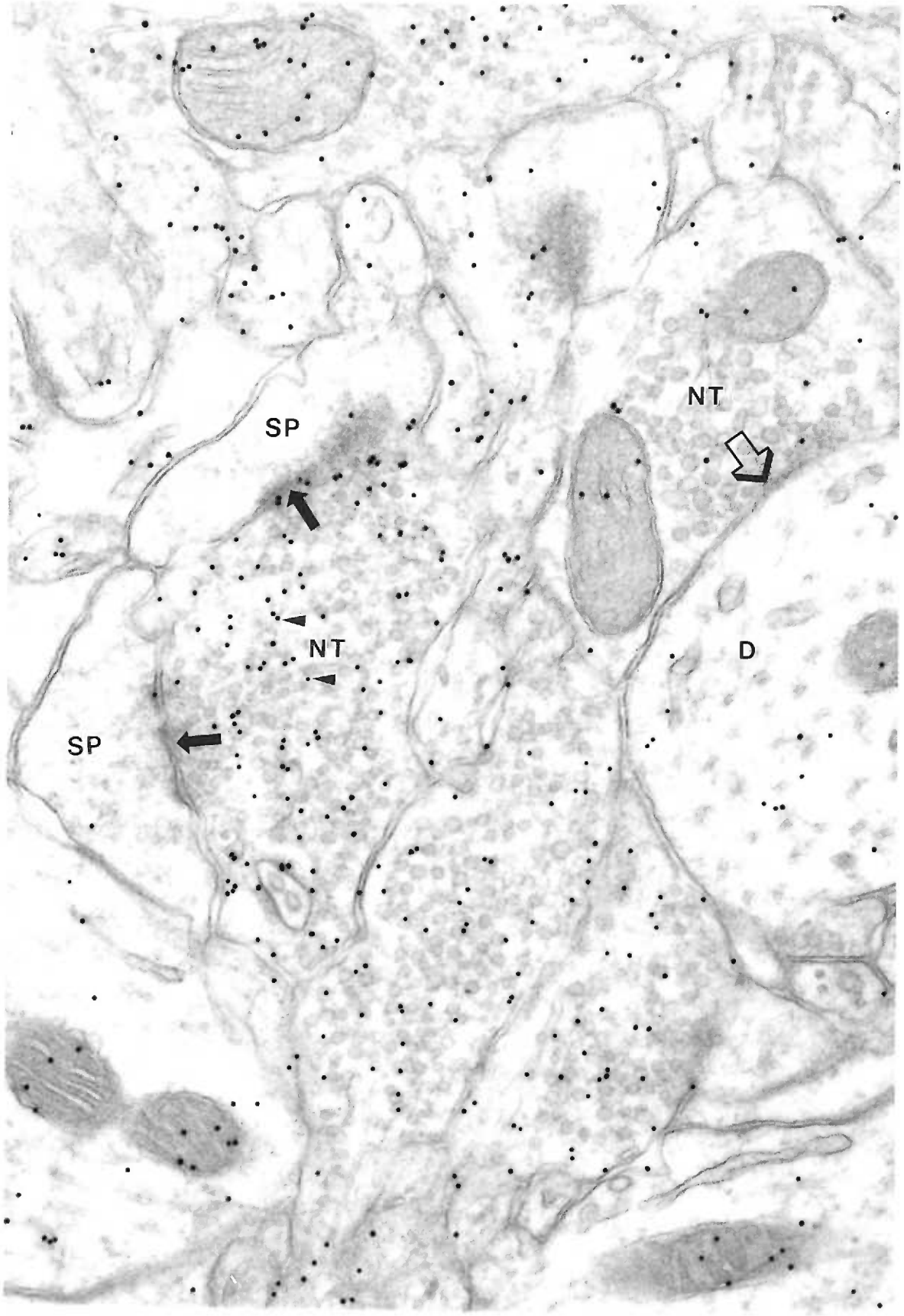

SP
NT
NT
SP
D
D

Figure 6. Electron micrograph (initial magnification: X25,000) of immunolabeled synapses in the DL CD/P following SAL administration. Note the concentration of immuno-gold labeling in the presynaptic asymmetrical terminal as opposed to the presynaptic symmetrical synapse (arrow head = gold particle, D = dendrite, SP = dendritic spine, NT = presynaptic terminal, closed arrow = asymmetrical contact, open arrow = symmetrical contact).


SP
NT
NT
D
SP

Gold Particle Density

Data were collected over three different experiments and particle density values were standardized (see Appendix B). Adjusted particle density scores are presented in Table 3. Analysis by one-way ANOVA revealed significant differences in the density of presynaptic immunolabeling within the VL CD/P ($F_{6,41} = 2.7$, $P = 0.03$), MCTX ($F_{6,36} = 2.7$, $P = 0.03$), and shell of NAC ($F_{6,37} = 2.7$, $P = 0.03$). No significant changes were detected within the DL CD/P ($F_{6,47} = 1.1$, $P = 0.36$). Post-hoc analysis by Peritz' F test (Harper, 1984) revealed significant differences between the SAL and METH treated animals 12 hrs after drug administration (Figure 7) in the MCTX ($P < 0.05$) and VL CD/P ($P < 0.05$). In both these regions, the density of GLU immunolabeling was decreased by approximately 35%. Although there were no statistically significant decreases in DL CD/P, there was a trend toward decreased labeling at the 12 hr time point ($P = 0.10$). At this time point, the density of GLU immunolabeling was decreased by 26%. Within the shell of the NAC, there were no differences between SAL and METH treated animals. The significant ANOVA can be attributed to a difference between the day 7 and day 14 groups ($P < 0.05$).

Presynaptic Terminal Area

A non-specific decrease in GLU immunoreactivity could be caused by swelling of the presynaptic terminals. Therefore, terminal area was analyzed to determine if changes in immunolabeling were due to alterations in the size of these terminals. Area means for each group are presented in Table 4. In

Table 3. Gold particle density (Mean num/μm^2 ± SEM) within the asymmetric nerve terminals of 4 different brain regions, and the number of animals per group (n), calculated at various times following SAL or METH treatment. Data were analyzed by one-way ANOVA followed by Peritz F Test (* $p < 0.05$ compared to SAL).

Group	MCTX	DL CD/P	VL CD/P	NAC
SAL	85.48 ± 8.05 (7)	110.09 ± 8.60 (9)	110.07 ± 11.68 (7)	108.39 ± 11.38 (7)
2 hrs	65.95 ± 5.41 (4)	89.08 ± 13.34 (8)	109.38 ± 8.40 (8)	119.43 ± 18.44 (5)
12 hrs	56.94 ± 4.73* (5)	81.13 ± 11.49 (8)	69.17 ± 6.23* (8)	87.21 ± 17.79 (5)
24 hrs	79.77 ± 7.16 (7)	98.12 ± 5.52 (9)	95.73 ± 11.54 (8)	92.02 ± 8.62 (7)
3 days	89.99 ± 8.79 (7)	113.09 ± 11.02 (7)	112.06 ± 9.25 (7)	92.26 ± 11.39 (7)
7 days	84.38 ± 6.91 (7)	87.13 ± 11.03 (7)	115.49 ± 16.37 (4)	77.81 ± 8.70 (7)
14 days	92.07 ± 5.12 (6)	104.72 ± 20.42 (6)	118.06 ± 15.58 (6)	138.48 ± 14.52 (6)

Figure 7. Decreased gold particle density, expressed as percent of SAL treated controls, within asymmetrical nerve terminals following METH treatment (5 mg/kg). Differences within the DL CD/P, VL CD/P, MCTX, and NAC were analyzed by one-way ANOVA followed by Peritz F test (* $p < 0.05$ compared to SAL). The hatched lines represent the SEM of the control groups.

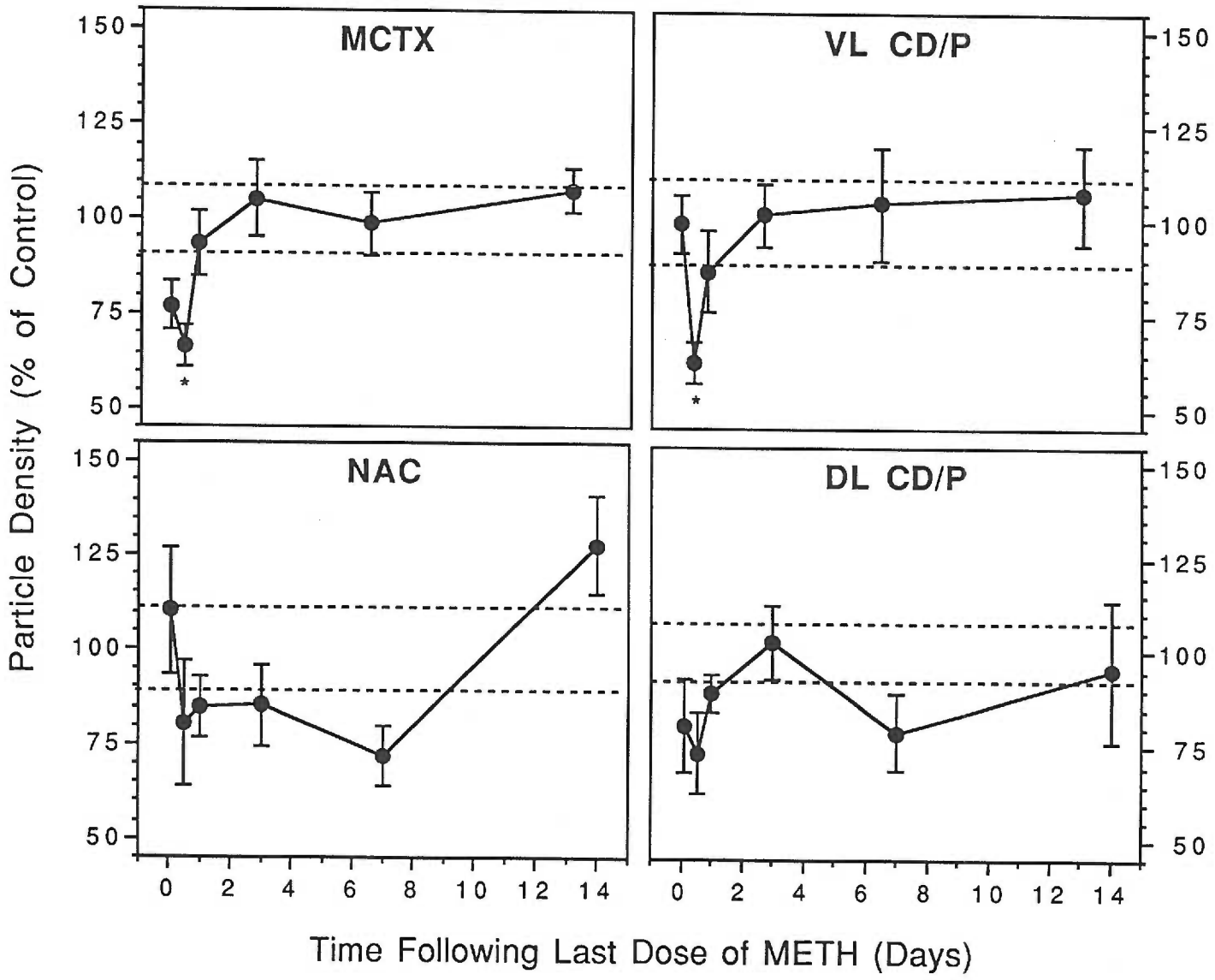

MCTX
VL CD/P
NAC
DL CD/P
Particle Density (% of Control)
Time Following Last Dose of METH (Days)
150
125
100
75
50
0
2
4
6
8
10
12
14
*
*

Table 4. Presynaptic terminal areas (Mean μm^2 ± SEM, n per group) within 4 different brain regions, and the number of animals per group (n), calculated at various times following SAL or METH treatment. Data were analyzed by one-way ANOVA. No significant differences were detected.

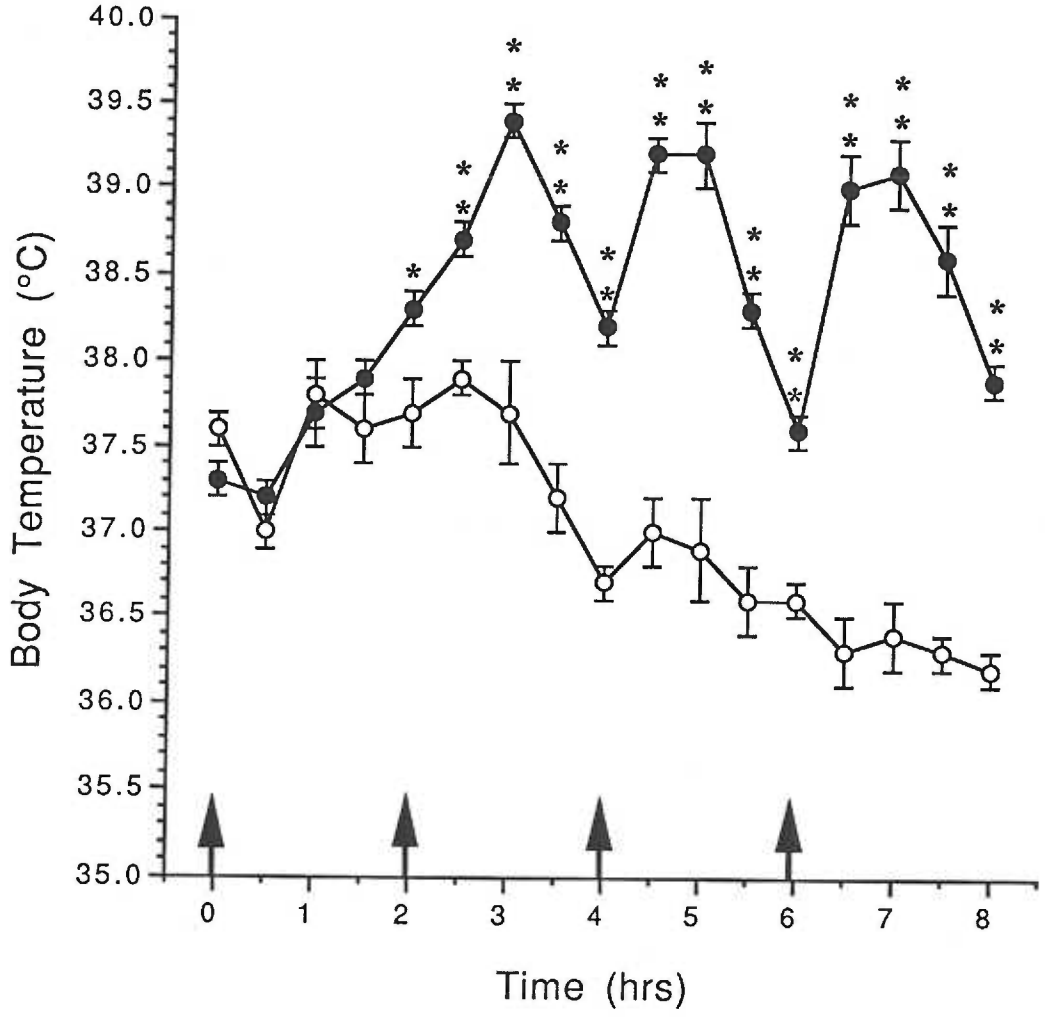
Group	MCTX	DL CD/P	VL CD/P	NAC
SAL	0.325 ± 0.019 (7)	0.372 ± 0.031 (9)	0.352 ± 0.028 (7)	0.450 ± 0.034 (7)
2 hrs	0.325 ± 0.019 (4)	0.341 ± 0.008 (8)	0.296 ± 0.013 (8)	0.511 ± 0.041 (5)
12 hrs	0.327 ± 0.017 (5)	0.329 ± 0.018 (8)	0.326 ± 0.015 (8)	0.442 ± 0.033 (5)
24 hrs	0.311 ± 0.007 (7)	0.314 ± 0.007 (9)	0.315 ± 0.013 (8)	0.423 ± 0.024 (7)
3 days	0.336 ± 0.014 (7)	0.329 ± 0.014 (7)	0.294 ± 0.012 (7)	0.433 ± 0.025 (7)
7 days	0.359 ± 0.012 (7)	0.352 ± 0.021 (7)	0.296 ± 0.032 (4)	0.467 ± 0.024 (7)
14 days	0.360 ± 0.022 (6)	0.328 ± 0.021 (6)	0.308 ± 0.014 (6)	0.542 ± 0.024 (6)

contrast to the finding that decreases in GLU immunoreactivity occur 12 hrs following drug administration, the effects of METH do not vary across time. No significant changes in presynaptic terminal area were found by one-way ANOVA in the DL CD/P ($F_{6, 47} = 1.1$, $P = 0.36$), VL CD/P ($F_{6, 41} = 1.4$, $P = 0.24$), MCTX ($F_{6, 36} = 1.4$, $P = 0.25$), or shell of the NAC ($F_{6, 37} = 2.2$, $P = 0.07$). This indicates that the decrease in GLU immunolabeling was not due to an increase in terminal size, but rather was the result of a loss of presynaptic GLU immunoreactivity.

Experiment 3: METH-Induced Hyperthermia

Peak body temperature for METH treated animals (39.8 ± 0.13°C) was significantly higher compared to SAL treated animals (37.0 ± 0.08°C) as analyzed by Student's t test ($t = 15.2$, $df = 21$, $P < 0.0001$). Analysis by two-way ANOVA revealed that there was a significant interaction between time and drug treatment ($F_{16, 336} = 18.74$, $P < 0.0001$), indicating that the degree of hyperthermia depended upon the time at which body temperature was taken. Analysis of simple effects revealed that METH treated animals had significant increases in body temperature that began 2 hrs after the first injection of METH ($P < 0.01$) and lasted throughout the rest of the experiment ($P < 0.0005$). Figure 8 shows that the elevation of body temperature following METH treatment was phasic, and peaked approximately 1 hr following each drug injection. In addition, there was a decline in body temperature across time within the SAL treated animals.

Figure 8. Rectal temperature (Mean ± SEM) of rats receiving repeated injections (arrows) of SAL (open circles) or 5 mg/kg METH (closed circles). Data were analyzed by a two-factor mixed design ANOVA (* $P < 0.01$; ** $P < 0.0005$ compared to SAL)


Body Temperature (°C)
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
0
1
2
3
4
5
6
7
8
Time (hrs)

Experiment 4: Analysis of Catecholamine Content

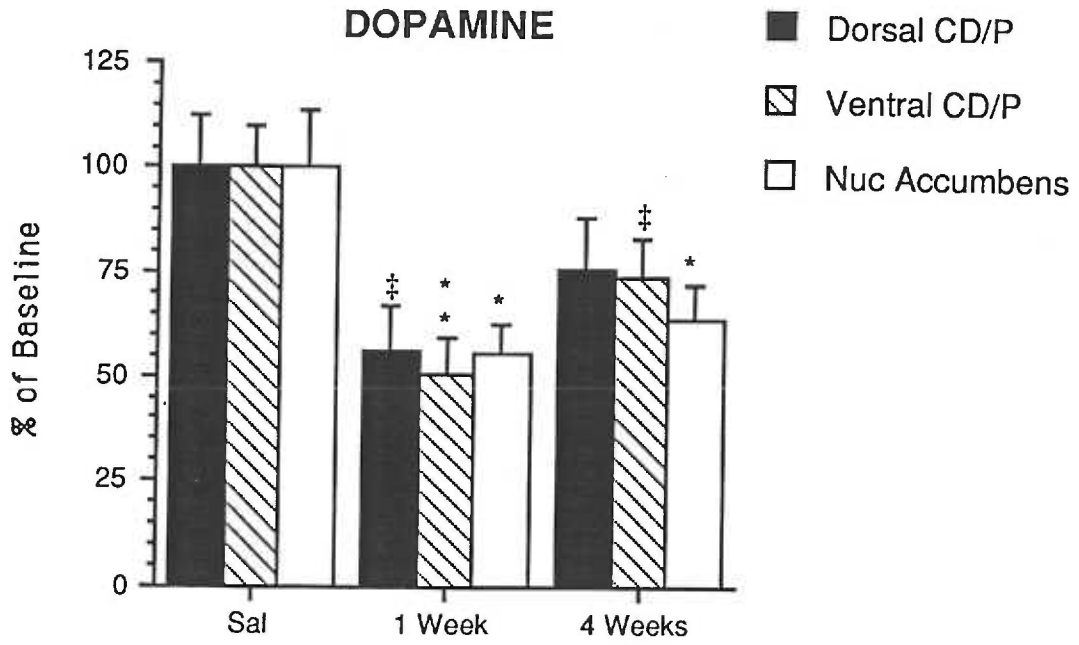
There were no significant effects of drug treatment on the content of NE, DOPAC, or HVA. Mean catecholamine content is presented in Table 5. NE content (Figure 9) within the dorsal CD/P ($F_{2,26} = 1.0$, $P = 0.40$), ventral CD/P ($F_{2,26} = 1.6$, $P = 0.22$), and NAC ($F_{2,26} = 1.1$, $P = 0.34$) was not affected by drug treatment. In addition, the content of HVA (Figure 10) was not affected by drug treatment in the dorsal CD/P ($F_{2,26} = 2.6$, $P = 0.09$), ventral CD/P ($F_{2,26} = 2.9$, $P = 0.08$), or NAC ($F_{2,26} = 1.8$, $P = 0.19$). Finally, non-significant decreases in DOPAC content were found within the dorsal CD/P ($F_{2,26} = 2.9$, $P = 0.07$), ventral CD/P ($F_{2,26} = 3.2$, $P = 0.06$), and NAC ($F_{2,26} = 1.9$, $P = 0.16$). In contrast, METH treatment did induce significant changes in DA content (Figure 9) within the ventral CD/P ($F_{2,26} = 7.1$, $P = 0.0034$) and NAC ($F_{2,26} = 5.5$, $P = 0.010$). Although DA content within the dorsal CD/P was decreased, these changes did not reach the more stringent criteria ($F_{2,26} = 3.6$, $P = 0.04$).

Post-hoc analysis by Newman-Keul's test revealed that DA content was significantly decreased, compared to SAL treated controls, in the ventral CD/P ($P < 0.01$), and NAC ($P < 0.05$) 1 week following METH treatment (Figure 9). DA depletion within the dorsal CD/P also approached significance at this time point ($P < 0.05$). Four weeks following drug treatment, DA content remained depleted only within the NAC ($P < 0.05$), although there was a trend for decreased DA content within the ventral CD/P ($P < 0.10$). METH administration also resulted in moderate, but not significant, depletions in DA metabolites (Figure 10). Decreases in DOPAC content approached

Table 5. Catecholamine content (Mean ng/mg protein ± SEM) following SAL or METH treatment. Animals given METH were killed 1 week or 4 weeks after drug treatment. Data were analyzed by one-way ANOVA followed by Newman-Keul's (** $P < 0.01$, * $P < 0.05$, ‡ $P < 0.10$, compared to respective SAL control).

Catechol.	Region	SAL	1 Week	4 Weeks
DA	Dorsal CD/P	99.33 ± 12.19	55.73 ± 10.37‡	75.39 ± 12.58
	Ventral CD/P	66.25 ± 6.53	33.29 ± 5.78**	48.67 ± 6.52‡
	NAC	51.10 ± 7.00	28.18 ± 3.75*	32.63 ± 3.75*
NE	Dorsal CD/P	1.79 ± 0.54	2.61 ± 0.55	1.64 ± 0.51
	Ventral CD/P	5.37 ± 0.57	4.28 ± 0.44	4.50 ± 0.33
	NAC	22.51 ± 4.52	20.73 ± 4.45	14.55 ± 1.34
DOPAC	Dorsal CD/P	20.78 ± 1.48	15.49 ± 2.05‡	16.40 ± 1.41‡
	Ventral CD/P	19.62 ± 1.33	14.85 ± 1.93‡	14.71 ± 1.30‡
	NAC	35.32 ± 5.82	27.54 ± 4.53	22.70 ± 1.98
HVA	Dorsal CD/P	10.61 ± 0.82	7.85 ± 0.89‡	9.04 ± 0.91
	Ventral CD/P	10.99 ± 0.62	7.73 ± 1.10‡	9.11 ± 1.18
	NAC	9.66 ± 1.30	7.43 ± 1.16	6.89 ± 0.68

Figure 9. DA and NE content, expressed as percent of basal levels (Mean ± SEM), in the dorsal CD/P (closed bars), ventral CD/P (striped bars), and NAC (open bars). Animals were treated with SAL or METH. Drug treated rats were examined 1 week or 4 weeks following METH administration. Data were analyzed by one-way ANOVA followed by Newman-Keul's (* $P < 0.05$, ** $P < 0.01$, ‡ $P < 0.10$, compared to SAL).


DOPAMINE
Dorsal CD/P
Ventral CD/P
Nuc Accumbens
% of Baseline
125
100
75
50
25
0
Sal
1 Week
4 Weeks

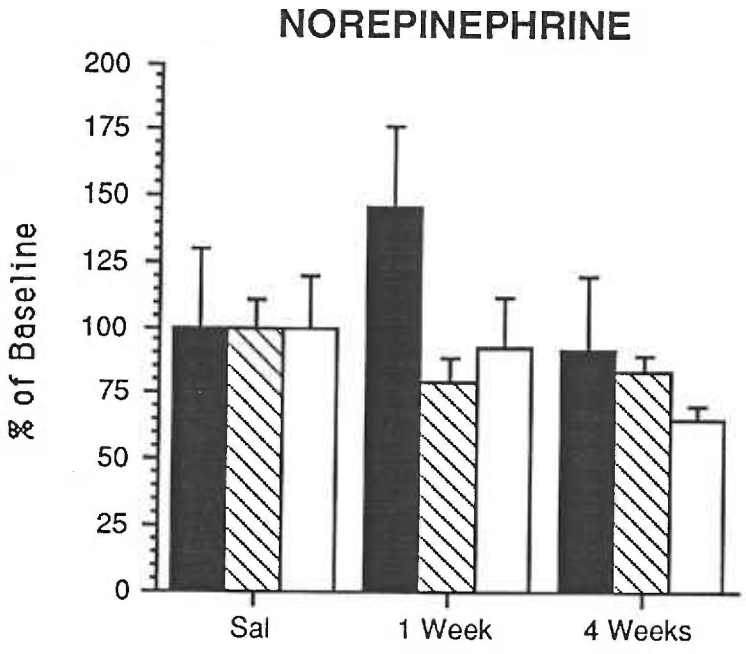
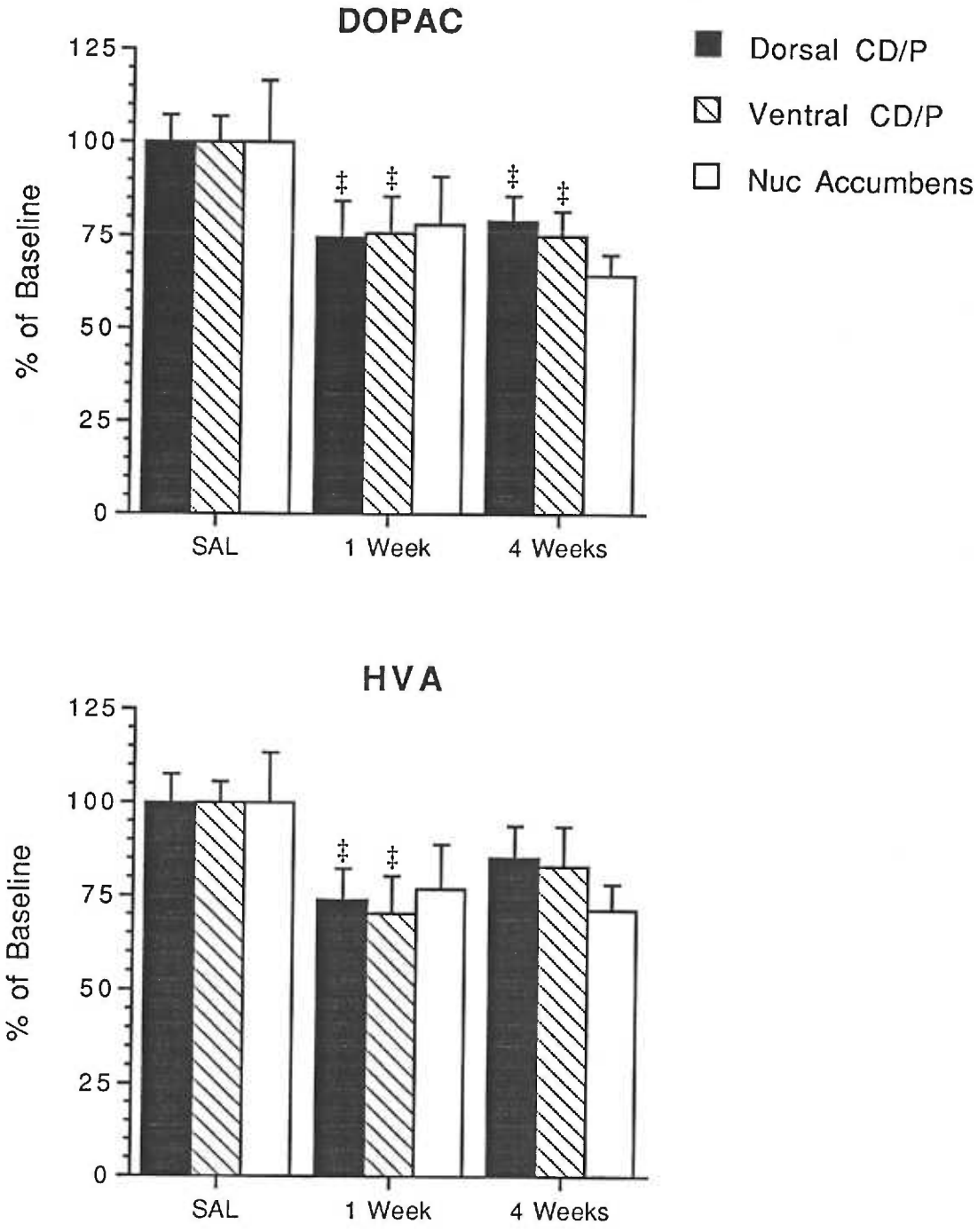

NOREPINEPHRINE
% of Baseline
200
175
150
125
100
75
50
25
0
Sal
1 Week
4 Weeks

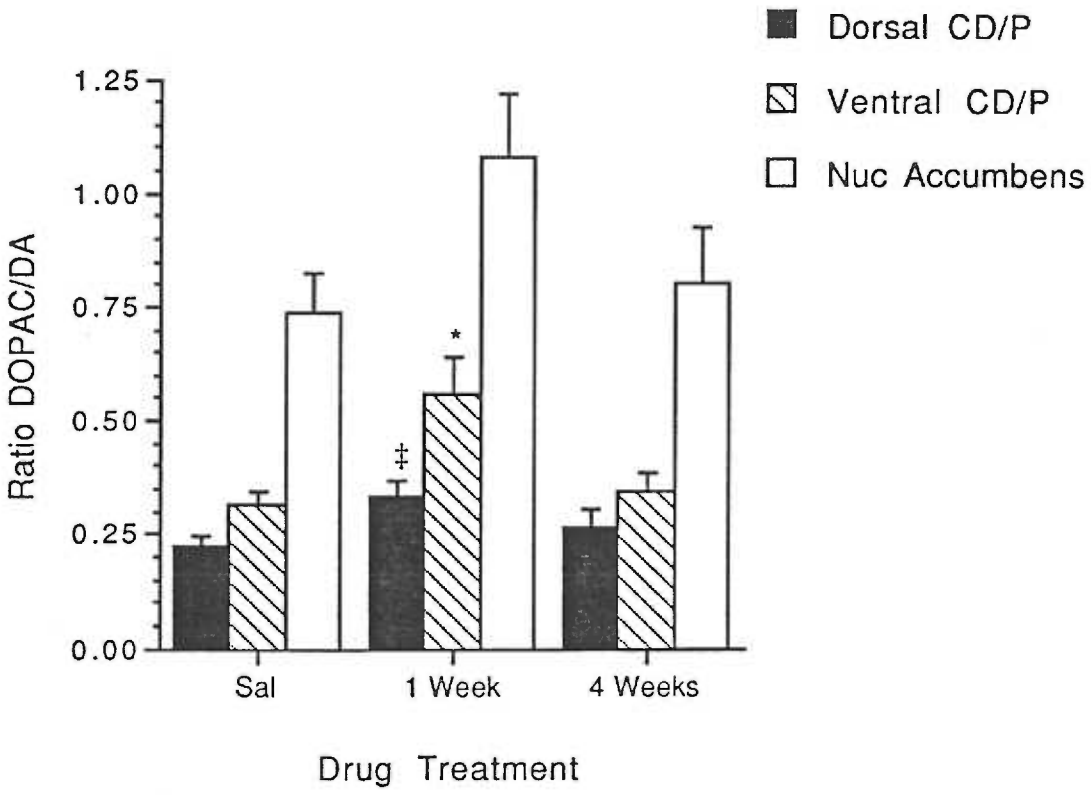
Figure 10. DOPAC and HVA content, expressed as percent of basal levels (Mean ± SEM), in the dorsal CD/P (closed bars), ventral CD/P (striped bars), and NAC (open bars). Animals were treated with SAL or METH. Drug treated rats were examined 1 week or 4 weeks following METH administration. Data were analyzed by one-way ANOVA followed by Newman-Keul's. There were no significant differences between groups (‡ $P < 0.10$).


DOPAC
HVA
% of Baseline
125
100
75
50
25
0
SAL
1 Week
4 Weeks
‡
Dorsal CD/P
Ventral CD/P
Nuc Accumbens

significance at both time points in the dorsal CD/P ($P < 0.10$) and ventral CD/P ($P < 0.10$). In addition, decreases in HVA content approached significance 1 week following METH treatment in the dorsal CD/P ($P < 0.10$) and ventral CD/P ($P < 0.10$).

The ratio of DOPAC/DA was significantly altered within the ventral CD/P ($F_{2,26} = 5.5$, $P = 0.01$) but not within the dorsal CD/P ($F_{2,26} = 2.7$, $P = 0.09$), or NAC ($F_{2,26} = 2.3$, $P = 0.12$) (Figure 11). Post-hoc analysis by Newman-Keul's found that within the ventral CD/P, the DOPAC/DA ratio was significantly increased by 77% 1 week following METH treatment ($P < 0.05$). The ratio within the dorsal CD/P was increased by 47% 1 week following METH administration and approached significance ($P < 0.10$). The apparent increase in the ratio within the NAC did not approach significance. In addition, the ratio of DOPAC/DA returned to basal levels by 4 weeks post drug.

Figure 11. The ratio of DOPAC/DA (Mean ± SEM) in the dorsal CD/P (closed bars), ventral CD/P (striped bars), and NAC (open bars). Animals were treated with SAL or METH. Drug treated rats were examined 1 week or 4 weeks following METH administration. Data were analyzed by one-way ANOVA followed by Newman-Keul's (* $P < 0.05$, ‡ $P < 0.10$, compared to SAL).


Dorsal CD/P
Ventral CD/P
Nuc Accumbens
Ratio DOPAC/DA
1.25
1.00
0.75
0.50
0.25
0.00
*
‡
Sal
1 Week
4 Weeks
Drug Treatment

DISCUSSION

Experiment 1: Examination for Terminal Degeneration

The introduction reviewed evidence supporting the theory that METH-induced DA depletion within the CD/P is the result of specific destruction of DA containing terminals arising from the nigrostriatal pathway, and that the damage may be caused by the endogenous formation of oxidative DA metabolites. In the current study, no evidence of dark degeneration was found in any area at any time following METH administration. Animals treated with METH did show pronounced hyperthermia in the range found to correlate with DA depletion (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994), indicating that DA depletion likely occurred in these animals.

The lack of terminal degeneration, coupled with the mainly transient nature of the DA depletion in comparably treated animals, suggests that moderate doses of METH may inhibit DA biosynthesis in the absence of widespread terminal loss. However, the modest depletion of DA remaining after 4 weeks suggests that a small percentage of DA terminals may be lost following moderate doses of METH, although ultrastructural evidence for such a loss is lacking. DA depletion resulting from administration of 6-OHDA is known to be dose dependent, and degenerating terminals (as measured by silver staining) are not found following a dose that results in a 40% decrease in striatal DA content (Hedreen and Chalmer, 1972). Although large doses of METH (100 mg/kg) are required to induce the formation of detectable levels of 6-OHDA (Seiden and Vosmer, 1984;

Marek *et al.*, 1990a), low doses of METH could lead to an increase in oxidative metabolites that reduce DA biosynthesis without degeneration.

AMPH treatment results in the accumulation of axonal catecholamines, suggesting that axonal transport may be compromised (Ellison *et al.*, 1978; Nwanze and Jonsson, 1981; Jonsson and Nwanze, 1982). In addition, Kogan *et al.* (1976) reported that METH administration (15 mg/kg every 6 hrs, 4 times) initially reduced TH activity in both the SN and CD/P. They found that levels of the enzyme recovered first in the cell bodies (after four days), then in the terminals (after eight days) at a rate consistent with that of axonal transport. A transient loss of the rate limiting enzyme, TH, without concurrent terminal death, could result in DA depletion that lasts at least 1 week. However, Ellison and Switzer (1993) reported dense silver staining of degenerating terminals and axons following administration of a slightly higher dose of METH (6 mg/kg every 2 hrs, 4 times) suggesting widespread destruction of dopaminergic afferents. Furthermore, the distribution of darkened degenerating terminals following continuous AMPH treatment was found to be quite sparse compared to that seen following silver staining (Ryan *et al.* 1990 and personal communication). This raises the possibility that (1) terminals could be damaged and lost without presenting the typical degenerating profile or (2) silver staining techniques may detect damaged as well as dying terminals.

Silver staining of degenerating nerve fibers has been used extensively in mapping the CNS. In this method, ionic silver is

reduced and deposited in sub-microscopic clumps selectively within terminals and axons. These silver deposits are enlarged by photographic development to the point where they become visible by light microscopy (Giolli and Karamanlidis, 1978). Although dark degeneration is not the only recognized form of neuronal degeneration, silver impregnating methods do deposit stain in darkened profiles. In addition, the regional distribution of silver staining overlaps with the distribution of cell types known to undergo dark degeneration. Together, this evidence indicates that silver impregnating methods stain terminals undergoing dark degeneration as opposed to other types of degeneration (Guillery, 1970). Recently it has been suggested that silver staining methods have become so refined that they may be able to pick up changes that are reversible (Discussion following Seiden *et al.*, 1993). On the other hand, the sensitivity of the silver impregnating methods may be higher such that damaged terminals are more readily seen. Either of these phenomena would explain the discrepancy between the presence of silver staining and lack of degeneration seen at the electron microscopic level.

Experiment 2: Analysis of GLU Immunoreactivity

These experiments were designed to test the hypothesis that the GLU overflow, measured be microdialysis, is correlated with decreased amounts of presynaptic neuronal GLU. METH treatment induces a delayed overflow of GLU in the CD/P that continues to rise after DA levels have returned to baseline (Nash and Yamamoto, 1992;

Abekawa *et al.*, 1994). If METH induces the release of neuronal GLU, then the concentration of presynaptic GLU should decrease. Indeed, GLU immunoreactivity was significantly decreased within the VL CD/P 12 hours following METH administration (Figure 7). These results correlate temporally with the *in vivo* microdialysis findings of a delayed GLU overflow that lasts at least 8-10 hours after drug treatment (Nash and Yamamoto, 1992; Nash and Yamamoto, 1993; Abekawa *et al.*, 1994). Although it cannot be concluded that the decrease in GLU immunoreactivity is directly due to increased neuronal release as opposed to decreased GLU synthesis or reuptake, the results lend strong support to the hypothesis that neuronally derived GLU plays an important role in METH-induced neurotoxicity.

In addition, these studies found that the anatomical distribution of decreased GLU immunoreactivity is consistent with activation of the thalamocorticostriatal loop (Figure 1). Activation of this loop would result in the release of GLU within the CD/P and superficial layers of the MCTX without affecting the shell of the NAC. Examination of GLU immunoreactivity found that significant decreases in immunolabeling occurred within the MCTX and the VL CD/P (Figure 7). Although decreases within the DL CD/P were not significant, the time course of the changes in labeling resembles that found within the VL CD/P and MCTX. In contrast, the time course of GLU immunolabeling within the shell of the NAC appears to differ from that found in the CD/P and MCTX. This finding indicates that the METH-induced decrease in GLU immunoreactivity is not a

generalized effect, but is specific to the known circuitry of the dorsal striatum.

Although METH did not appear to alter GLU immunoreactivity within the shell of the NAC, when compared to SAL treated animals, significant decreases in DA levels within the NAC were found. This may be explained by the fact that DA content was measured in whole NAC. Dissecting out the shell from the core in fresh tissue is very difficult, and because no DA depletion was expected to occur in the NAC, the entire region was examined. In view of the morphological and pharmacological differentiation of the NAC into the core and shell (Heimer *et al.*, 1991; Deutch and Cameron, 1992), it is not unreasonable to assume that regional variations in the vulnerability of the NAC to METH-induced toxicity may exist. In fact, Ricaurte *et al.* (1982) reported that high doses of METH (50 mg/kg every 8 hrs, 3 times) resulted in the degeneration of terminals, as analyzed by silver staining, within the core but not the shell of the NAC. The finding that GLU immunoreactivity is not decreased within the shell of the NAC, coupled with the findings of Ricaurte *et al.* (1982), lend support to the hypothesis that the shell of the NAC can be functionally distinguished from the core. It would be interesting to determine if METH affected the density of terminal GLU immunoreactivity within the core of the NAC since this region is believed to be functionally related to the dorsal striatum.

Studies examining the increase in extracellular GLU following repeated METH administration have found that GLU begins to rise approximately 2 hrs after the last dose of METH and continue to rise

for at least 4 hrs (Nash and Yamamoto, 1992; Abekawa *et al.*, 1994; Stephans and Yamamoto, 1994). Administration of AMPH in combination with the MAO inhibitor iprindole resulted in GLU overflow lasting at least 8 hours (Nash and Yamamoto, 1993). The results from the current study suggest that GLU release continues to occur 12 hours following METH administration. In addition, the density of GLU immunolabeling approached basal levels within 24 hours, indicating that the initial loss of GLU immunoreactivity was transient (Figure 7).

Eisch *et al.* (1996) have recently reported that administration of METH, at the same dose used in the current study, resulted in a transient loss of NMDA binding sites only within the ventral and lateral regions of the CD/P. These changes were present 1 week, but not 1 month, following METH treatment and were attributed to regulatory responses that occur following augmented GLU release. The finding that NMDA binding was altered in ventral but not dorsal CD/P lends support to our finding that GLU immunoreactivity was significantly decreased in VL DC/P but not in the DL CD/P. Such a dichotomy is consistent with the evidence that the VL CD/P is more vulnerable to the neurotoxic effects of METH. In contrast to the results of the current study, NMDA binding within the MCTX was not affected by METH administration. Eisch *et al.* (1996) attributed this to the belief that METH does not induce GLU release within the MCTX. Although a low dose of METH (2 mg/kg) does not induce GLU overflow in the medial prefrontal CTX (Stephans and Yamamoto, 1995), the

possibility of GLU efflux within the MCTX following a neurotoxic dose of METH has not been examined by microdialysis.

Experiment 3: METH-Induced Hyperthermia

The body temperature of SAL treated rats was found to significantly decline over time. Rectal temperature was first measured two hours before lights were normally turned on. Because body temperature is known to be elevated during the dark cycle (Fioretti *et al.*, 1974), this decline in the body temperature of SAL treated rats is likely due to a circadian effect. This effect may have also masked any increase in body temperature following the first injection of METH. Regardless of these effects, animals treated with METH showed pronounced hyperthermia in the range found to correlate with DA depletion (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994; Taraska and Finnegan, 1995). As can be seen from Figure 8, body temperature peaked 1 hr after each of the last three METH injections, and was continuously elevated for at least six hours. On the basis of previous reports documenting the high correlation between elevated rectal temperatures and maximal DA depletion following METH administration (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994), it is assumed that animals used in the electron microscopic and immunocytochemical analyses did receive a neurotoxic dose of METH. It is also likely that these animals had DA depletions that were similar to those measured in the animals whose DA content was analyzed by HPLC.

Although the hyperthermic effects of psychostimulants have long been postulated to be involved in the lethal effects of these drugs in both rodents (Craig and Kupferberg, 1972) and humans (Jordan and Hampson, 1960; Callaway and Clark, 1994), exploration of their specific role in METH-induced DA depletion is still in the early stages of development. Bowyer *et al.* (1992), have shown that the degree of DA depletion induced by METH can be altered by artificial manipulation of the environmental temperature. For example, placing an animal in a cold environment (4°C) was protective, while increased room temperature (28°C) enhanced DA depletion (Bowyer *et al.*, 1993; Bowyer *et al.*, 1994).

Although the mechanisms underlying this phenomenon have not been examined, some interesting parallels between METH-induced hyperthermia and heat-induced ischemia may be drawn. METH overdose can induce ischemia in humans (Yen *et al.*, 1994), and increased body temperatures in the range induced by METH are known to result in ischemia (i.e., heat-stroke) (Kao *et al.*, 1994). The onset of heat-stroke is accompanied by an increase DA release (Lin *et al.*, 1995). In addition, ischemia induces GLU release, and ischemic damage can be blocked by pretreatment with MK-801 (Globus *et al.*, 1988; Kochar *et al.*, 1991). Finally, Torp *et al.* (1991) found that ischemia induced a decrease in GLU immunolabeling in pyramidal cell bodies in the hippocampus. These studies present intriguing evidence suggesting that METH toxicity and ischemia may have similar underlying mechanisms. In addition, this data suggests that perhaps high doses of METH may result in heat-induced ischemia and

that ischemia, in turn, may play a role in METH-induced toxicity. However, no studies examining this possibility have yet been undertaken.

Experiment 4: Analysis of Catecholamine Content

These results are consistent with previous data demonstrating a significant loss of striatal DA (Table 1), without reduction of NE, 1 week following METH treatment (Figure 9) (Wagner *et al.*, 1979; Lucot *et al.*, 1980). Although DA metabolites were not significantly decreased following METH administration, there was a consistent 25% reduction of both DOPAC and HVA (Figure 10). The moderate decrease in DA metabolites is similar to that previously reported (Table 2).

The results from the current study indicate that recovery from DA depletion does occur following a moderate dose of METH. Four weeks following METH treatment, DA content had returned to 65-75% of basal levels and was no longer significantly depleted in the CD/P (Figure 9). This finding is similar to that of Bowyer *et al.* (1992) who reported that METH treatment resulted in an initial 60% decrease in DA content 1 week after drug treatment, DA returned to 70% of basal levels 2 weeks after drug administration. Furthermore, there was a trend towards a decrease in striatal levels of DOPAC and HVA 4 weeks following METH administration (Figure 10). The fact that moderate decreases in the level of striatal DA and DA metabolites have been found to remain both at 2 weeks following METH treatment (Bowyer *et al.*, 1992) and 4 weeks following METH

suggests that DA content has sufficiently recovered beyond the point that compensatory increases in DA turnover are needed.

As discussed above, DA content was significantly decreased by approximately 50% 1 week following METH administration. Interestingly, this decrease was similar in all three regions studied (Figure 9). Furthermore, this finding was replicated in both the main study and the pilot study (Appendix 1). It is generally believed that the NAC is less vulnerable to the neurotoxic effects of METH. This finding of METH-induced toxicity within the NAC, although rare, is not novel. O'Dell *et al.* (1991) reported that this dose of METH resulted in a 43% decrease in DA content within the NAC. This suggests that under certain circumstances METH toxicity can occur within the NAC.

Although previous studies have found differences between the dorsal and ventral regions of the CD/P in terms of their neurochemistry and vulnerability to METH (Ellison *et al.*, 1978; Jonsson and Nwanze, 1982; Marshall and Navarrete, 1990; Eisch *et al.*, 1992), we found that METH has similar DA depleting effects in both areas. Although the decrease in the dorsal CD/P did not reach significance, the magnitude of the effect was similar in both regions of the CD/P. One possible explanation for this difference may be ascribed to how the dorsal and ventral regions of the CD/P were defined in this study. In order to examine regional differences in METH toxicity, Marshall *et al.* (1990) and Eisch *et al.* (1992) divided the CD/P into twelve subregions, while the current studies used only two. Perhaps by pooling these subregions, subtle

differences in the regional specificity of METH-induced toxicity were masked. The finding that GLU immunoreactivity is decreased to a greater degree in VL CD/P as opposed to DL CD/P is more consistent with studies examining specific subregions of the striatum.

SUMMARY AND CONCLUSIONS

Previous studies examining METH-induced neuronal damage have, in general, used doses that are much higher than those required for DA depletion to occur. It has been suggested that METH-induced DA depletion results from the specific destruction of DA containing terminals arising from the nigrostriatal pathway, and that the damage may be caused by the endogenous formation of oxidative DA metabolites. It was hypothesized that administration of a moderate dose of METH, known to induce DA depletion, would result in the degeneration of terminals within the CD/P but not within the shell of the NAC. In addition, it was hypothesized that METH would induce damage at a rate similar to that seen following oxidative damage. However, no evidence of degeneration was found in any area at any time following METH administration. Animals treated with METH did show pronounced hyperthermia in the range found to correlate with DA depletion (Bowyer *et al.*, 1992), indicating that DA depletion likely occurred in animals used for the ultrastructural analysis. This lack of degeneration suggests that moderate doses of METH may inhibit DA biosynthesis in the absence of widespread terminal loss. However, this result conflicts with studies showing dense silver

staining following similar schedules of METH administration (Ellison and Switzer, 1993) and suggests that terminals could be damaged and lost without presenting a typical degenerating profile. As an example, catecholamine containing terminals often occur as varicosities along the length of the axon. If these *boutons en passant* were damaged and vesicles were no longer apparent, it would not be possible to identify them as terminals.

METH administration results in a delayed overflow of GLU that appears to be a crucial step in the neurotoxic cascade. The origin of this extracellular GLU is unknown and could come from several sources. It was hypothesized that METH administration may result in the release of neuronally derived GLU from corticostriatal afferents via the activation of the thalamocorticostriatal loop. Consistent with this hypothesis, there was a decrease in GLU immunolabeling within asymmetrical presynaptic terminals 12 hrs after METH administration. These changes were found in the VL CD/P and MCTX, but not within the shell of NAC. Although decreases within the DL CD/P were not significant, the time course of the changes in labeling closely matched those found within the VL CD/P and MCTX. These findings indicate that METH does not induce generalized decreases in presynaptic GLU content, but is specific to the known circuitry of the dorsal striatum. These results correlate temporally with the *in vivo* microdialysis findings of a delayed GLU overflow (Nash and Yamamoto, 1992). In addition, the density of immunolabeling approached basal levels within 24 hours, indicating that the initial decrease in the density of terminal GLU

immunoreactivity was transient. These results lend strong support to the hypothesis that neuronally derived GLU plays an important role in METH-induced neurotoxicity.

The fact that METH administration results in DA depletion within the striatum is well documented (Table 1). It was hypothesized that administration of METH, at the same dose used in the electron microscopic and immunocytochemical analyses, would induce DA toxicity as previously reported by others. The results are in agreement with previous studies demonstrating a significant loss of striatal DA 1 week following METH treatment. Although DA metabolites were not significantly decreased following METH administration, there was a consistent 25% reduction of both DOPAC and HVA. Recovery from DA depletion did occur in the CD/P. However, moderate decreases in the level of striatal DA and DA metabolites were found to remain 4 weeks following METH administration. This suggests that low doses of METH may have small but long-lasting effects on the basal ganglia. The present study found similar DA depleting effects all three striatal regions examined. This is in contrast to previous reports that METH has heterogeneous effects within the striatum. One possible explanation for this difference may be ascribed to how the different regions were defined in this study.

Increased neurotransmitter turnover, as measured by an increase in the DOPAC/DA ratio, is a measure of increased neurotransmitter release known to occur in animals with partial striatal lesions. In the current study, the DOPAC/DA ratio was

significantly increased 1 week following METH treatment in the ventral CD/P, and a trend towards an increased ratio was apparent in the dorsal CD/P. This suggests that DA turnover was increased in order to compensate for the loss of presynaptic DA levels. The fact that the increase in DA turnover was no longer present 4 weeks after METH administration suggests that DA content had sufficiently recovered beyond the point that compensatory increases in DA turnover were needed.

In conclusion, the lack of degeneration, coupled with the mainly transient nature of DA depletion in comparably treated animals, suggests that moderate doses of METH may inhibit DA biosynthesis without widespread terminal loss. In addition, the results indicate that a decrease in presynaptic GLU is temporally and anatomically correlated with the METH-induced GLU overflow believed to play a role in DA depletion. This supports the hypothesis that neuronally derived GLU may participate in the neurotoxic effects of METH.

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APPENDIX A.
Comparison of High and Low METH Regimens

Rationale

A wide variety of METH doses and regimens have been employed in the study of METH-induced DA depletion (Table 1). There is some evidence that METH-induced toxicity is variable in that it can be affected by differences in the time of year, number of rats per cage, room temperature, bedding material, and other factors that may fluctuate between research facilities (John Marshall, personal communication). In order to determine which schedule of METH results in greater DA depletion or synaptic degeneration in our facility, experiments comparing the effects of 2 moderate regimens of METH administration were undertaken. These drug schedules were chosen because they have been found to induce similar depletions of DA in the CD/P 3-7 days following drug treatment (Bowyer *et al.*, 1992; Stephans and Yamamoto, 1994).

Methods and Results

Subjects

Male Sprague-Dawley rats (Harlan, 200-300 g, age 2-3 months) were housed 2-3 to a cage and maintained on a 12 hr light/dark cycle with food and water available ad lib.

Drug administration.

Animals treated with vehicle (SAL) or 10 mg/kg METH (s.c., in normal saline) were given drug every 2 hrs for a total of 3

injections. Animals treated with 5 mg/kg METH received drug every 2 hrs for a total of 4 injections. Ambient temperature remained at a constant 22 ± 1°C throughout the experiment.

Catecholamine Levels

Animals were treated with 5 mg/kg METH (n = 6), 10 mg/kg METH (n = 11) or an equivalent volume of vehicle (n = 6). Five of the animals treated with 10 mg/kg METH did not survive the procedure. Animals were killed by decapitation 7 days following drug or vehicle administration and brains were removed and dissected for analysis of DA, NE and DOPAC content. Preparation of tissue and procedures for HPLC analysis were identical to those previously described.

Analysis of catecholamine content (Table 6) was identical to that described in the Statistical Analysis section. DA, DOPAC, and NE content were analyzed by one-way ANOVA, with drug treatment (SAL, 5 mg/kg METH, or 10 mg/kg METH) as the between-subjects factor. No changes in NE content were detected within the dorsal CD/P ($F_{2,15} = 1.6$, $P = 0.23$), ventral CD/P ($F_{2,15} = 0.1$, $P = 0.89$), or NAC ($F_{2,15} = 1.3$, $P = 0.31$). In addition, levels of DOPAC were not affected by drug treatment within the dorsal CD/P ($F_{2,15} = 0.04$, $P = 0.96$), ventral CD/P ($F_{2,15} = 0.5$, $P = 0.59$), or NAC ($F_{2,14} = 2.6$, $P = 0.11$). In contrast, drug treatment did significantly decrease DA content within the ventral CD/P ($F_{2,15} = 6.4$, $P = 0.01$). Although DA content was decreased within the dorsal CD/P ($F_{2,15} = 4.1$, $P = 0.04$), and the NAC ($F_{2,15} = 4.4$, $P = 0.03$), these changes did not reach the more stringent significance level of $\alpha = 0.017$. Post-hoc analysis by Newman-Keul's test found that animals treated with

Table 6. Catecholamine content (Mean mg/g tissue ± SEM) following SAL or METH treatment. Animals given METH treated with either 5 mg/kg (low dose) or 10 mg/kg (high dose) and were killed 1 week after drug treatment. Data were analyzed by one-way ANOVA followed by Newman-Keul's (* $P < 0.05$, ‡ $P < 0.10$, compared to respective SAL control).

Catechol.	Region	SAL	Low METH	High METH
DA	Dorsal CD/P	5426.74 ± 963.69	1497.29 ± 667.82‡	2909.44 ± 1237.26‡
	Ventral CD/P	4338.11 ± 1219.69	1024.28 ± 352.34*	1129.42 ± 203.59*
	NAC	2212.87 ± 574.82	550.80 ± 324.55‡	832.31 ± 276.34‡
NE	Dorsal CD/P	172.06 ± 25.31	217.53 ± 45.62	329.50 ± 96.56
	Ventral CD/P	342.26 ± 72.07	295.14 ± 124.34	285.10 ± 47.13
	NAC	430.04 ± 87.03	735.15 ± 233.86	425.08 ± 114.54
DOPAC	Dorsal CD/P	678.41 ± 22.33	740.47 ± 186.95	742.77 ± 255.97
	Ventral CD/P	681.41 ± 91.76	546.60 ± 106.10	722.94 ± 164.49
	NAC	1034.08 ± 316.80	325.83 ± 178.42	500.39 ± 172.93

either the high or low dose of METH showed comparable DA depletions within the ventral CD/P ($P < 0.05$) (Figure 12). DA content within the dorsal CD/P and NAC approached significance following either dose of METH ($P < 0.05$). There were no differences between the high and low dose of METH.

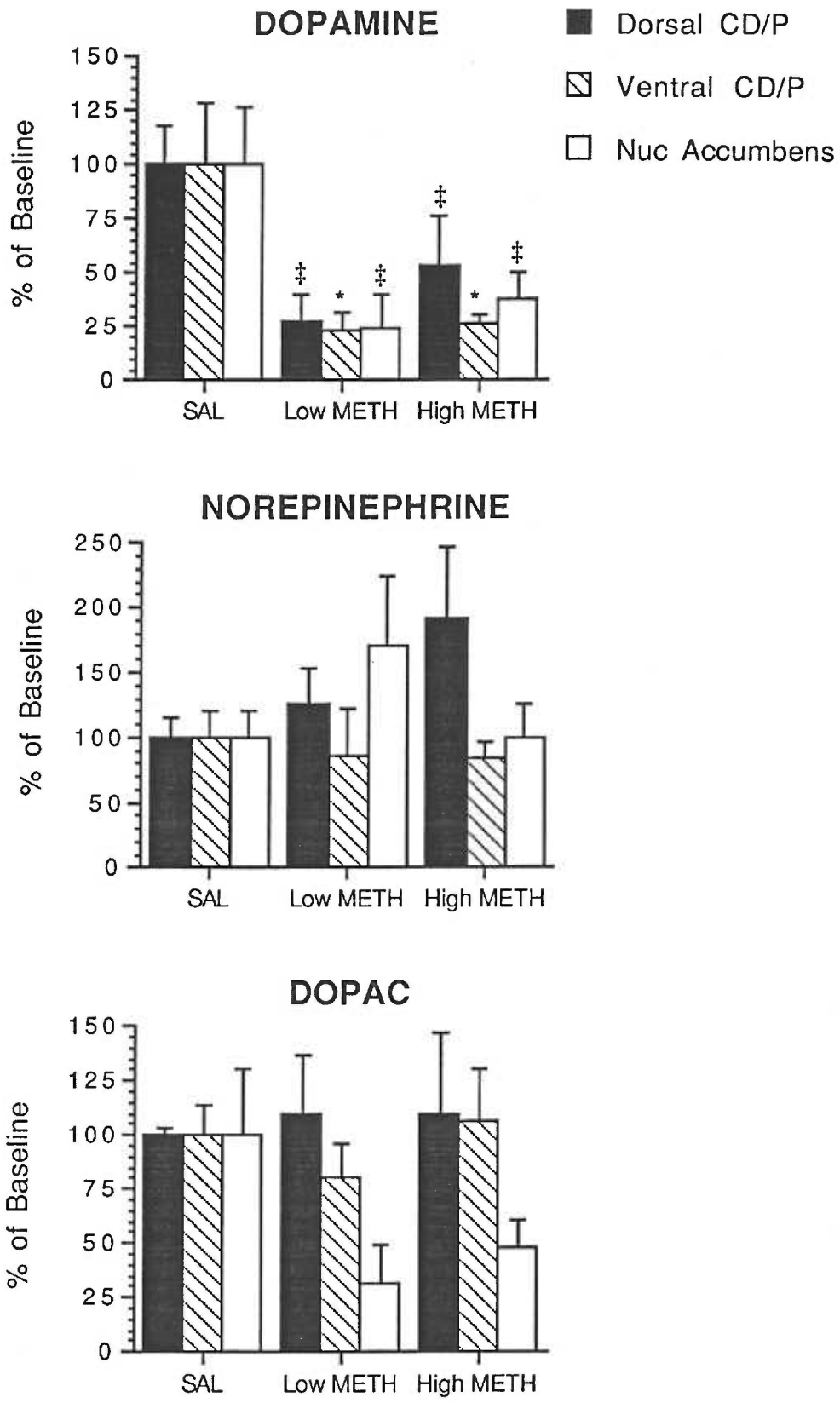
Electron Microscopy

Animals were treated with 5 mg/kg METH (n = 6), 10 mg/kg METH (n = 9) or an equivalent volume of vehicle (n = 3). Six of the animals treated with the high dose of METH did not survive the procedure. Five of these died after the second injection of METH. Rats treated with METH were killed by cardiac perfusion 24 hours (n = 1-2), 3 days (n = 1-2), or 7 days (n = 1-2) following the last METH injection. SAL treated animals (n = 3) were killed 5 days after treatment. Preparation of tissue and electron microscopic procedures were identical to those previously described in the Methods section. No evidence of synaptic or neuronal degeneration was observed in the surviving animals that had been treated with either the low or high METH regimen.

Discussion

Similar to previous findings (Table 1), METH administration did induce a significant depletion of DA content within the ventral CD/P, and there was a trend towards depletion within the dorsal CD/P and NAC. No evidence of synaptic degeneration was found, and there were no differences in catecholamine depletion between animals administered the low or high dose regimen of METH. The only

Figure 12. Catecholamine levels, expressed as percent of basal levels (Mean ± SEM), in the dorsal CD/P (closed bars), ventral CD/P (striped bars) and NAC (open bars) were measured by HPLC. Rats were treated with repeated injections of SAL, 5 mg/kg METH (Low METH) or 10 mg/kg METH (High METH). DA (top graph), NE (middle graph) and DOPAC (bottom graph) content were analyzed by one-way ANOVA followed by Newman-Keul's (* $P < 0.05$ compared to SAL; ‡$P < 0.10$).


DOPAMINE
Dorsal CD/P
Ventral CD/P
Nuc Accumbens
% of Baseline
150
125
100
75
50
25
0
‡
*
‡
‡
*
‡
SAL
Low METH
High METH
NOREPINEPHRINE
% of Baseline
250
200
150
100
50
0
SAL
Low METH
High METH
DOPAC
% of Baseline
150
125
100
75
50
25
0
SAL
Low METH
High METH

difference between the METH treated groups was in terms of lethality. Animals treated with the low dose of METH all survived, but 55% of animals treated with the high dose of METH did not survive. In order to maximize DA depletion and minimize animal death, the low dose of METH was used in all further studies.

APPENDIX B.
Standardization of Immunocytochemical Data

Rationale

Data were collected over three experiments that differed slightly in tissue preparation and immunolabeling protocols (Table 7). In the first experiment (EXP 1), rats were perfused with a HEPES based fixative. Although this perfusion method resulted in excellent tissue preservation, the HEPES interfered with the immunoreactivity of the tissue. Consequently, the density of immunolabeling was low. Phosphate based fixative (PBS) has been found to interfere less with the immunoreactivity of the tissue (Meshul, personal communication). Therefore, animals in the second experiment (EXP 2) were perfused with PBS based fixative. Although immunolabeling in EXP 2 was approximately 150% higher than that found in EXP 1, perfusion with PBS based fixative resulted in high levels of tissue precipitate. For this reason the third group of animals (EXP 3) was perfused with the HEPES based fixative. Additionally, tissue from EXP 3 was incubated in primary GLU antibody that was from a different lot (the first lot of antibody was no longer available). This second lot of GLU antibody had slightly different binding characteristics and consequently immunolabeling for EXP 3 was intermediate between EXP 1 and EXP 2.

Analysis of control particle density levels by one-way ANOVA revealed significant differences between experiments ($F_{2, 27} = 15.14$, $P < 0.0001$). Post-hoc analysis by Newman-Keul's showed

Table 7. Particle density (Mean num/μm^2 ± SEM, n) of control animals for each experiment before (raw) and after (corrected) standardization. Data were collapsed over brain region.

Experiment	Raw Control Density	Corrected Control Density
EXP 1	53.25 ± 4.34 (6)	101.19 ± 5.06 (6)
EXP 2	130.28 ± 8.12 (20)	103.23 ± 7.44 (20)
EXP 3	91.22 ± 4.26 (4)	111.66 ± 4.25 (4)

that the density of labeling differed between all three groups ($P < 0.05$). In order to be able to combine particle density across experiments, the data were standardized.

Methods

Weighted means ($\overline{X}_w$) were determined for each experiment by pooling data from control animals within each experiment. A grand weighted mean ($\overline{X}_{gw}$) was determined by pooling data from all control animals. The correction factor was calculated as the difference between the grand weighted mean and each experimental weighted mean ($\overline{X}_{gw} - \overline{X}_w$). This factor, ascertained separately for specific brain regions, was added to the individual density means within each experiment. Table 8 shows the standardization procedure for the DL CD/P.

Results and Discussion

Analysis of corrected means by one-way ANOVA revealed no significant differences between experiments ($F_{2, 27} = 0.18$, $P = 0.84$). This indicated that standardizing the data successfully reduced the differences between experiments and allowed direct comparison of particle density between groups from all three experiments.

Table 8 . Standardization of immunocytochemical data for statistical analysis: Example of the DL CD/P.

Animal	EXP	n	ΣX	Raw $\overline{X}$	Corrected $\overline{X}$
1	1	37	2214.89	59.86	112.49
2	1	27	1519.73	56.29	108.92
3	2	21	2558.35	121.83	76.32
4	2	18	2903.87	161.33	115.82
5	2	27	4425.90	163.92	118.41
6	2	17	2035.31	119.72	74.21
7	2	21	4352.25	207.25	161.74
8	3	31	3047.81	98.32	119.00
9	3	35	2912.48	83.21	103.89

EXP	n_w	ΣX_w	$\overline{X}_w$	Correction Factor
1	64	3734.63	58.35	52.63
2	104	16275.68	156.50	-45.51
3	66	5960.28	90.31	20.68

N	ΣX_g	$\overline{X}_{gw}$
234	25970.59	110.99