Effects of a Neurotoxic Dose of Methamphetamine on Presynaptic GABA and Glutamate Immunoreactivity

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AUC	Area Under the Time vs. Body Temperature Curve
CD	Caudate
COLD	MA + Hypothermia Treated Rats
DA	Dopamine
DHBA	Dihydrobenzylamine
DLCD	Dorsolateral Caudate
DOPAC	Dihydroxyphenyl Acetic Acid
ENK	Enkephalin
GABA	γ-Aminobutyric acid
GAD	Glutamate Decarboxylase
GP	Globus Pallidus
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HOT	Saline + Hyperthermia Treated Rats
HPLC	High Performance Liquid Chromatography
MA	Methamphetamine
METH	MA treated rats
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
NMDA	N-Methyl-D-aspartate
mRNA	Messenger Ribonucleic Acid
6-OHDA	6-Hydroxydopamine
SAL	Saline Treated Rats
TBST	Tris Buffered Saline with Triton X-100
VLCD	Ventrolateral Caudate

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ABSTRACT

Methamphetamine (MA) is a popular drug of abuse that damages the nigrostriatal dopamine (DA) system in both humans and animals (Seiden *et al.*, 1975/76; Ricaurte *et al.*, 1980; Steranka and Sanders-Bush, 1980; Wilson *et al.*, 1996). This damage appears to be mediated both by drug-induced hyperthermia and the formation of reactive oxygen species. Although long-term changes in the DA system following MA treatment have been well characterized, alterations in non-dopaminergic systems are less well understood. These experiments were designed to test the hypothesis that high-dose MA treatment alters presynaptic immunoreactivity for γ-aminobutyric acid (GABA) and glutamate, two of the major neurotransmitter systems associated with basal ganglia function. These studies also tested the hypothesis that immunocytochemical changes are only found in animals with severe DA loss (i.e. > 80%), and that they are not the result of prolonged hyperthermia alone.

Sprague Dawley rats were treated with either saline or 15 mg/kg MA every 6 hrs for a total of 4 doses. To examine the role of hyperthermia in nigrostriatal damage, rats were randomly assigned to 1 of 4 treatment groups. Animals in the SAL and HOT groups were both given normal saline, but the HOT group was exposed to hyperthermia similar to that seen following MA treatment. Animals in the METH and COLD groups were both given MA, but rats in the COLD group were prevented from becoming hyperthermic so that their body temperature did not differ from the SAL group. Animals were killed after 1 or 4 weeks, and examined for alterations in GABA and glutamate presynaptic immunoreactivity and morphology.

Dynamic changes in the density of presynaptic GABA labeling were seen in both the caudate (CD) and globus pallidus (GP) of rats in the METH group. GABA immunoreactivity was decreased after 1 week and increased after 4 weeks. Although the initial decrease was present in both the dorsolateral and ventrolateral regions of the CD (DLCD and VLCD), the delayed increase was present only in the VLCD. Terminal area was also increased after 4 weeks within the VLCD. In glutamate immunolabeled tissue, there was a significant increase in the percent of asymmetrical synapses with perforated post synaptic densities in the METH group 4 weeks after MA treatment. This morphological change, found in both the DLCD and VLCD, was not associated with altered immunoreactivity or terminal size. No changes in GABA or glutamate immunoreactivity or terminal size were found in the HOT or COLD groups.

To determine if immunocytochemical changes were correlated with the degree of MA-induced toxicity, striatal DA content was determined in additional animals. MA administration resulted in a severe (>80%) and long-lasting decrease in striatal DA content in the METH treatment group. Cooling was protective in that DA loss in the COLD group was less extensive (35-65% loss), while hyperthermia alone in the HOT group did not deplete DA. In addition, rats in the METH treatment group had a 4-5 fold increase in the ratio of dihydroxyphenyl Acetic Acid (DOPAC) to DA, reflecting an increase in DA metabolism. Thus, changes in presynaptic GABA immunoreactivity appear to occur only following a severe loss of DA, but not following a partial DA lesion. These results agree with previous 6-hydroxydopamine (6-OHDA) studies, and provide further evidence that a majority of DA must be lost

before compensatory changes in both dopaminergic and non-dopaminergic systems occur.

A loss of DA input to the striatum should result in the disinhibition of the striatopallidal output pathway. The decrease in GABA immunoreactivity found in the current study may reflect depleted stores due to increased activity of these striatal output neurons. Similarly, the augmented GABA immunolabeling found after 4 weeks could reflect a delayed increase in the synthesis and content of GABA that is known to occur following DA loss induced by 6-OHDA. These studies provide the first evidence that high-dose MA treatment can result in long-lasting alterations in GABAergic systems. The changes seen in the current study resemble those found in animals following 6-OHDA-induced DA loss (Ingham *et al.*, 1997; Meshul *et al.*, 1997) and in patients with Parkinson's disease (Perry *et al.*, 1983; Tossman *et al.*, 1986; Segovia and Garcia-Munoz, 1987). It is not known if early MA abuse is a risk factor in Parkinson's disease, but it is possible that such damage to the nigrostriatal DA system could result in an earlier onset of symptoms in individuals predisposed to develop Parkinson's. Thus, further analysis of MA-induced alterations in the extrapyramidal motor circuit may have important clinical implications.

INTRODUCTION

A. Background and Significance

Methamphetamine (MA), is a potent psychostimulant that has recently reemerged as a popular drug of abuse (Derlet and Heischober, 1990; Sato *et al.*, 1992). Chronic use of MA can lead to a form of psychosis which is virtually indistinguishable from schizophrenia (Sato *et al.*, 1992). Psychosis is not solely attributed to the acute psychogenic action of MA since symptoms can last up to a month after drug use has ended, and reoccurrence has been reported without substance abuse (Sato *et al.*, 1983; Sato, 1992). Undefined neuronal damage produced gradually during chronic MA use is assumed to be the cause of psychostimulant-psychosis and the apparent long-lasting sensitization to the actions of the drug (Sato, 1992).

Administration of psychostimulants to both rodents and non-human primates can produce long lasting effects on the central nervous system. Of particular interest is the finding that MA induces long lasting depletions of the catecholamine dopamine (DA) specifically within the striatum. This neurotransmitter system has been implicated in several human disorders, including schizophrenia, Huntington's disease, and Parkinson's disease (Seiden *et al.*, 1975/76; Lucot *et al.*, 1980; Ricaurte *et al.*, 1980). Damage to the nigrostriatal DA system is known to effect differentially the GABAergic striatal output pathways. Such studies have focused upon the 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesion models of Parkinson's disease, and little is known about the consequences of MA-induced DA loss with respect to it's impact on the rest of the extrapyramidal motor circuit.

Unlike 6-OHDA and MPTP, MA is generally believed to be an soma-sparing neurotoxin in that damage is usually restricted to the DA-containing boutons in the striatum (Ricaurte *et al.*, 1980; Ryan *et al.*, 1990; Di Monte *et al.*, 1996). There is also evidence for recovery of DA synthesis and content in the months following MA administration (Fields *et al.*, 1991; Melega *et al.*, 1997b). In addition, 6-OHDA and MPTP are usually administered centrally and induce a unilateral lesion of the nigrostriatal pathway, while DA loss following systemic MA administration results in bilateral damage. Together, this evidence suggests that MA-induced toxicity may not have the same consequences as DA loss mediated by a 6-OHDA or MPTP lesion. Since MA is an abused drug, it is important to know if there are long-lasting consequences of MA usage. The primary focus of this work is to describe morphological and immunocytochemical changes within specific areas of the basal ganglia following MA-induced nigrostriatal damage, and to relate these changes to disorders associated with basal ganglia dysfunction.

B. Effects and Mechanisms of MA Toxicity

Neurotoxic Effects of MA Administration

MA is the N-methyl substituted derivative of amphetamine. Both compounds have similar effects on neurochemistry and behavior, although MA is more potent, has greater efficacy in the central nervous system, and has fewer peripheral actions (Lake and Quirk, 1984; Nichols, 1984; Hoffman and Lefkowitz, 1990). Although amphetamine is primarily a DA neurotoxin, MA has the ability to damage both dopaminergic and serotonergic nerve terminals (Hotchkiss *et al.*, 1979; Ricaurte *et al.*, 1980). The effects of high-dose MA administration on the nigrostriatal DA system include a long-

lasting depletion in DA content (Seiden *et al.*, 1975/76; Lucot *et al.*, 1980), decreased tyrosine hydroxylase activity (Fibiger and McGeer, 1971; Hotchkiss and Gibb, 1980; Pu and Vorhees, 1993), and a loss of DA uptake sites (Steranka and Sanders-Bush, 1980; Wagner *et al.*, 1980). Similar to studies examining dopaminergic damage in animals (Table I), postmortem analysis of tissue taken from humans who died following chronic MA use (daily to monthly for 1 to 23 years) found a 50-55% reduction of DA, and a 30-50% reduction in DA transporter number in the striatum (Wilson *et al.*, 1996).

In rats, high doses of MA have been shown to induce terminal degeneration, seen with reduced silver staining techniques (Ricaurte *et al.*, 1982). In addition, continuous administration of amphetamine for 3-5 days, or repeated injections of MA (4 doses of 6 mg/kg given every 2 hrs) has been reported to induce degeneration of axons and presynaptic terminals within the striatum of rats (Ryan *et al.*, 1990; Ellison and Switzer, 1993). In contrast, MA does not appear to damage DA cell bodies in the midbrain (Ricaurte *et al.*, 1980; Jonsson and Nwanze, 1982; Woolverton *et al.*, 1989; Ryan *et al.*, 1990; Di Monte *et al.*, 1996). However, Sonsalla *et al.* (1996) have found neuronal loss in the substantia nigra pars compacta of mice whose striatal DA depletion exceeded 90%, indicating that MA can damage dopaminergic cell bodies. In addition, MA induces cell death when administered *in vitro* to ventral midbrain DA neurons (Kontur *et al.*, 1987; Cubells *et al.*, 1994). Nevertheless, since cell body loss has not been found to occur in rats, it is generally accepted that MA-induced damage to the nigrostriatal DA system in these animals is limited to the loss of functional dopaminergic terminals in the striatum.

Table I. Representative Studies and Drug Doses Resulting in MA-Induced DA Depletion in Rats

Reference	Dose and Regimen	DA	Time
Abekawa *et al.*, 1994	4 mg/kg every 2 hr, 4 doses	-57%	5 days
O'Dell *et al.*, 1994	4 mg/kg every 2 hr, 4 doses	-31%	1 week
Marshall *et al.*, 1993	4 mg/kg every 2 hrs, 4 doses	-51%	1 week
Bowyer *et al.*, 1992	5 mg/kg every 2 hrs, 4 doses	-60%	3 days
Burrows & Meshul, 1997	5 mg/kg every 2 hrs, 4 doses	-50%	1 week
Burrows & Meshul, 1997	5 mg/kg every 2 hrs, 4 doses	-26%	4 weeks
Finnegan & Karler, 1992	10 mg/kg every 2 hrs, 4 doses	-76%	1 week
Stephans & Yamamoto, 1994	10 mg/kg every 2 hrs, 3 doses	-45%	1 week
Ricaurte *et al.*, 1982	12.5 mg/kg every 8 hrs, 3 doses	-6%	3 weeks
Ricaurte *et al.*, 1980	12.5 mg/kg every 12 hrs, 8 doses	-21%	3 weeks
Green *et al.*, 1992	15 mg/kg every 3 hrs, 4 doses	-75%	3 days
Baldwin *et al.*, 1993	15 mg/kg every 2 hrs, 4 doses	-36%	3 days
Ritter *et al.*, 1984	15 mg/kg every 6 hrs, 5 doses	-87%	18 hrs
Morgan & Gibb, 1980	15 mg/kg every 6 hrs, 5 doses	-80%	7 days
Robinson *et al.*, 1990	15 mg/kg every 6 hrs, 5 doses	-81%	7 days
Ricaurte *et al.*, 1980	50 mg/kg every 12 hrs, 8 doses	-48%	3 weeks
Lucot *et al.*, 1980	100 mg/kg every 24 hrs, 4 doses	-47%	16 weeks

Mechanisms of Toxicity: Oxidative Stress and Hyperthermia

The formation of striatal 6-OHDA following neurotoxic regimens of MA (Seiden and Vosmer, 1984; Marek *et al.*, 1990) has lead to the proposal that MA-induced damage is mediated by the endogenous formation of reactive oxygen species. In addition, recent studies have found that high-dose MA treatment induces hydroxyl radical formation and lipid peroxidation in the striatum (Wallace *et al.*, 1997; Yamamoto and Zhu, 1997). Pretreatment with DA synthesis inhibitors, antioxidants, or spin trapping agents have been found to decrease the formation of 6-OHDA or hydroxyl radicals that occur following MA treatment, and also to protect against MA-mediated DA loss (Schmidt *et al.*, 1985; De Vito and Wagner, 1989; Cappon *et al.*, 1995; Wallace *et al.*, 1997). In addition, drugs which increase the formation of 6-OHDA or hydroxyl radicals enhance MA-induced DA loss (De Vito and Wagner, 1989; Marek *et al.*, 1990). These studies suggest that DA loss induced by 6-OHDA and MA may share similar underlying mechanisms, and that long-lasting DA depletion observed following MA may result in similar morphological and neurochemical changes.

One obvious difference between MA and 6-OHDA is the role that hyperthermia plays in mediating the effects of MA-induced lethality and neurotoxicity (Zalis *et al.*, 1967; Craig and Kupferberg, 1972; Singer and Armstrong, 1976; Callaway and Clark, 1994). Bowyer *et al.* (1992), have shown that the degree of DA depletion induced by MA can be altered by manipulation of the environmental temperature. For example, placing an animal in a cold environment (4°C) during drug administration was protective, whereas increasing the ambient temperature (28°C) enhanced DA depletion (Bowyer *et al.*, 1993; 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). However, core body temperatures over 41.6°C can result in ischemic damage to striatal neurons (Knochel, 1989; Kao *et al.*, 1994; Lin *et al.*, 1995), and MA overdose in humans is often associated with seizures, transient ischemia, and cardiovascular collapse that have been attributed to severe hyperthermia (41.5-43.0 C°) (Callaway and Clark, 1994; Yen *et al.*, 1994). In addition, MA-induced hyperthermia does result in the induction of a heat shock protein (HSP-72) whose expression is found only in metabolically stressed cells (Kuperman *et al.*, 1997). Therefore, it is quite possible that some of the long-lasting damage resulting from high-dose MA exposure could be attributed to prolonged hyperpyrexia.

The source of psychostimulant-induced hyperthermia is not known, but has been postulated to be mediated by several factors, including increased locomotor activity, peripheral vasoconstriction, and by the central release of DA. Both D1 and D2 receptors are believed to play a role in thermoregulation. Although administration of either D1 or D2 receptor agonists alone does not greatly affect core temperature, co-administration of both D1 and D2 receptor agonists results in a 3-4°C increase in body temperature (Nagashima *et al.*, 1992; Verma and Kulkarni, 1993). This effect can be blocked by prior administration of either a D1 or D2 receptor antagonist, further demonstrating the synergistic nature of D1/D2 receptor interactions (Nagashima *et al.*, 1992). Similarly, pretreatment with either a D1 or D2 receptor antagonist decreases both MA-induced hyperthermia and protects against DA loss (Sonsalla *et al.*, 1986; Marshall *et al.*, 1993; Staal *et al.*,

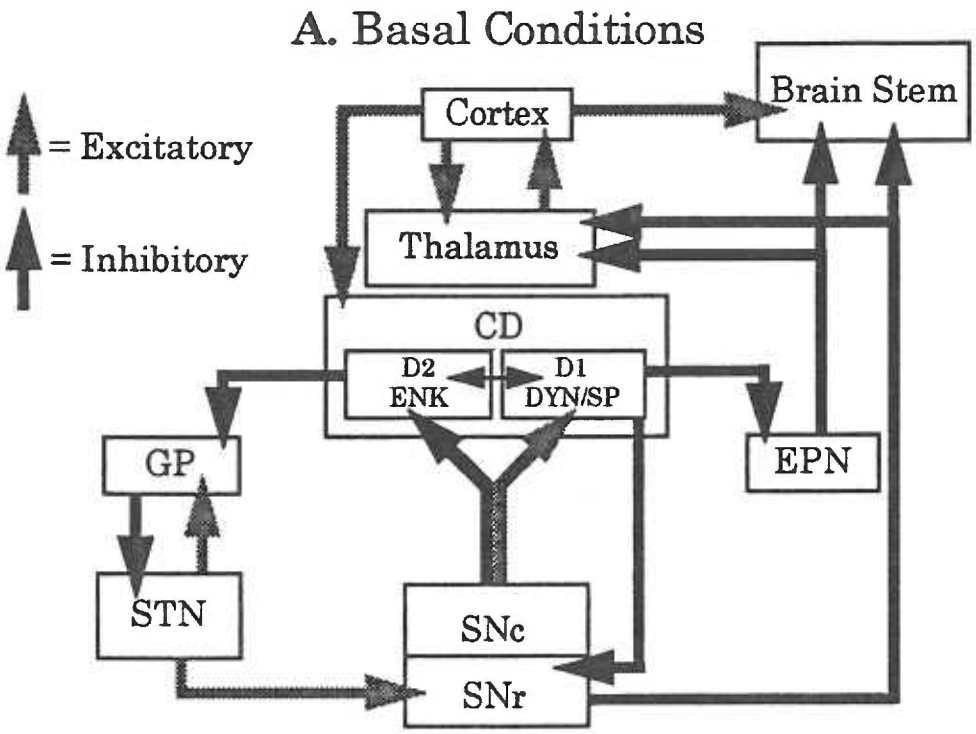
1996). Together, this evidence indicates that combined D1 and D2 receptor stimulation could account for at least part of the hyperthermic effects of MA.

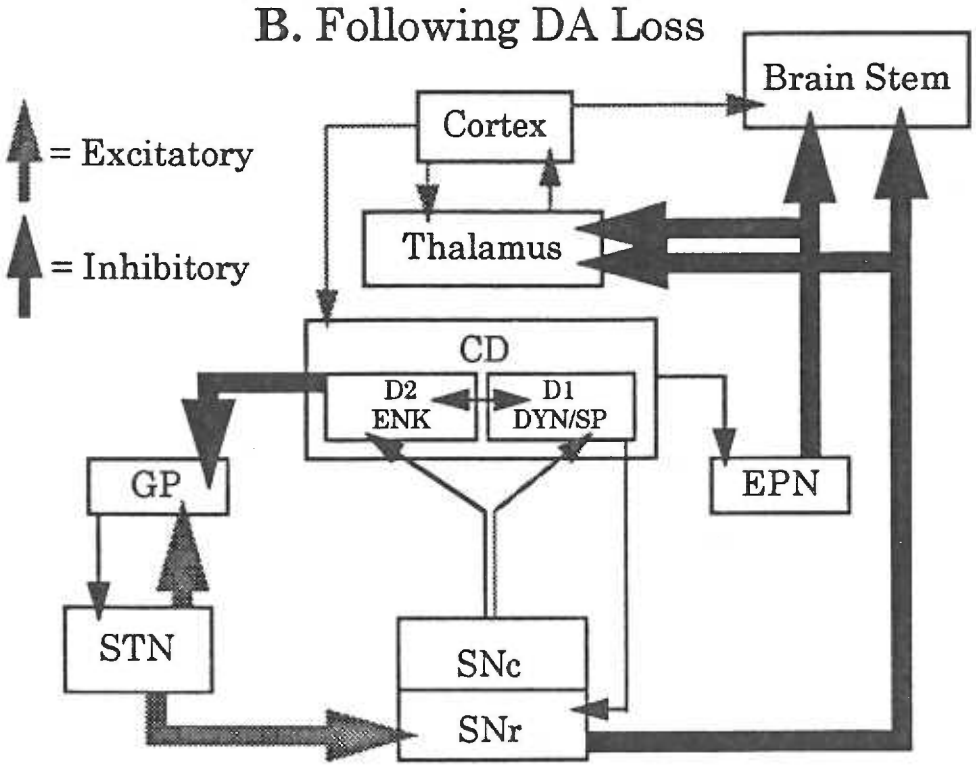
It is likely that hyperthermia contributes to MA toxicity by increasing the overall state of metabolic stress, and enhancing both the formation and damaging effects of DA-mediated free-radical production. Evidence for this action comes from studies demonstrating that prevention of MA-hyperthermia, by treating animals in a cold environment, attenuates both oxygen radical production and the toxic effects of the drug (Fleckenstein *et al.*, 1996). Furthermore, drugs which protect against MA toxicity invariably do so by alleviating oxidative stress (antioxidants, free-radical scavengers, nitric oxide synthase inhibitors, or spin trapping agents) and/or by inducing hypothermia (MK-801, haloperidol) (Farfel and Seiden, 1995; Itzhak and Ali, 1996; Staal *et al.*, 1996). However, hyperthermia may contribute to MA toxicity by directly enhancing DA release. Bowyer *et al.* (1992) have reported that administration of MA in a cold environment (4°C) does attenuate DA overflow compared to rats treated at room temperature (23°C). Together, this evidence suggests that MA induced hyperthermia may exacerbate MA-induced damage by directly enhancing DA overflow and/or by indirectly increasing metabolic stress within the striatum.

C. Effects of DA Deafferentation on Basal Ganglia Function

Damage to the nigrostriatal DA system results in altered synaptic morphology and neurochemistry within several of the nuclei and neurotransmitter systems associated with the extrapyramidal motor loop (Figure 1A). Therefore, the basic organization and neurochemistry of the

Figure 1. Diagram of the extrapyramidal motor loop showing **A.** basal conditions, and the **B.** hypothetical consequences of nigrostriatal DA loss. The thickness of the arrows is proportional to the amount of activity in that pathway (i.e., thicker arrows = increased activity, thinner arrows = decreased activity). This figure was adapted from Meshul *et al.* (1992) and includes information from several additional sources (Fallon and Moore, 1978; Nakanishi *et al.*, 1987; Alexander and Crutcher, 1990; Kawaguchi *et al.*, 1990; Smith and Bolam, 1990; Gerfen, 1992). Abbreviations: CD, caudate; DYN, dynorphin; ENK, enkephalin; EPN, entopeduncular nucleus; GP, globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SP, substance P; STN, subthalamic nucleus.


A. Basal Conditions
= Excitatory
= Inhibitory
Cortex
Brain Stem
Thalamus
CD
D2
ENK
D1
DYN/SP
GP
EPN
STN
SNc
SNr


B. Following DA Loss
= Excitatory
= Inhibitory
Cortex
Brain Stem
Thalamus
CD
D2
ENK
D1
DYN/SP
GP
EPN
STN
SNc
SNr

basal ganglia will be reviewed, followed by an overview of dysfunction caused by DA deafferentation.

Organization of the Basal Ganglia: An Overview

The basal ganglia is a collection of diverse nuclei which function to control the planning and generation of voluntary movement (Graybiel, 1990). Dysfunction within this pathway plays an important role in the generation of symptoms in several human disorders, including Parkinson's disease, Huntington's disease, and the tardive diskinesia seen following neuroleptic treatment. Regulation of this extrapyramidal motor loop occurs via two striatal output systems, the direct and indirect pathways, which act in parallel to control the excitatory thalamocortical input to the striatum (Figure 1A). The direct pathway consists of striatal output neurons which send projections to both the substantia nigra pars reticulata and entopeduncular nucleus. In contrast, the indirect output pathway consists of a separate circuit with neurons projecting, in turn, to the globus pallidus (GP), subthalamic nucleus, and substantia nigra pars reticulata. Neurons in the entopeduncular nucleus and substantia nigra pars reticulata then send afferents to the thalamus and brainstem.

Under resting conditions striatal neurons are quiescent while the output stations (GP, substantia nigra pars reticulata and entopeduncular nucleus) are tonically active and inhibit the thalamus and brainstem. Thus, the overall output signal of the basal ganglia is inhibitory during rest. Immediately preceding movement, DA release in the striatum results in the activation of the direct pathway and inhibition of the indirect pathway, with the overall effect being the disinhibition of the thalamus and brainstem,

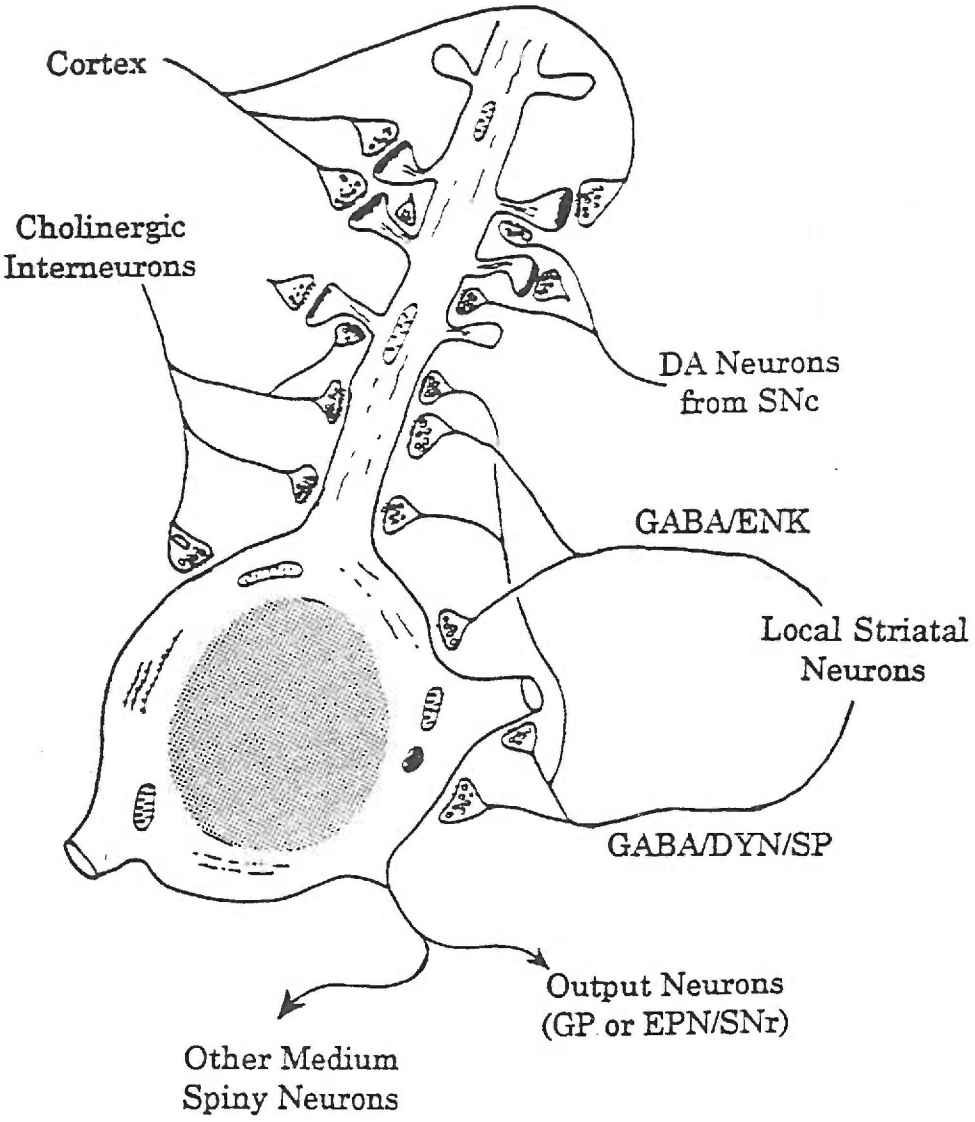
leading to the initiation of movement (Chevalier and Deniau, 1990; Gerfen, 1992). Overactivity of the striatopallidal pathway under resting conditions, resulting in decreased thalamocortical feedback (Figure 1B), may interfere with the generation of movement and underlie parkinsonian symptoms (Miller and DeLong, 1987; Albin *et al.*, 1989; Klockgether and Turski, 1989; Mitchell *et al.*, 1989).

Neurochemistry of the Striatal Output Pathways

Most neurons in the striatum (90-95%) are glutamate decarboxylase (GAD)-positive medium spiny neurons (Wilson and Groves, 1980; Penny *et al.*, 1986; Gerfen, 1992), while the remaining cells are aspiny somatostatin or cholinergic interneurons (Gerfen, 1984; Gerfen *et al.*, 1987). These medium spiny neurons receive synaptic inputs from both local and extrastriatal neurons containing a variety of neurotransmitters (Figure 2). Of importance are the inhibitory γ-aminobutyric acid (GABA) synapses that arise from other medium spiny neurons, primarily as collaterals from the striatal projection cells and/or local interneurons (Somogyi *et al.*, 1981; Smith and Bolam, 1990). These inhibitory afferents form symmetrical synapses with the cell bodies and dendritic shafts of medium spiny neurons (Smith and Bolam, 1990) and play an important role in mediating striatal output.

The primary striatal output pathways (direct and indirect) are comprised of separate but equal numbers of GAD-positive medium spiny neurons (Gerfen, 1992) that can be distinguished from each other on the basis of neuropeptide content and DA receptor populations (Izzo *et al.*, 1987). Output neurons projecting to the GP via the indirect pathway are enkephalin (ENK) rich and are inhibited by dopaminergic input via D2 receptor

Figure 2. Synaptic organization of inputs to striatal medium spiny neurons. Note that GABA inputs synapse upon the soma and dendritic shafts while cortical inputs synapse exclusively upon spines. This figure was modified from Figure 3 in Smith and Bolam (1990). Abbreviations: DYN, dynorphin; ENK, enkephalin; EPN, entopeduncular nucleus; GP, globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SP, substance P.


Cortex
Cholinergic
Interneurons
DA Neurons
from SNc
GABA/ENK
Local Striatal
Neurons
GABA/DYN/SP
Output Neurons
(GP or EPN/SNr)
Other Medium
Spiny Neurons

stimulation (Figure 1). In contrast, output neurons projecting to the entopeduncular nucleus and substantia nigra pars reticulata via the direct pathway are dynorphin and substance P rich, and are excited by dopaminergic input via D1 receptor stimulation (Alheid and Heimer, 1988; Gerfen and Young, 1988; Gerfen *et al.*, 1990). In addition to DA-mediated release, the synthesis of the GABA co-localized peptides is also under the influence of dopaminergic activity. DA inhibits ENK synthesis but stimulates both substance P and dynorphin synthesis (Gerfen *et al.*, 1990; Gerfen *et al.*, 1991; Nisenbaum *et al.*, 1994). Similarly, when DA is removed ENK synthesis increases while substance P and dynorphin synthesis is inhibited (Young *et al.*, 1986; Gerfen, 1992).

Postmortem studies of tissue taken from patients with Parkinson's disease have found increased GAD activity in the substantia nigra pars reticulata (Javoy-Agid *et al.*, 1981) and increased GABA content in the putamen (Perry *et al.*, 1983). In addition, there is evidence for increased GABA release in GP in Parkinson's disease (Tossman *et al.*, 1986; Segovia and Garcia-Munoz, 1987). Together, this evidence points to a basic dysfunction of the GABAergic striatal output system following a loss of DA neurons. Therefore, much emphasis has been placed on identifying GABA dysfunction in the chronic DA-depletion animal models of Parkinson's disease (i.e., 6-OHDA and MPTP). Because the GABAergic striatal output pathways can be distinguished on the basis of neuropeptide content, studies examining ENK, dynorphin and substance P content and function have led to important insights into the complex changes that occur in the functional circuitry of the basal ganglia following DA deafferentation.

Effects of DA Deafferentation On GABAergic Systems

Similar to changes seen in Parkinson's disease, GABA content, synthesis, and stimulated release are enhanced in both the striatal and GP output neurons following 6-OHDA treatment (Segovia and Garcia-Munoz, 1987; Lindefors *et al.*, 1989; Gerfen *et al.*, 1991; Kincaid *et al.*, 1992). In addition, metabolic and electrophysiological evidence indicates increased activity in striatopallidal neurons following DA removal (Schultz and Ungerstedt, 1978; Wooten and Collins, 1981; Pan and Walters, 1988; Mitchell *et al.*, 1989). These changes are generally found 3-4 weeks following destruction of the nigrostriatal tract and may be very long-lasting (Table II). Although increased GABA activity has been found both within the caudate/putamen (CD) and GP (i.e., the indirect pathway), altered GAD levels in the substantia nigra (i.e., the direct pathway) are not always seen (Vincent *et al.*, 1978; Segovia and Garcia-Munoz, 1987). In fact, GAD activity within the other target of the direct pathway, the entopeduncular nucleus, is actually decreased by 38% one month following DA loss (Segovia and Garcia-Munoz, 1987). This evidence supports the proposed differential effect that DA loss would have on the striatal output pathways in that 6-OHDA-induced lesions appear to increase GABA output to the GP and decrease GABA output to the substantia nigra pars reticulata and entopeduncular nucleus, resulting in an overall decrease in thalamocortical activity (Figure 1B) (Greenamyre, 1993).

Several studies have found that the neuropeptides within the direct and indirect pathways are also differentially regulated following DA deafferentation (Table III). Such alterations are confined to the medium spiny neurons since peptide markers of aspiny interneurons remain unaffected by DA loss (Thal *et al.*, 1983). As would be predicted by the

Table II. *Effects of Nigrostriatal 6-OHDA Lesion on the Tissue Content, Immunoreactivity, Morphology, and Stimulated Release of GABA in the Striatal Output Systems.*

Reference	1-2 Weeks Post-Lesion	3-4 Weeks Post-Lesion	2-12 Months Post-Lesion
Lindefors *et al.*, 1989		↑ GABA Content & Over-flow in CD	
Vincent *et al.*, 1978		↑ GAD in CD but not SN	
Segovia & Garcia-Munoz, 1987		↑ GAD in CD, GP and SN ↓ GAD in EPN	
Nitsch & Riesenberg, 1995			↑ Num of GAD-IR Dendrites and Boutons in CD
Meshul *et al.*, 1997		↑ GABA-IR in CD	↑ GABA-IR in CD
Vernier *et al.*, 1988		↑ GAD mRNA in CD	Normal GAD mRNA in CD
Schultz & Ungerstedt, 1978	↑ Striatal Cell Activity		Normal Striatal Cell Activity

Abbreviations: CD, caudate; EPN, entopeduncular nucleus; GAD, glutamate decarboxylase; GP globus pallidus; IR, immuno-reactivity; SN, substantia nigra

Table III. *Effects of Nigrostriatal 6-OHDA Lesion on the Tissue Content, Immunoreactivity, Morphology, and Stimulated Release of Neuropeptides in the Striatal Output Systems*

A. Enkephalin	**1-2 Weeks Post-Lesion**	**3-4 Weeks Post-Lesion**	**2-12 Months Post-Lesion**
Ingham *et al.*, 1991	No Effect	↑ ENK Area & Length of Active Zone	↑ ENK Area & Length of Active Zone
Thal *et al.*, 1983	No Effect	↑ ENK in CD	↑ ENK in CD
Voorn *et al.*, 1987	↑ ENK-IR in CD	↑ ENK-IR in CD	↑ ENK-IR in CD
Vernier *et al.*, 1988		↑ ENK mRNA	↑ ENK mRNA
Ingham *et al.*, 1997		↑ ENK-IR in CD and GP	

B. Substance P	**1-2 Weeks Post-Lesion**	**3-4 Weeks Post-Lesion**	**2-12 Months Post-Lesion**
Lindefors *et al.*, 1989		↓ SP Content in CD & SN ↓ Stim. Release of SP in SN	
Voorn *et al.*, 1987	No Effect on SP-IR	No Effect on SP-IR	No Effect on SP-IR

Abbreviations: CD, caudate; ENK, enkephalin; GP globus pallidus; IR, immunoreactivity; SN, substantia nigra; SP, substance P

inhibitory influence DA has on ENK production, increases in GABA levels following DA removal are paralleled by increases in ENK synthesis and content (Vernier *et al.*, 1988). 6-OHDA-induced lesions result in a more than doubling of striatal ENK levels starting 2 weeks after destruction of DA neurons and lasting at least 2 months. Although some evidence suggests that GABAergic function does eventually return to basal levels, increased ENK activity can last out to a year following DA loss (Schultz and Ungerstedt, 1978; Thal *et al.*, 1983; Voorn *et al.*, 1987; Vernier *et al.*, 1988).

Ingham *et al.* (1991) found that the area of striatal ENK-immunopositive presynaptic boutons was increased by greater than 50% four weeks following a 6-OHDA-induced lesion. In addition, the length of the active zone, which may be correlated with an increased probability of neurotransmitter release, was larger in ENK-immunopositive profiles. ENK immunoreactive boutons were also larger and more numerous within the GP of DA denervated rats (Ingham *et al.*, 1997). Together with the neurochemical evidence of enhanced ENK content and synthesis, these morphological changes suggest that more neurotransmitter may be released per nerve terminal following DA deafferentation. These effects appear to occur specifically within the striatopallidal projection neurons. In contrast, the biosynthesis and striatal levels of substance P and dynorphin are decreased or unchanged in the CD and substantia nigra pars reticulata following DA depletion (Pettibone and Wurtman, 1980; Voorn *et al.*, 1987; Lindefors *et al.*, 1989; Graybiel, 1990). Additional evidence suggests that stimulated release of substance P is also decreased in striatonigral neurons (Lindefors *et al.*, 1989). These 6-OHDA-induced changes in ENK and substance P are in agreement with the opposing influence that nigrostriatal dopaminergic

afferents have on striatal output pathways. DA loss induced by 6-OHDA or MPTP decreases the firing rate of the D1 receptor/substance P mediated direct pathway (Miller and DeLong, 1987; Mitchell *et al.*, 1989) and increases the firing rate of the D2 receptor/ENK mediated pathway (Pan and Walters, 1988; Mitchell *et al.*, 1989). The overall result is overactivation of the subthalamic nucleus and inhibition of the thalamus and brainstem (Figure 1B), possibly leading to tremor and rigidity characteristic of Parkinson's disease (Gerfen, 1992; Gerlach and Riederer, 1996).

DA Deafferentation Alters Glutamatergic Systems

In contrast to the clear-cut changes in GABA systems seen following 6-OHDA, the effects of DA loss on excitatory glutamate pathways are somewhat contradictory in nature (Table IV). Loss of the nigrostriatal DA input to the CD would theoretically result in the disinhibition (i.e., increased activity) of the indirect pathway, and decreased excitation (i.e., decreased activity) of the direct pathway, leading to increased GABA release within the motor thalamus (Figure 1). Although acute GABA release within the thalamus would be expected to decrease activation of the corticostriatal pathway, chronic administration of GABA within the motor thalamus has been shown to increase basal levels of extracellular glutamate within the CD (Meshul *et al.*, 1996), suggesting that long-term increases in GABA input to the thalamus may actually lead to an enhancement of corticostriatal activity. In fact, basal glutamate levels within the CD, measured by *in vivo* microdialysis, are enhanced one month following 6-OHDA administration (Calabresi *et al.*, 1993; Meshul *et al.*, 1997). In addition, there is an increase in the percent of asymmetrical synapses containing a perforated postsynaptic density

Table IV. *Effects of Nigrostriatal 6-OHDA Lesion on Glutamate Immunoreactivity, Morphology, Receptor Levels, and Release in the Striatal Output Systems*

Reference	1-2 Weeks Post-Lesion	3-4 Weeks Post-Lesion	2-12 Months Post-Lesion
Weihmuller *et al.*, 1993		↓ NMDAR's in the CD	
Wüllner *et al.*, 1994	↓ NMDAR's in CD No Effect in SN		↑ NMDAR's in CD ↓ NMDAR's in SN
Porter *et al.*, 1994			↓ AMPAR's in EPN & SN ↓ NMDAR's in CD & GP
Wüllner *et al.*, 1993	↓ AMPAR's in GP & SN ↓ NMDAR's in SN		No Effects
Meshul *et al.*, 1997		↓ Glutamate-IR in CD ↑ Basal glutamate Overflow ↑ % of Perf. Synapses	↑ Glutamate-IR in CD ↓ Basal glutamate Overflow ↑ % of Perf. Synapses
Ingham *et al.* 1991, & 1993		↑ Length of Active Zone ↓ Spine Density in CD	
Tremblay *et al.*, 1995 Ulas and Cotman, 1996	↑ NMDAR mRNA in CD		

Abbreviations: AMPAR, AMPA type receptor; CD, caudate; EPN, entopeduncular nucleus; GP globus pallidus; NMDAR, NMDA type receptor; SN, substantia nigra

(Meshul *et al.*, 1997), an increase in the length of the active zones associated with asymmetrical synapses (Ingham *et al.*, 1991), and a small but significant decrease (5-10%) in [3H]glutamate binding to N-methyl-D-aspartate (NMDA) receptors (Weihmuller *et al.*, 1993). This evidence indicates that an increase in the activity of excitatory synapses within the CD can be found 1 month following DA deafferentation.

Not all studies agree with the hypothesis that DA loss increases corticostriatal activity. Binding of [H3]glutamate or [H3]MK-801 to NMDA receptors two or more months following 6-OHDA treatment has been reported to either increase or decrease relative to unlesioned control animals (Porter *et al.*, 1994; Wüllner *et al.*, 1994a). In addition, Meshul *et al.* (1997) have reported a complete reversal of extracellular glutamate levels (from increased to decreased) and a shift in the density of presynaptic glutamate immunoreactivity (from decreased to increased) that occurs between 1-3 months following 6-OHDA-induced lesion. Other studies suggest that there may be a loss of corticostriatal glutamatergic input 1 month following DA deafferentation. Medium spiny neurons within the CD show an overall decrease in the number of dendritic spines within the neuropil and a concurrent decrease in the number of spines per length of dendritic shaft (Ingham *et al.*, 1993). Although increases in the length of active zones of asymmetrical synapses have been reported, these asymmetrical terminals do not show increased size following a 6-OHDA-induced lesion (Ingham *et al.*, 1991; Meshul *et al.*, 1997). It is possible that although some synaptic reorganization occurs, remaining glutamatergic terminals show higher levels of activity. Such studies, however, suggest that the effects of DA loss on glutamate activity are both complicated and dynamic. Initial compensatory

increases in corticostriatal activity may be responsible for the fast behavioral recovery (i.e., reversal of anorexia, adipsia, and akinesia) that occurs within the first 2 weeks post-lesion in rodents, while longer term decreases in glutamate activity parallel changes seen in Parkinson's disease.

Effects of Amphetamine and MA on GABAergic and Glutamatergic Systems

Studies of MA toxicity have concentrated on the source of DA loss, as opposed to the lasting effects that such damage can incur. As such, there are few reports describing MA's effects on GABA or glutamate systems. There is, however, evidence that MA may initially damage non-dopaminergic profiles. Ryan *et al.* (1990) reported that chronic administration of high doses of amphetamine (20-60 mg/kg/day for 3 days) induced the degeneration of glial, terminal, and axonal profiles in the CD, somatosensory cortex, and motor cortex of rats. The degenerating nerve terminals were associated with both symmetrical and asymmetrical synapses, indicating that destruction of non-catecholaminergic transmitter systems may have occurred.

MA and amphetamine activate both the GABAergic output pathways and the excitatory corticostriatal loop, indicating that the entire extrapyramidal motor circuit may be involved in psychostimulant-induced activation and toxicity. As such, administration of MA or amphetamine enhances extracellular striatal levels of both GABA (Porras and Mora, 1993) and glutamate (Nash and Yamamoto, 1992) as measured by *in vivo* microdialysis. Administration of MA increases the immunoreactivity for peptides associated with GABA synapses, also indicating activation of GABAergic striatal neurons. Although MA induces increased expression of preproenkephalin mRNA only within the CD (Wang and McGinty, 1996), both

substance P and dynorphin immunoreactivity are enhanced in the substantia nigra, entopeduncular nucleus, and CD 18 hrs after MA treatment (10-15 mg/kg every 6 hrs for 4-6 doses) (Ritter *et al.*, 1984; Hanson *et al.*, 1987). We have found that 12 hours following MA administration (5 mg/kg every 2 hrs for 4 doses), presynaptic immunogold labeling of glutamate in the ventrolateral caudate (VLCD) and motor cortex is transiently decreased (Burrows and Meshul, 1997). Eisch *et al.* (1996) reported that this same dose of drug resulted in a loss of NMDA binding sites only within the ventral and lateral regions of the CD. These changes were present 1 week, but not 1 month, following MA treatment and were attributed to regulatory responses that occur following augmented glutamate release.

These changes in GABA and glutamate functions support the hypothesis that MA treatment leads to an overall activation of the extrapyramidal motor circuitry, but it is not known how long these changes last or how these changes correlate with DA loss. Evidence indicates that MA treatment resulting in approximately a 50% loss of striatal DA content alters corticostriatal function for at least 1 week. In contrast, administration of MA at a dose known to induce DA loss (15 mg/kg every 6 hrs for 5 doses) does not alter striatal GAD activity in tissue where tyrosine hydroxylase activity is decreased by 50% (Hotchkiss *et al.*, 1979). Daily injections of 5.4 mg/kg amphetamine for 45 days does increase GAD activity within the substantia nigra, but the effect of this dose on DA levels is not known (Perez-de la Mora *et al.*, 1990).

Effects of a Partial or Severe Lesion on DA Function

Compensatory presynaptic changes within the nigrostriatal DA system that are known to occur following 6-OHDA treatment include increased synthesis, metabolism, and fractional release of DA in remaining terminals (Agid *et al.*, 1973; Hefti *et al.*, 1980; Altar *et al.*, 1987). In general, it is believed that the loss of the majority of DA terminals is required for these compensatory changes to occur. For example, Robinson *et al.* (1990) reported that MA or 6-OHDA treatment resulting in less than 80% loss of DA content had no effect on basal extracellular levels of DA or on DA release that occurred in response to a MA challenge. Similarly, increases in DA synthesis following 6-OHDA do not occur unless DA loss exceeds 60% (Hefti *et al.*, 1980), and MA-treated animals with only 30-50% loss of striatal DA content do not have altered DA metabolism (Ricaurte *et al.*, 1982; Nash and Yamamoto, 1992; Nash and Yamamoto, 1993). Compensatory increases in DA turnover (ratio of dihydroxyphenyl Acetic Acid (DOPAC) to DA) and upregulation of D2 receptors following amphetamine or MA have both been reported in animals with larger DA depletions (Ritter *et al.*, 1984; Fields *et al.*, 1991; Bowyer *et al.*, 1993). If changes in non-dopaminergic pathways are part of the array of compensatory changes that occur following DA loss, then long-term alterations in GABAergic and glutamatergic systems may not occur in MA-treated animals with only a partial DA loss. However, changes in morphology and immunocytochemistry have only been examined in animals presumed to have severe DA depletions. It is not known what alterations, if any, occur following a partial lesion.

In addition to induction of a partial (≈50%) lesion within the nigrostriatal DA system, there is evidence for recovery from MA-induced DA

loss (Table I). As an example, Melega *et al.* (1997b) reported the gradual recovery of fluoro-DOPA uptake (a measure of DA synthesis) following MA administration in monkeys. DA synthesis was decreased by 70% after 1 month, 45% after 6 months, 20% after 12 months, and had control levels of fluoro-DOPA uptake 24 months following MA exposure. Coupled with the lack of midbrain DA cell loss usually reported (Ricaurte *et al.*, 1980; Seiden and Ricaurte, 1987; Di Monte *et al.*, 1996), this evidence suggests that DA terminals lose their catecholamine phenotype at least temporarily. The loss of terminal function may result in compensatory changes that differ from the effects of the physical loss of DA terminals and cell bodies. In addition, recovery of presynaptic DA content may preclude any compensatory changes that may occur following long-term DA loss.

D. Rationale: Approaches for Identifying MA-Induced Alterations

Use of pre-embedding immunocytochemistry has demonstrated that 6-OHDA lesions result in a qualitative increase in ENK immunoreactivity at the light microscopic level, and altered morphology of ENK immunoreactive boutons at the electron microscopic level (Ingham *et al.*, 1991; Ingham *et al.*, 1993; Ingham *et al.*, 1997). Although pre-embedding immunocytochemical analyses can provide qualitative information regarding where neurochemical alterations occur, and can indicate the direction of these modifications, the degree to which these changes occur cannot be accurately assessed. A quantitative technique employing post-embedding colloidal-gold immunocytochemistry allows for the detection of subtle changes in the density of neurotransmitter immunoreactivity within presynaptic terminals. Using these methods, primary antibodies raised against the amino acid

neurotransmitters glutamate and GABA can be used to quantify changes in presynaptic immunoreactivity following drug treatment. Ji *et al.* (1991) found that gold particles are enriched over synaptic vesicles, and reported a strong correlation between particle density and synaptic vesicle density within immuno-labeled nerve terminals. Similarly, we have generally found that gold-particles not associated with synaptic vesicles account for less than 10% of terminal immunoreactivity (Meshul *et al.*, 1997; Burrows, unpublished observation). For these reasons, changes in presynaptic immunoreactivity are believed to reflect changes in the vesicular neurotransmitter pool, as opposed to the cytosolic (or metabolic) pools of amino acids.

Recent evidence gained using this immunocytochemical technique suggest that the density of presynaptic glutamate and GABA immunoreactivity are differentially regulated within the striatum one and three months following a 6-OHDA-induced lesion (Meshul *et al.*, 1997). These data correlate with biochemical studies and indicate that the enhancement of GABA function may be related to increased presynaptic neurotransmitter levels as suggested by an increase in nerve terminal immunoreactivity. In addition, these data have added to the evidence suggesting changes in corticostriatal excitatory activity may be due to alterations in the morphology and neurotransmitter content of presynaptic boutons (Meshul *et al.*, 1997), as well as to a decrease in the number of available postsynaptic targets (Ingham *et al.*, 1993).

It is likely that long-lasting regulatory changes in striatal GABA and glutamate following MA or amphetamine treatment have not been reported because of the relative intactness of the DA system (i.e., ≈ 50% of DA content remaining). By selectively examining the density of presynaptic GABA and

glutamate immunoreactivity using ultrastructural immunocytochemistry, we were able to detect subtle changes in nerve terminal amino acid content that may not be measurable in gross tissue homogenates or by examination of the tissue at the light microscopic level. Such shifts in neuronal transmitter density may be involved in compensatory activities that allow for the maintenance of function. In addition, the technique allows for morphological analysis of boutons with identified neurotransmitter content. Nerve terminal changes that can be measured using this technique include altered terminal area, and the size and type of synaptic contact. Identification of morphological alterations can provide important insights into understanding how biochemical modifications may affect synaptic structure and function, as can be seen in recent studies characterizing such ultrastructural changes following DA deafferentation (Ingham *et al.*, 1991; Ingham *et al.*, 1993; Ingham *et al.*, 1997; Meshul *et al.*, 1997).

As discussed above, 6-OHDA-induced lesions of the nigrostriatal pathway result in biochemical, histochemical and morphological alterations in non-dopaminergic profiles. Although long-term changes in the dopaminergic system following MA treatment have been well characterized, alterations in non-dopaminergic systems are less well understood. These experiments were designed to test the hypothesis that high-dose MA treatment, known to deplete striatal DA content, results in altered presynaptic immunoreactivity of two of the major neurotransmitter systems associated with basal ganglia function. Furthermore, these studies tested the hypothesis that neurochemical changes are only found in animals with severe DA loss, and that they are not the result of prolonged hyperthermia alone. In particular, the GABAergic and glutamatergic systems were studied since

these are known to be altered by DA deafferentation. The specific aims of this study were to 1) quantify characterize the effects of MA administration on the synaptic morphology and the density of nerve terminal immunoreactivity for the excitatory neurotransmitter glutamate, and the inhibitory neurotransmitter GABA, within three regions of the basal ganglia, 2) determine if these neurochemical and morphological changes are transient or long-lasting, and 3) investigate the role of hyperthermia in MA toxicity.

MATERIALS AND METHODS

A. General Methods

To insure that MA administration resulted in a severe DA depletion lasting at least one month, a high dose of MA (15 mg/kg) was given repeatedly over a 24 hour period (every six hours for four doses). This dose regimen induces the most dramatic decreases in DA content (Table I) and tyrosine hydroxylase activity (Morgan and Gibb, 1980; Ritter *et al.*, 1984; Robinson *et al.*, 1990; Johnson *et al.*, 1994), and had a much lower mortality rate compared to other doses examined (25 or 50 mg/kg every 12 hrs) in which almost all animals died following 1-3 injections (Burrows, unpublished observation).

The severity of MA-toxicity is also dependent upon the age of the animal: neonatal and 1 month old rats show no toxicity, 2 month old rats show moderate toxicity, and 3 month old rats show severe toxicity (Lucot *et al.*, 1982; Taraska and Finnegan, 1995). Animals older than 6 months do show severe toxicity, but also have very high mortality rates (60-100% mortality following 5-16 mg/kg MA every 2 hrs for 4 doses; Lucot *et al.*, 1982;

Taraska and Finnegan, 1995; Burrows and Meshul, 1996). To maximize DA depletion while minimizing the lethal effects of MA, all rats used in these studies were between 3 and 4 months of age.

Most measures of MA toxicity (i.e., DA levels, tyrosine hydroxylase 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. MA-induced hyperthermia correlates with DA depletion (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994) and can be used as an independent measure of toxicity. Therefore, animals used in the immunocytochemical and morphological analyses had their core body temperature monitored throughout MA administration. In order to demonstrate conclusively that the dose and schedule of MA administration used in these studies did induce neurotoxicity, catecholamine content was assessed in an identically treated group of rats. In addition, these animals also had their core body temperatures monitored in order to have a measure that could be directly compared across groups.

B. Subjects and Drug Administration

Male Sprague-Dawley rats (Harlan, 350-400 g, age 3 months) were housed, 2 animals per cage, in clear plastic shoe boxes with corncob bedding. Animals were maintained on a 12 hr light/dark cycle (lights on at 6:00 am) with food and water available *ad libitum* throughout the experiments. Room temperature was 22 ± 1°C for all experiments. MA was dissolved in 0.9% saline at a concentration of 15 mg/ml. All animals received 4 injections (each 6 hours apart) of either MA (15 mg/kg s.c.) in normal saline or an equivalent volume of vehicle. Rectal temperatures were measured every 30 min for 5 hrs

following each injection using an RET-2 copper-constantan (type T) thermocouple rectal probe (Physitemp Instruments, Inc.) and a TH-8 thermalert thermometer (Sensortek, Inc.).

Rats (n = 150) were randomly assigned to 1 of 4 treatment groups. Rats in the SAL group (n = 24) were given normal saline at room temperature (22°C). The METH group (n = 65) was given MA at room temperature (22°C) and allowed to become hyperthermic. Bowyer et al. (1994) has reported that cooling animals whose body temperature exceeds 41.3°C results in a significant decrease in mortality rates without preventing DA loss. Therefore, if core body temperature exceeded 41.3°C, animals were cooled by placing them in an empty shoe box seated over wet ice for 20-30 min. This usually resulted in a 2-3°C drop in core body temperature. Animals in the HOT group (n = 31) were given normal saline and had their cages placed on a heating pad in an incubator (ambient temperature 28-30°C). The heating pad was turned on/off to manipulate body temperature in order to match the levels of hyperthermia experienced by the animals in the METH group (range of bedding temperature was 26-50°C, mean = 34.8°C). When rectal temperature exceeded 41.3°C, rats in the HOT group were cooled exactly like the METH group. Rats in the COLD group (n = 30) were given MA at room temperature (22°C) but prevented from becoming hyperthermic. If rectal temperature exceeded 38.5°C, animals were cooled by being placed in an empty shoe box seated over wet ice for 20-30 min.

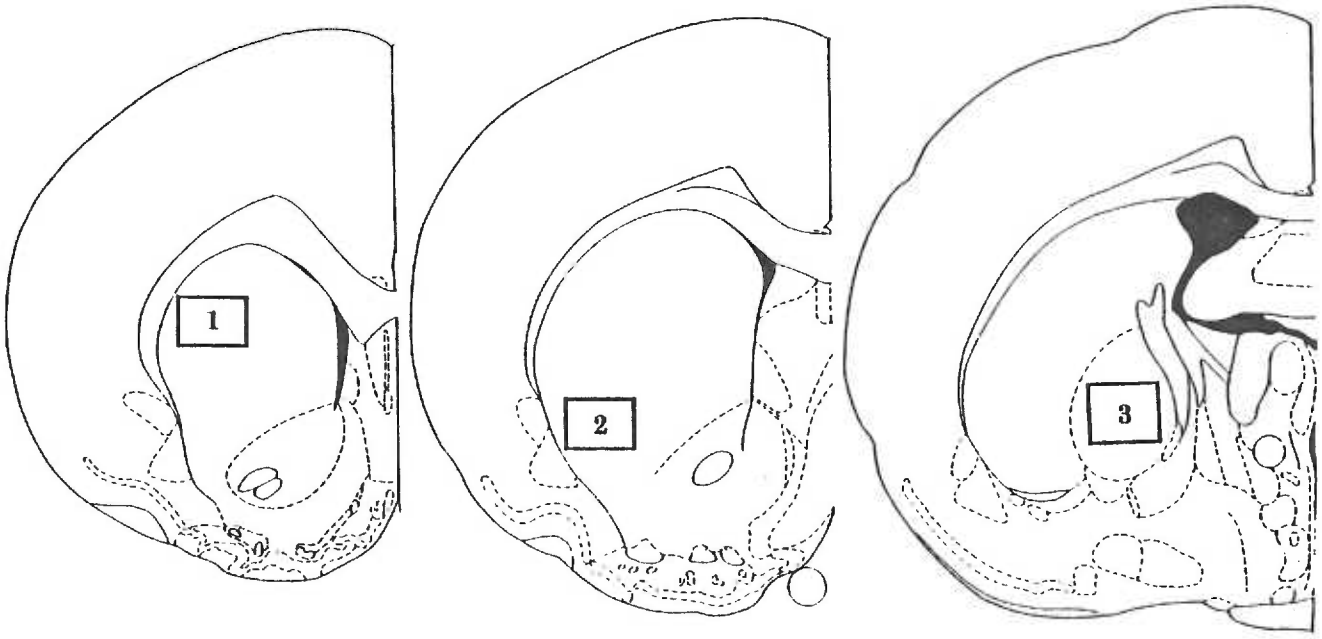
Severe depletion of striatal DA is known to induce anorexia and adipsia (Singer and Armstrong, 1976; Jicha and Salamone, 1991). In previous studies it was noted that some drug treated animals remained severely dehydrated and died 2-3 days post-MA. Therefore, body weight was

monitored daily for 4 days following drug treatment, then once each week until killed. If animals appeared lean and/or dehydrated they were treated with 4 ml normal saline with 5% dextrose i.p. and/or 2-3 ml Ensure brand diet supplement by gavage twice daily for 2-4 days. In addition, these animals were offered fruit-flavored gelatin and wet cat food in order to encourage eating.

C. Ultrastructural Immunocytochemistry

One or four weeks after drug treatment, animals were anesthetized with 1 ml/kg rat cocktail (5% ketamine, 2% xylazine, 1% acepromazine) and perfused intracardially with fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.1% picric acid in 0.1 M HEPES pH 7.3). This process rapidly fixes the brain by cross-linking proteins and was necessary in order to ensure excellent preservation of the tissue. Additionally, since the primary antibodies were raised against glutaraldehyde-glutamate (or GABA) conjugates, a high glutaraldehyde content was required for optimal immunolabeling. Following perfusion, brains were removed and placed in cold fixative overnight (12-17 hrs). Tissue was prepared according to the procedure of Meshul *et al.* (1994). Brains were washed three times for 30 min each in HEPES buffer. Brain slices (400 mm) were cut using a vibratome. Pieces of tissue, approximately 1 mm square, from the DLCD, VLCD, and GP (Figure 3) were dissected and washed in cold HEPES 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 filtered water and placed into 0.5% aqueous uranyl acetate for 30 min at room temperature. Tissue was dehydrated through an increasing series of

Figure 3. Diagram illustrating the regions excised for immunocytochemical analysis. Tissue (approximately $1mm^2$) was taken from the DLCD (1), VLCD (2), and GP (3). Adapted from the atlas by Paxinos and Watson (1986), plates 12 (AP +1.60 mm from bregma), 15 (AP +0.70 mm from bregma), and 22 (AP -0.92 mm from bregma).


1
2
3
1mm

ethanols, cleared in propylene oxide, and polymerized in Embed 812/Spurr for no longer than 24 hrs at 60°C.

Post-embedding immuno-gold electron microscopy was performed 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 antibody solution (rabbit polyclonal glutamate Ab 1:250,000 with 1.0 mM aspartate, or rabbit polyclonal GABA Ab 1:7000) and incubated overnight in a moist chamber at room temperature. Both antibodies have been previously characterized (Hepler *et al.*, 1988). 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 1:50 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 Inc., Foster City CA). Pre-absorption controls demonstrating the specificity of immunolabeling using these antibodies have been previously described (Meshul *et al.*, 1994).

Ten photomicrographs (initial magnification X25,000), taken randomly throughout the neuropil were analyzed for each animal. GABA-immunolabeled presynaptic terminals contained round or pleomorphic vesicles and formed symmetrical synaptic contacts with dendritic shafts or

cell bodies (Smith and Bolam, 1990). Glutamate-immunolabeled boutons contained multiple small clear round vesicles and formed asymmetrical synaptic contacts. In the rat, glutamate afferents of corticostriatal origin synapse exclusively with dendritic spines, while those of thalamostriatal origin synapse upon dendritic shafts (Dubé *et al.*, 1988). Therefore, only terminals making an axospinous contact were analyzed. The number of gold particles per asymmetric terminal making contact with a dendritic spine (glutamate immunolabeling), or per symmetric terminal making contact with a dendritic shaft or cell body (GABA immunolabeling), were counted, and the area of these terminals was measured using ImagePro Plus analysis software (Media Cybernetics, Silver Spring MD). These antibodies are known to crossreact with Krebs cycle intermediates found within mitochondria (Phend *et al.*, 1992). Therefore, gold-labeling found within mitochondria in GABA- or glutamate-positive presynaptic terminals was analyzed separately as a measure of metabolic activity.

D. Analysis of Dopamine and DOPAC Levels

Animals were killed by decapitation one or four weeks after drug administration. Brains were prepared for analysis by high performance liquid chromatography (HPLC) using the method of Feller *et al.* (1993). Briefly, the lateral CD was dissected out (+1.70 to -0.40 mm from bregma) and divided into dorsal and ventral halves. Tissue was quick frozen in liquid nitrogen, and stored at -80°C until assayed. Brains were then thawed at 4°C and sonicated in 670 µl mobile phase with 30 ng dihydrobenzylamine (DHBA) as an internal standard. Mobile phase contained (per liter) 6.9 g sodium phosphate, pH = 3.7, 250 mg heptane sulfonic acid, 80 mg EDTA and 7%

MeOH v/v. Samples were rapidly frozen in methanol/dry ice, thawed on wet ice, and centrifuged at 48,000 x g at 4°C for 30 min The supernatant was stored at -80°C until needed.

Prior to HPLC analysis, the supernatant was thawed at 4°C and centrifuged at 12,000 x g for 15 min. Samples (10 or 20 µl of centifuged supernatant) were injected into a HP 1090 liquid chromatograph system (dual piston pump, 20µl injection loop) (Hewlett Packard, San Fernando CA). DA and DOPAC were separated on a catecholamine R-80 column (reverse phase-C-18, ESA Inc., Bedford MA) 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). DA and DOPAC content within each sample were expressed as a percent of the internal standard recovered. Catecholamine levels, measured as peak height determined by HPLC analysis, were used to calculate the amount in each sample (pg/ml) from a 6 point standard curve.

E. Drugs

Glutamate and GABA antibodies (raised in rabbit, conjugated to glutaraldehyde) were supplied by Arnel (Brooklyn, NY). 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.).

STATISTICAL ANALYSIS

A. Manipulation of Core Body Temperature

In order to examine the magnitude of the hyperthermic response across dose (4 injections or total effect) and treatment condition (SAL, METH, HOT, COLD), the area under the time versus body temperature curve (AUC) was determined for the 5 hr period following each injection of saline or MA. AUC was analyzed by mixed two-factor ANOVA with repeated injections as the within-subjects factor (Doses 1-4), and treatment condition (SAL, METH, HOT, COLD) as the between-subjects factor. The significance level was set at $\alpha = 0.05$. The total AUC summed across dose was analyzed by one-way ANOVA with treatment condition (SAL, METH, HOT, COLD) as the between-subjects factor. Significant main effects and interactions were further analyzed by Newman-Keul's test.

B. Ultrastructural Immunocytochemistry

Tissue from all four treatment groups taken from a given brain region and time point were processed together in each immunocytochemical run. Background labeling of postsynaptic processes varied across immuno-run, but not between groups within a given run (Table V). In order to compare data across runs, all data were analyzed as percent of control. This completely eliminated differences between runs, allowing for a more direct comparison between both time points and brain regions, and for easier interpretation of the data (Figure 4).

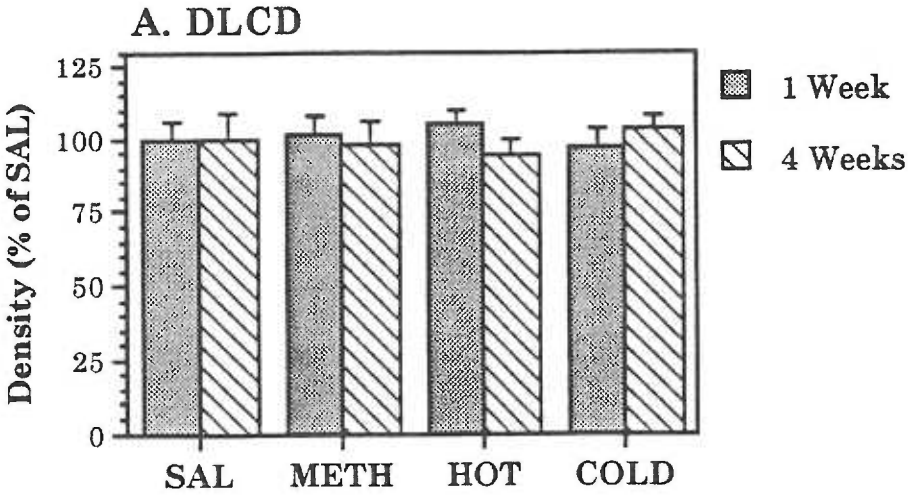
Three rats from the SAL group were discarded due to poor perfusions, leaving only 3-4 animals in the 1 week immunocytochemical study. In order to

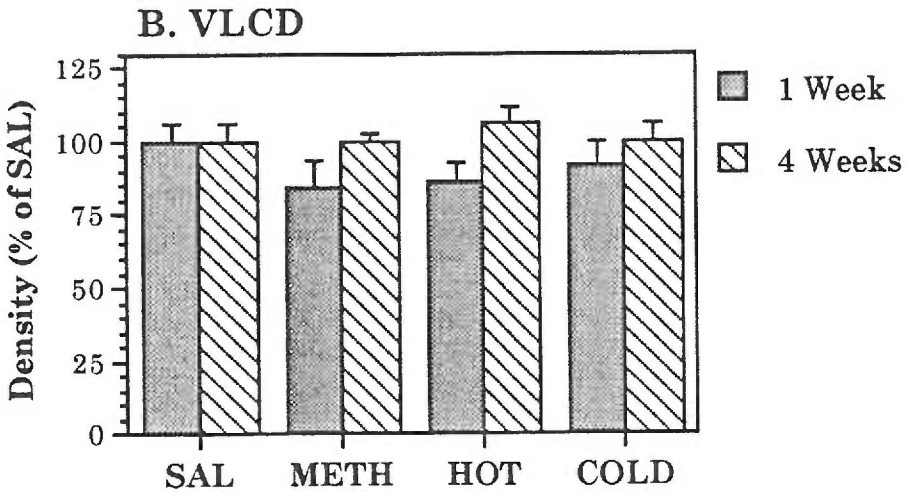
Table V. Background (Postsynaptic) GABA Immunolabeling

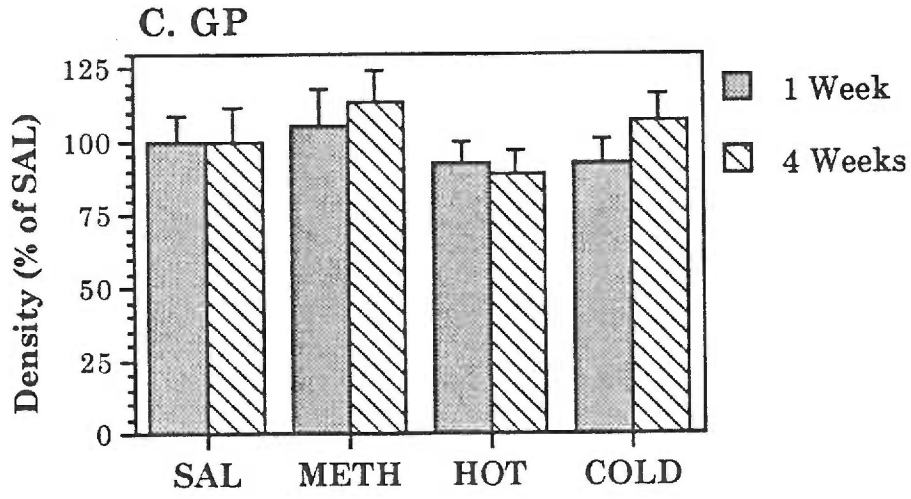
Region	Group	1 Week	4 Weeks
		RUN A	RUN B
DLCD	SAL	19.40 ± 1.23 (7)	27.92 ± 2.33 (7)
	METH	19.73 ± 1.23 (7)	27.39 ± 2.20 (11)
	HOT	20.47 ± 0.79 (6)	26.41 ± 1.36 (6)
	COLD	18.89 ± 1.18 (7)	28.90 ± 1.15 (7)
		RUN C	RUN D
VLCD	SAL	19.31 ± 1.11 (6)	26.80 ± 1.56 (7)
	METH	16.22 ± 1.80 (7)	26.67 ± 0.81 (11)
	HOT	16.58 ± 1.23 (6)	28.40 ± 1.37 (7)
	COLD	17.69 ± 1.65 (7)	26.84 ± 1.55 (7)
		RUN E	RUN F
GP	SAL	11.29 ± 1.24 (8)	19.79 ± 1.71 (7)
	METH	12.74 ± 1.23 (8)	20.77 ± 2.47 (10)
	HOT	10.07 ± 0.85 (6)	18.36 ± 1.34 (6)
	COLD	12.06 ± 1.07 (6)	18.26 ± 1.63 (6)

Background labeling for GABA (mean number gold particles per μm^2 ± SEM, n per group). Tissue from the four treatment groups within a given region and time point were processed together in an immunocytochemical run. No significant differences were found within a given immuno-run, but analysis by ANOVA revealed differences between runs.

Figure 4. Density of postsynaptic (Background) GABA immunolabeling, expressed as % of the SAL control group (Mean ± SEM, n = 6-11 animals per group), in DLCD (A), VLCD (B), and GP (C). Labeling was analyzed 1 week (closed bars) or 4 weeks (striped bars) following treatment. No significant differences were found.


A. DLCD
Density (% of SAL)
125
100
75
50
25
0
SAL
METH
HOT
COLD
1 Week
4 Weeks


B. VLCD
Density (% of SAL)
125
100
75
50
25
0
SAL
METH
HOT
COLD
1 Week
4 Weeks


C. GP
Density (% of SAL)
125
100
75
50
25
0
SAL
METH
HOT
COLD
1 Week
4 Weeks

determine if 1 and 4 week SAL groups could be pooled for statistical analysis, tissue from 4 week SAL treated animals were processed alongside tissue from the 1 week time point. Analysis by Student's t-test revealed that there were no differences in immunolabeling between 1 and 4 weeks SAL groups in any of the immuno-runs (Table VI), demonstrating that immunolabeling in control animals was stable across time. Therefore, in order to increase the number of control animals (SAL group) being compared with the other three treatment groups, immunocytochemical data from control animals processed in the same immuno-run were collapsed across time.

Particle density within presynaptic terminals or mitochondria, percent of synapses with perforated postsynaptic densities (glutamate only), and terminal area were analyzed by two-way ANOVA with treatment group (SAL, METH, HOT, COLD) and time (1 or 4 weeks) as between-subjects factors. The significance level was set at $\alpha = 0.05$. Simple main effects were analyzed for significant interactions. Significant main effects were further characterized Newman-Keul's test.

C. Analysis of Dopamine and DOPAC Levels

DA, DOPAC, and the ratio of DOPAC/DA within the DLCD and VLCD were analyzed by two-way ANOVA with treatment group (SAL, METH, HOT, COLD) and time (1 or 4 weeks) as between-subjects factors. The significance level was set at $\alpha = 0.05$. Simple main effects were analyzed for significant interactions. Significant main effects were further characterized Newman-Keul's test.

Table VI. Presynaptic GABA and Glutamate Immunolabeling in the SAL Group

Region	Group	GABA	Glutamate
		RUN A	**RUN B**
DLCD	1 Week	72.81 ± 2.94 (4)	65.99 ± 5.51 (4)
	4 Weeks	73.59 ± 10.77 (3)	64.29 ± 5.60 (3)
	Combined	73.14 ± 4.36 (7)	65.26 ± 3.64 (7)
		RUN C	**RUN D**
VLCD	1 Week	64.42 ± 4.42 (3)	77.6 ± 6.36 (4)
	4 Weeks	62.84 ± 1.25 (3)	85.95 ± 6.85 (2)
	Combined	63.63 ± 2.08 (6)	80.39 ± 4.74 (6)
		RUN E	
GP	1 Week	47.22 ± 3.75 (4)	
	4 Weeks	51.37 ± 3.89 (4)	
	Combined	49.29 ± 2.62 (8)	

Particle density (mean number/μm^2 ± SEM, n per group) in presynaptic terminals analyzed for GABA or glutamate immuno-reactivity. Tissue from the 4 treatment groups within a given region and time point were processed together in an immuno-cytochemical run. No significant differences were found, within a given immuno-run, between SAL treated rats killed 1 or 4 weeks after treatment (Data analyzed by Student's t-test).

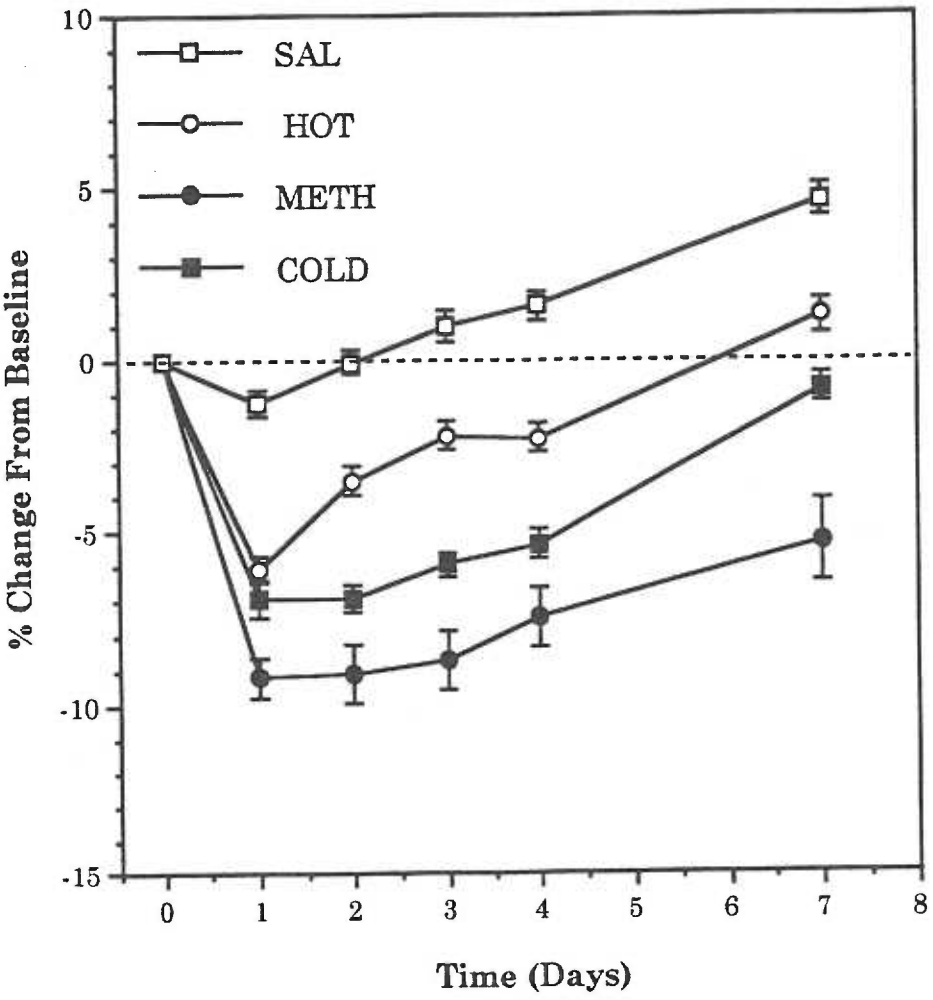
RESULTS

Preliminary evidence suggested that rats in the METH group which did not become severely hyperthermic had less severe DA depletions and no alterations in GABA immunolabeling (Appendix A). Therefore, rats in the METH group whose core body temperature did not exceed 41.0°C (9/63) were excluded from the study. Of the remaining animals in the METH treatment group, over half (25/45) became severely lean and dehydrated (decrease in body weight ranged from 2.67% to 23.75%), requiring post-experimental care (fluids and calories). It was noted that rats which received MA (METH and COLD groups) remained stereotypic throughout the experiment (i.e., at least 23 hrs). Rats in all 3 experimental groups lost weight compared to the SAL treatment group, although weight loss was greatest in the METH subjects (9.2% decrease at 24 hrs post MA), followed by the COLD group (7.0% decrease at 24 hrs post MA) (Figure 5). The mortality rate of the METH group was 13% (9/63 animals died within 24 hrs of MA treatment), while none of the animals in the HOT, COLD, or SAL groups died. Eight additional animals (1 COLD, 2 HOT, 3 SAL and 2 METH) were discarded due to poor tissue preservation.

A. Manipulation of Core Body Temperature

In order to separate the effects of drug alone from the effects of prolonged hyperpyrexia, some animals were exposed to hyperthermia in the absence of drug (HOT group) while others received MA but were kept cool (COLD group). Rats in the METH group developed severe hyperthermia, with body temperature peaking approximately 1-1.5 hrs following each injection of

Figure 5. Change in body weight (% change from baseline) in the first week following MA or saline treatment (Mean ± SEM, n = 21-45 animals per group). Note that animals in all treatment groups (METH, COLD, HOT) lost weight, but rats in the METH group had the greatest decrease in body weight.


SAL
HOT
METH
COLD
10
5
0
-5
-10
-15
0
1
2
3
4
5
6
7
8
% Change From Baseline
Time (Days)

15 mg/kg MA (Figure 6). Rats given repeated MA but kept cool (COLD group) had a body temperature profile similar to SAL treated rats (peak temperature 38.4 ± 0.05°C and 38.7 ± 0.06°C, respectively). Rats in the HOT group were given saline and placed on a heating pad to match the level of hyperthermia experienced by the METH group (peak temperature 41.7 ± 0.08°C and 41.8 ± 0.05°C, respectively). The magnitude of the hyperthermic response (AUC) in the METH and HOT groups were similar, and both were significantly greater that the hyperthermic response found in the SAL and COLD groups ($p < 0.001$) (Figure 7). In addition, there were no significant differences in the number of times that the body temperature of rats in the HOT and METH groups exceeded 41°C (3.3 times vs. 2.9 times; Student's *t*-test $t = 1.41$, $df = 72$, $p > 0.1$). Mixed two-factor ANOVA of the AUC following each dose revealed significant main effects for both treatment group and injection number, as well as a significant interaction between the two, indicating that the hyperthermic response varied across both dose and treatment group (Treatment group: $F_{3,120} = 479.6$, $p < 0.0001$; Dose number: $F_{3,360} = 14.5$, $p < 0.0001$; Interaction: $F_{9,360} = 7.721$, $p < 0.0001$). Analysis of the total AUC, summed across all 4 doses, by ANOVA also revealed a significant effect of treatment group ($F_{3,120} = 479.631$, $p < 0.0001$). There were no differences in the hyperthermic profiles of rats processed for analysis of DA content vs. immunocytochemistry (data not shown).

B. Ultrastructural Immunocytochemistry: GABA

Approximately 3000 nerve terminals (DLCD, $n \approx 700$; VLCD, $n \approx 700$; GP, $n \approx 1600$) from photomicrographs (1730) were examined for changes in

Figure 6. Core body temperature (Mean ± SEM, n = 21-45 animals per group) of rats receiving repeated injections (arrows) of 15 mg/kg MA (closed symbols) or saline (open symbols). Note that rats given MA but kept cool (COLD group) had a temperature response profile similar to vehicle treated rats (SAL group). Rats in the HOT group were given saline and placed on a heating pad to match the hyperthermic response of MA treated rats allowed to become hyperthermic (METH group).

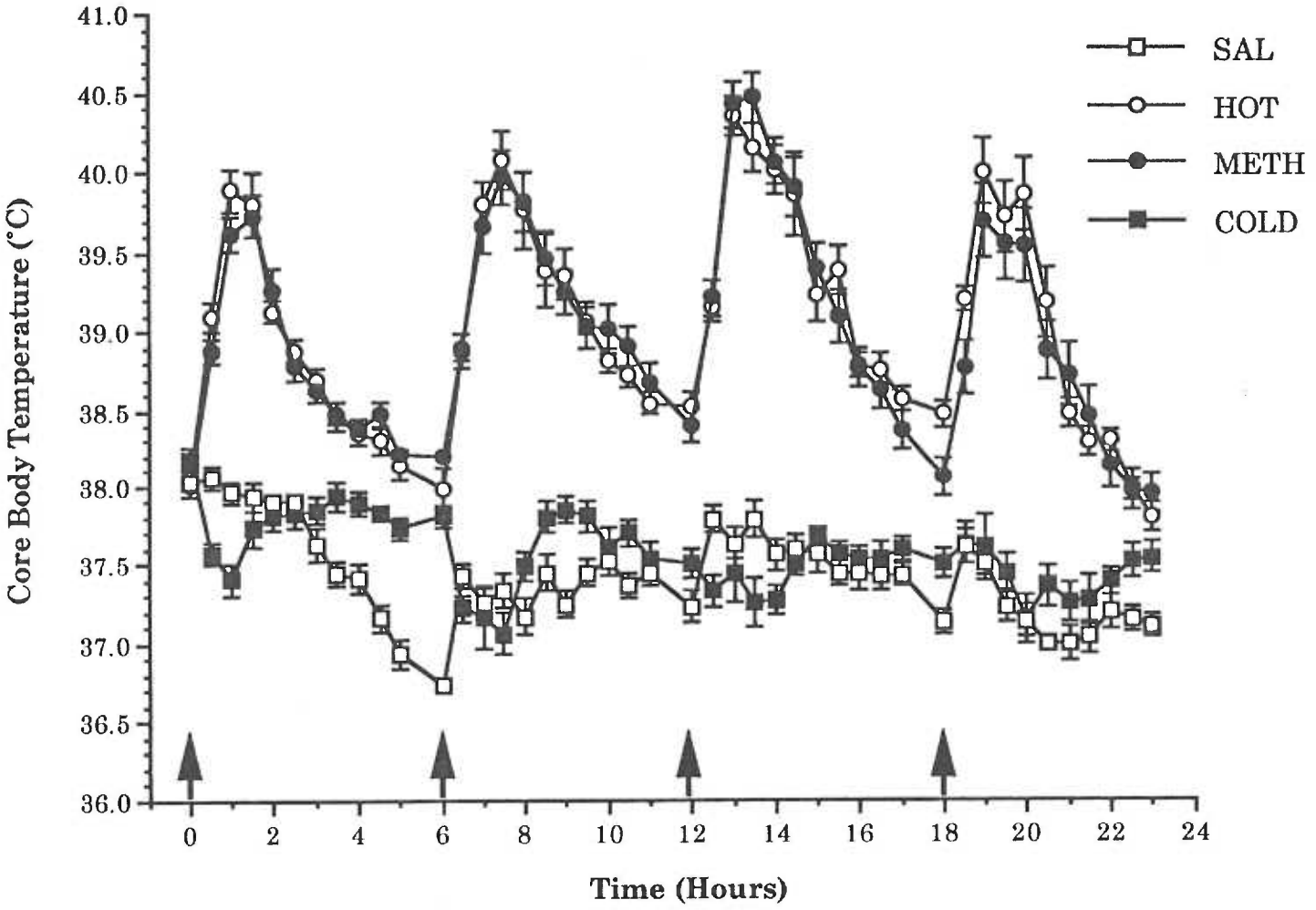
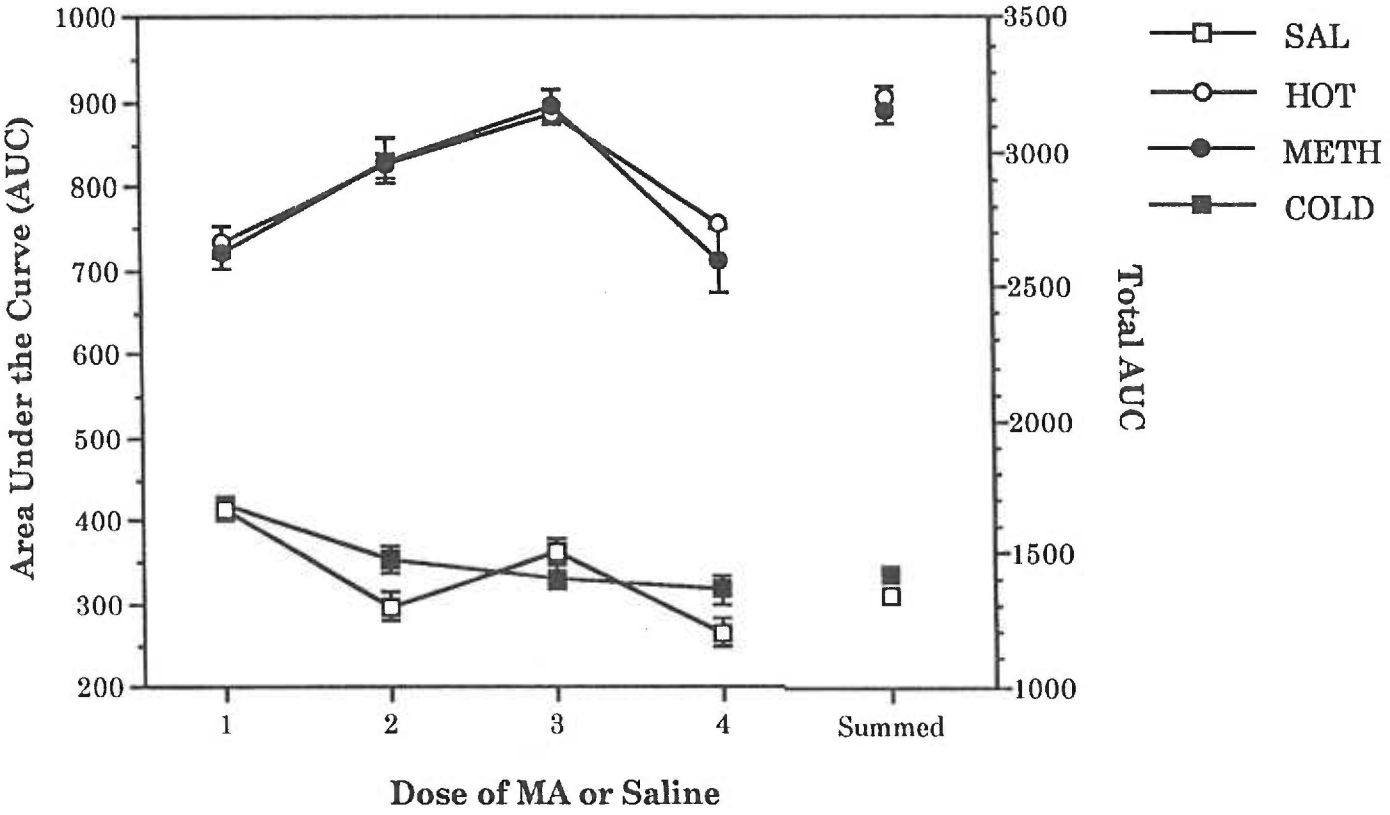

41.0
40.5
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
Core Body Temperature (°C)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (Hours)
SAL
HOT
METH
COLD

Figure 7. Area under the temperature vs. time curve (AUC) following MA or saline treatment (Mean ± SEM, n = 21-45 animals per group). Data are given for the AUC following each individual dose (left axis) or combined across dose (right axis). Note that AUC is not different between the METH and HOT groups, or between the COLD and SAL groups.

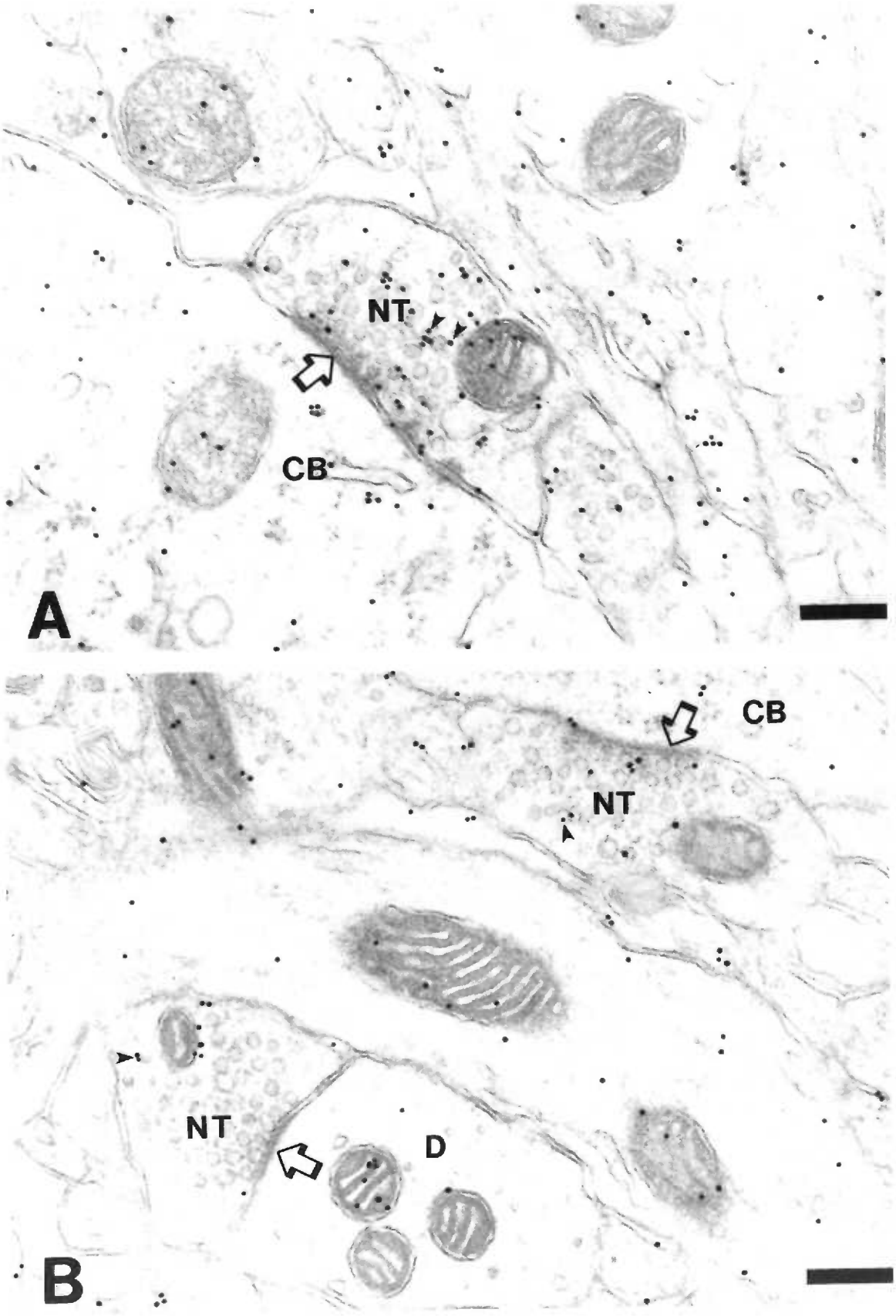

Area Under the Curve (AUC)
1000
900
800
700
600
500
400
300
200
3500
3000
2500
2000
1500
1000
Total AUC
1
2
3
4
Summed
Dose of MA or Saline
SAL
HOT
METH
COLD

GABA immunoreactivity. All of these terminals formed symmetrical synaptic contact with dendritic shafts or cell bodies. Roughly 12 terminals from each striatal region, and 28 terminals from the GP, were photographed and analyzed per animal. GABA labeled synapses in the VLCD and GP are shown in Figures 8 and 9.

Severe DA loss induced by 6-OHDA lesions increases the content and synthesis of GABA in striatopallidal neurons. Therefore, it was predicted that MA mediated DA loss would result in a long-lasting increase in presynaptic GABA immunoreactivity. Within the METH group, there were significant differences between the 1 and 4 week time points in all 3 brain regions ($p < 0.01$). An initial decrease in labeling (35-45%) was found in all 3 brain regions ($p < 0.01$) 1 week following MA treatment. GABA immunolabeling within the DLCD of the METH group had returned to basal levels 4 weeks after drug treatment, but was increased by 30-40% in both the VLCD ($p < 0.01$) and GP ($p < 0.01$) (Figure 10). Presynaptic GABA immunolabeling in the HOT and COLD groups did not differ from the SAL control group. Analysis of presynaptic GABA immunolabeling by two-way ANOVA revealed a significant interaction between treatment group and time following MA administration in all three brain regions (VLCD: $F_{3,50} = 15.3$, $p < 0.0001$; DLCD: $F_{3,50} = 4.0$, $p < 0.05$; GP: $F_{3,49} = 17.5$, $p < 0.0001$). ANOVA also revealed a significant main effect for group, indicating that GABA immunoreactivity varied across treatment group (VLCD: $F_{1,50} = 9.2$, $p < 0.005$; DLCD: $F_{1,50} = 23.2$, $p < 0.0001$; GP: $F_{1,49} = 21.8$, $p < 0.0001$).

Increases in the size of nerve terminals may be associated with increased synaptic activity. Analysis of the size of GABA-positive boutons by two-way ANOVA revealed significant a interaction between treatment group

Figure 8. Electron micrograph (initial magnification: X25,000) of GABA immunolabeled synapses in the VLCD from a rat 1 week post-saline (SAL group) **(A)**, 1 week post-MA (METH group) **(B)**, 4 weeks post-saline (SAL group) (C), or 4 weeks post-MA (METH group) **(D)**. Note the concentration of immuno-gold labeling in the presynaptic symmetrical terminal (arrow head = gold particle, NT = nerve terminal, double arrow = symmetrical contact).


NT
CB
A
CB
NT
NT
D
B

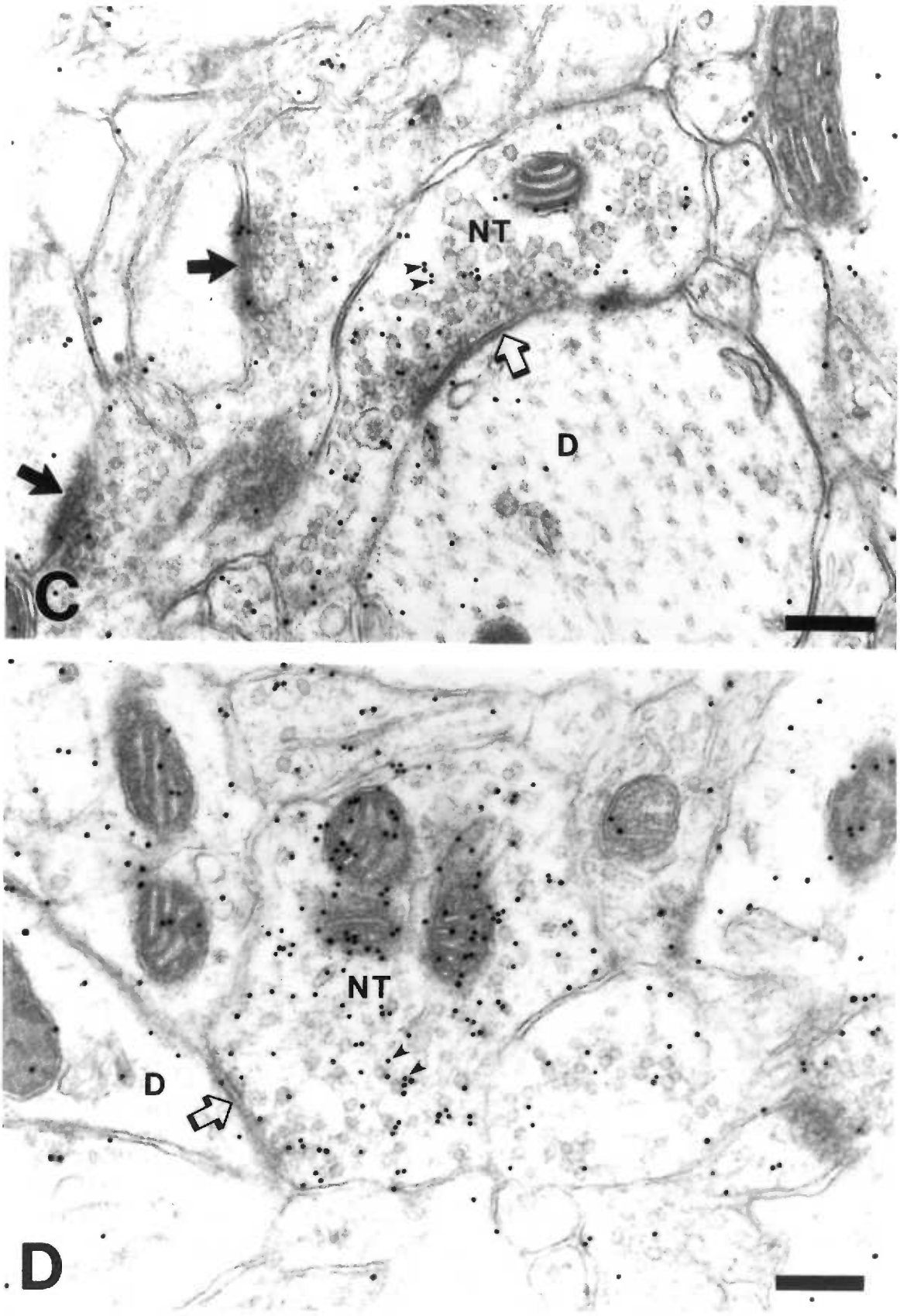

NT
D
C
NT
D
D

Figure 9. Electron micrograph (initial magnification: X25,000) of GABA immunolabeled synapses in the GP from a rat 1 week post-saline (SAL group) **(A)**, 1 week post-MA (METH group) **(B)**, 4 weeks post-saline (SAL group) **(C)**, or 4 weeks post-MA (METH group) **(D)**. Note the concentration of immuno-gold labeling in the presynaptic symmetrical terminal (arrow head = gold particle, NT = nerve terminal, double arrow = symmetrical contact).

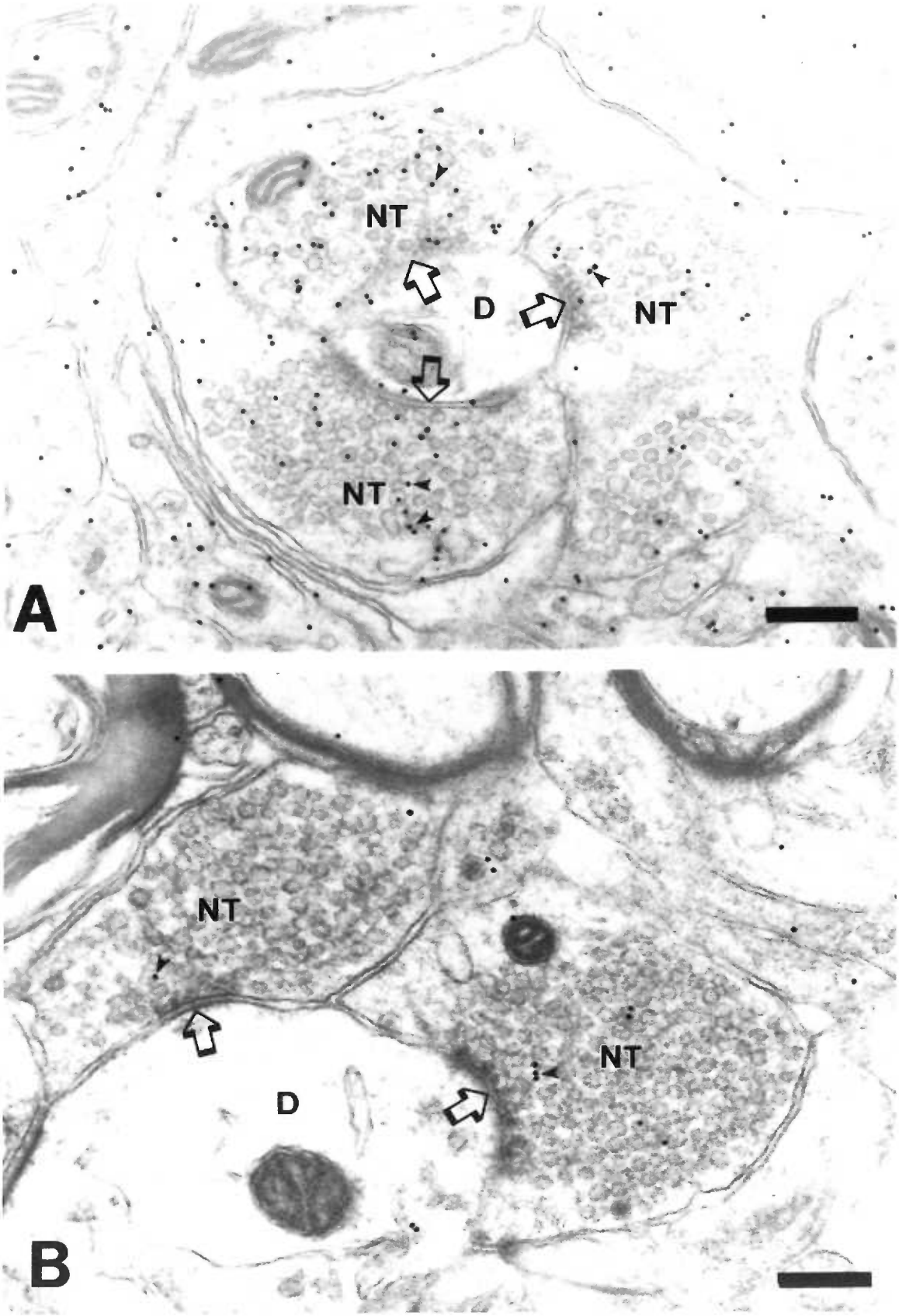
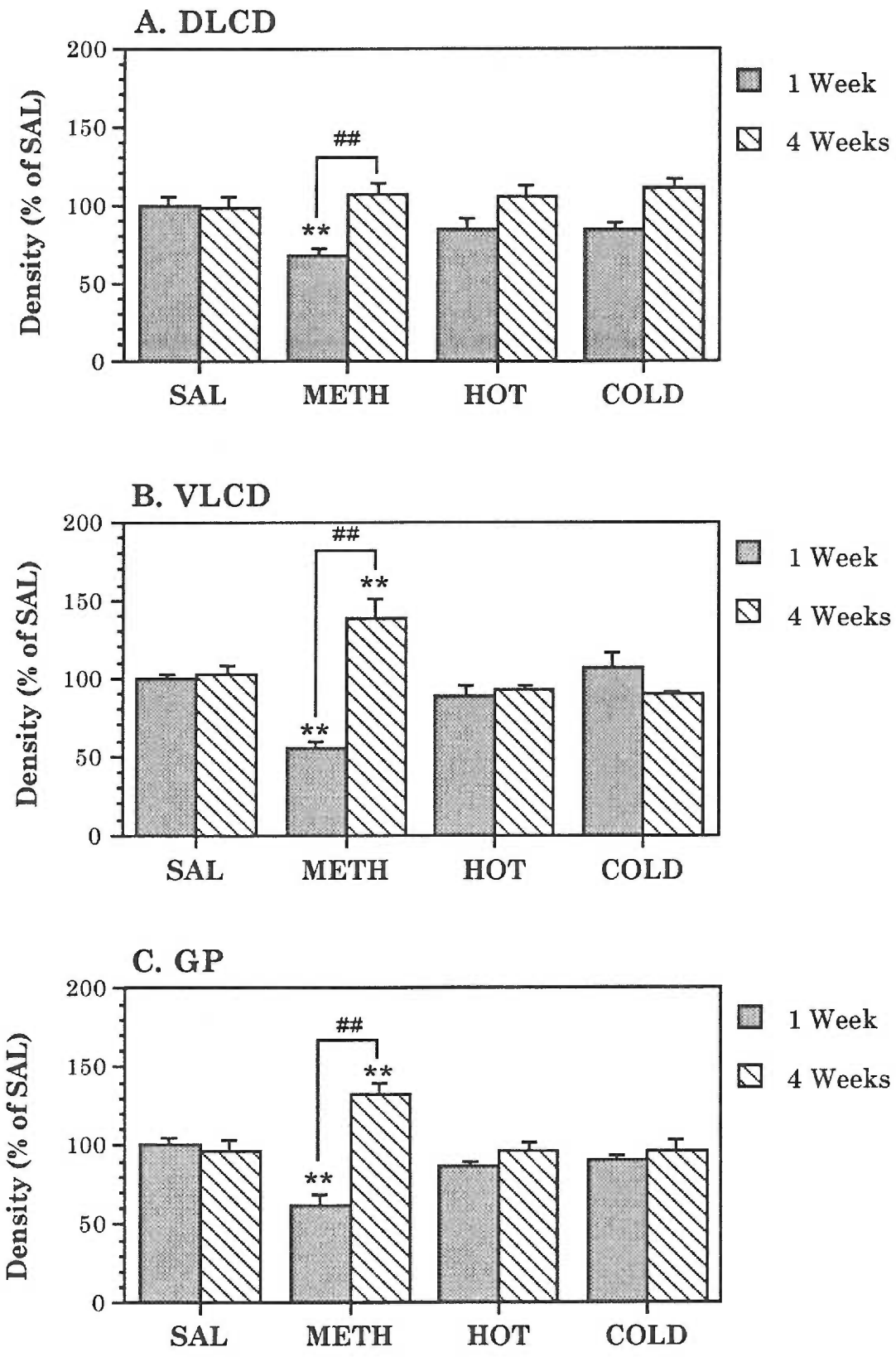

NT
D
NT
NT
A
NT
NT
D
B

Figure 10. Density of presynaptic GABA immunolabeling, expressed as % of the SAL control group (Mean ± SEM, n = 6-11 animals per group), in the DLCD **(A)**, VLCD **(B)**, and GP **(C)**. Labeling was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test, while interactions were characterized by simple main effects (** = $p < 0.01$ vs. SAL, ## = $p < 0.01$ One Week vs. Four Weeks). Data are also presented in Table IX (Appendix B).


A. DLCD
Density (% of SAL)
200
150
100
50
0
##
**
SAL
METH
HOT
COLD
1 Week
4 Weeks
B. VLCD
Density (% of SAL)
200
150
100
50
0
##
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks
C. GP
Density (% of SAL)
200
150
100
50
0
##
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks

and time following MA administration in the VLCD, and a trend in the GP (VLCD: $F_{3,50} = 3.8$, $p < 0.05$; GP: $F_{3,49} = 2.7$, $p = 0.058$). There was also a significant main effect for treatment group in the GP, although the source for this effect could not be found ($F_{3,49} = 3.0$, $p < 0.05$). Terminal size in the METH group (Figure 11) was significantly increased by 18% in the VLCD ($p < 0.01$) compared to the SAL group. In addition, there was a trend towards an increase in the GP ($p = 0.065$) of rats in the METH group. These changes were seen only 4 weeks following MA administration. The size of GABA-positive terminals did not differ from control levels in the HOT or COLD groups.

Since the GABA antibody is known to crossreact with Krebs cycle intermediates (Phend *et al.*, 1992), labeling within mitochondria associated with GABA-positive boutons was analyzed separately as a measure of metabolic activity. GABA immunolabeling was decreased in the mitochondria of both the METH ($p < 0.05$) and HOT ($p < 0.05$) groups by 34% (Figure 12). These changes were found only within the VLCD 1 week after either treatment (main effect of treatment group: $F_{3,50} = 3.5$, $p < 0.05$; group by time interaction: $F_{3,50} = 4.4$, $p < 0.01$).

C. Ultrastructural Immunocytochemistry: Glutamate

Electron photomicrographs of nerve terminals were analyzed for changes in glutamate immunoreactivity (1160 micrographs, DLCD, $n \approx 1500$ boutons; VLCD, $n \approx 1400$ boutons). All of these terminals formed asymmetrical synapses with dendritic spines, indicating that they were of corticostriatal origin (Dubé *et al.*, 1988). An average of 25 terminals from each striatal region were photographed and analyzed per animal. An example of glutamate immunolabeled synapses is shown in Figure 13. MA-induced

Figure 11. Area of GABA immuno-positive nerve terminals, (Mean ± SEM, n = 6-11 animals per group), in the in the DLCD **(A)**, VLCD **(B)**, and GP **(C)**. Area was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test, while interactions were characterized by simple main effects (* = $p < 0.05$ vs. SAL, # = $p < 0.05$ One Week vs. Four Weeks). Data are also presented in Table X (Appendix B).

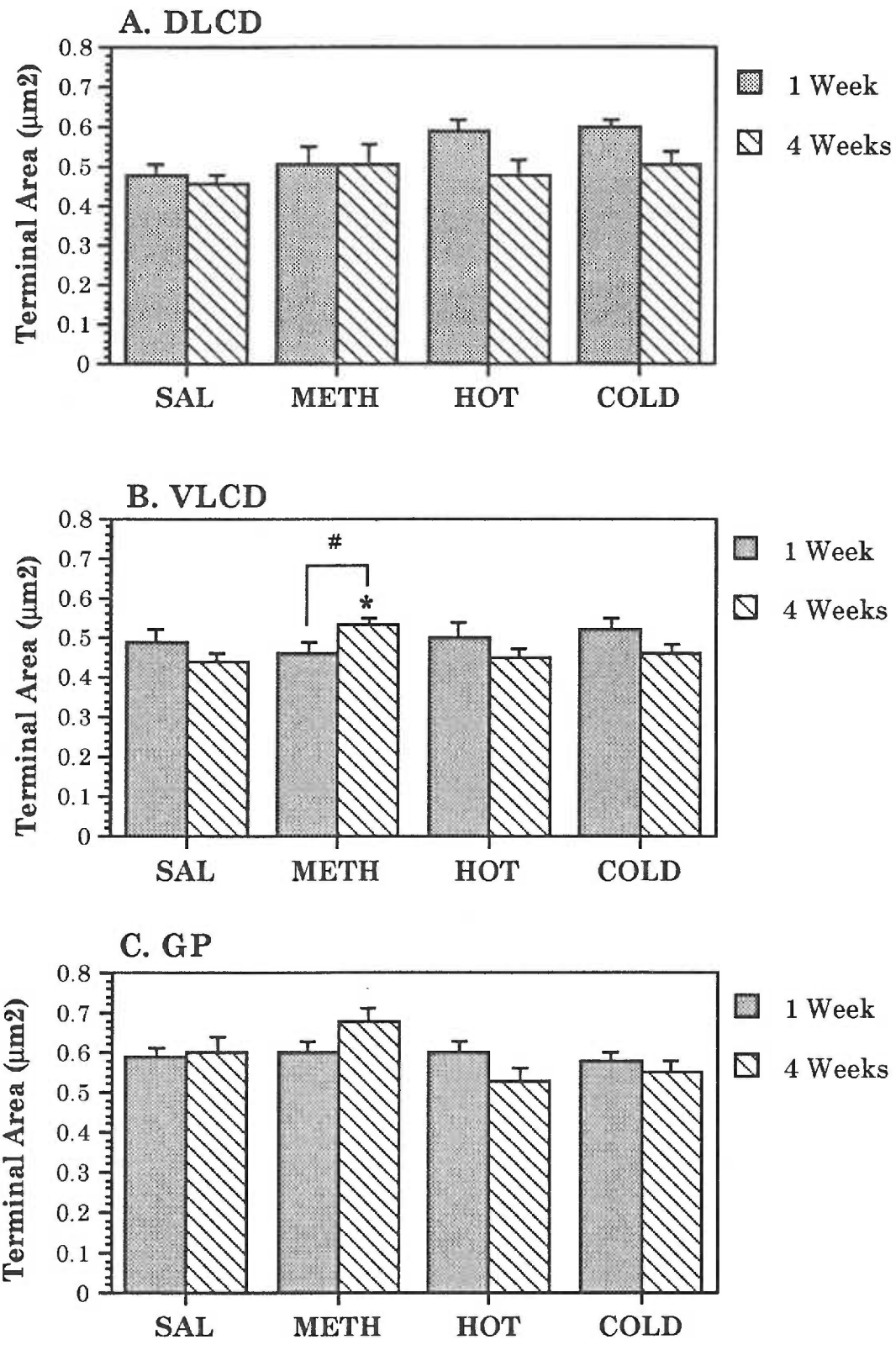

A. DLCD
Terminal Area (µm2)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
SAL
METH
HOT
COLD
1 Week
4 Weeks
B. VLCD
Terminal Area (µm2)
#
*
SAL
METH
HOT
COLD
1 Week
4 Weeks
C. GP
Terminal Area (µm2)
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 12. Density of GABA immunolabeling in mitochondria associated with immunolabeled nerve terminals, expressed as % of the SAL control group (Mean ± SEM, n = 6-11 animals per group), in the DLCD (A), VLCD (B), and GP (C). Labeling was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test, while interactions were characterized by simple main effects (* = $p < 0.05$ vs. SAL, # = $p < 0.05$ One Week vs. Four Weeks). Data are also presented in Table XI (Appendix B).

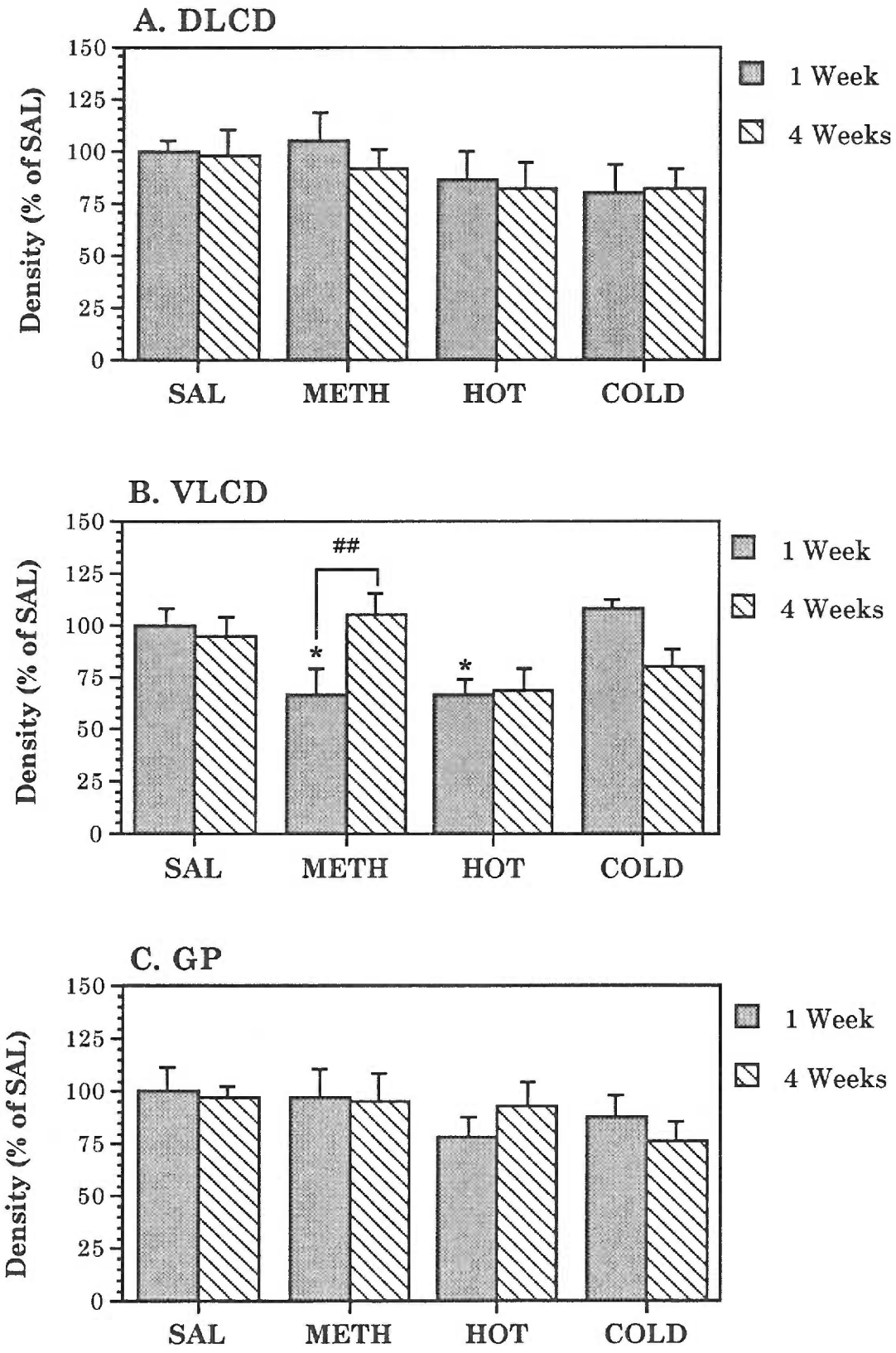
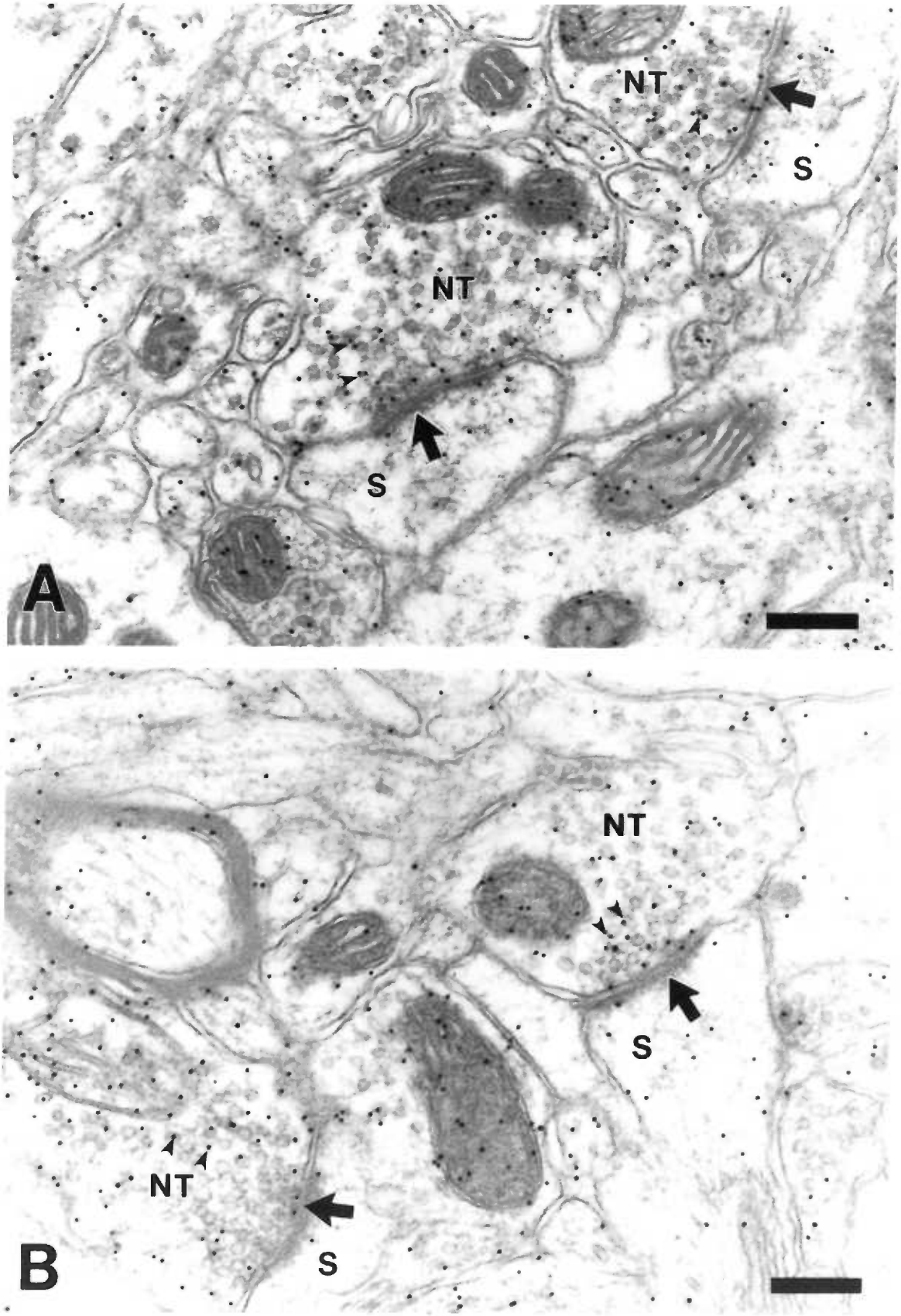

A. DLCD
Density (% of SAL)
150
125
100
75
50
25
0
SAL
METH
HOT
COLD
1 Week
4 Weeks
B. VLCD
Density (% of SAL)
##
*
*
SAL
METH
HOT
COLD
1 Week
4 Weeks
C. GP
Density (% of SAL)
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 13. Electron micrograph (initial magnification: X25,000) of glutamate immunolabeled synapses in the VLCD from a rat 1 week post-saline (SAL group) **(A)** and 1 week post-MA (METH group) **(B)**. Note the concentration of immuno-gold labeling in the presynaptic asymmetrical terminal (arrow head = gold particle, SP = spine, NT = nerve terminal, arrow = asymmetrical contact, Asterisk = denotes a perforated postsynaptic density).


NT
S
NT
S
A
NT
S
NT
S
B

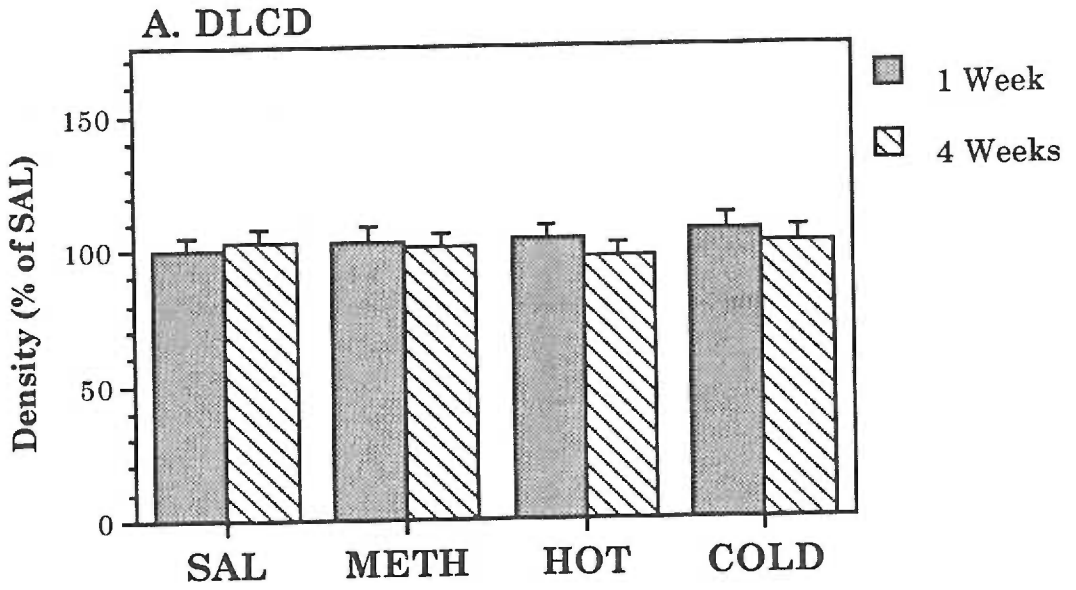
DA loss was predicted to increase corticostriatal activity, resulting in a decrease in presynaptic glutamate immunoreactivity. However, no differences in the density of presynaptic glutamate terminal immunoreactivity (Figure 14), mitochondrial labeling (Figure 15), or terminal area (Figure 16) were found to occur across either time or treatment group.

In both the DLCD and VLCD, the METH group had a 50-60% increase ($p < 0.05$) in the percent of synapses associated with perforated postsynaptic densities 4 weeks after MA treatment (Figure 17). Changes in the percent of synapses with perforated postsynaptic densities are believed to reflect increases in synaptic activity. These effects were not the result of a change in the total number of synapses analyzed (DLCD, $p = 0.31$; VLCD, $p = 0.67$). Analysis by two-way ANOVA revealed that increases in the percent of perforated synapses varied across time within treatment groups in both the VLCD ($F_{3,49} = 4.1$, $p < 0.05$), and DLCD ($F_{3,52} = 4.3$, $p < 0.01$). In addition, a significant main effect for treatment group was found in both regions (DLCD, $F_{3,52} = 5.4$, $p < 0.01$; VLCD, $F_{3,49} = 4.2$, $p < 0.05$). To determine if changes in glutamate immunolabeling occurred in a subset of synapse type, terminal area and particle density were analyzed separately for synapses with perforated or non-perforated postsynaptic densities (Table VII). Although perforated synapses were generally larger than non-perforated synapses, no differences in terminal labeling or area were found in either brain region examined.

D. Analysis of Dopamine and DOPAC Levels

Analysis by two-way ANOVA revealed significant main effects for treatment group in both striatal subregions, demonstrating that MA

Figure 14. Density of presynaptic glutamate immunolabeling, expressed as a percent of the SAL group (Mean ± SEM, n = 6-11 animals per group), in the DLCD (A), and VLCD (B). Labeling was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. No significant differences were found. Data are also presented in Table X (Appendix B).


A. DLCD
Density (% of SAL)
150
100
50
0
SAL
METH
HOT
COLD
1 Week
4 Weeks

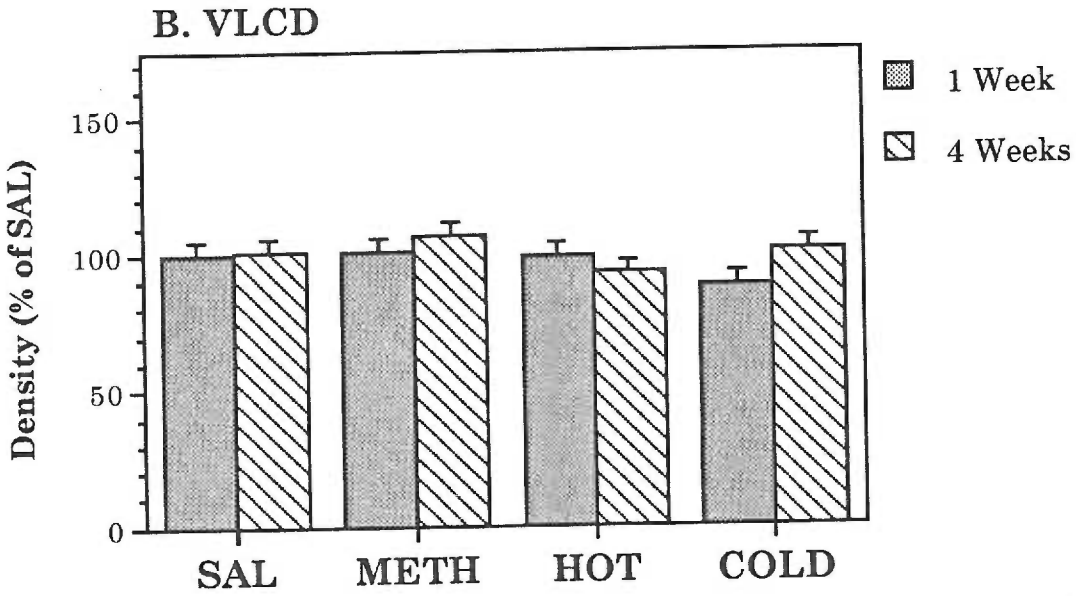
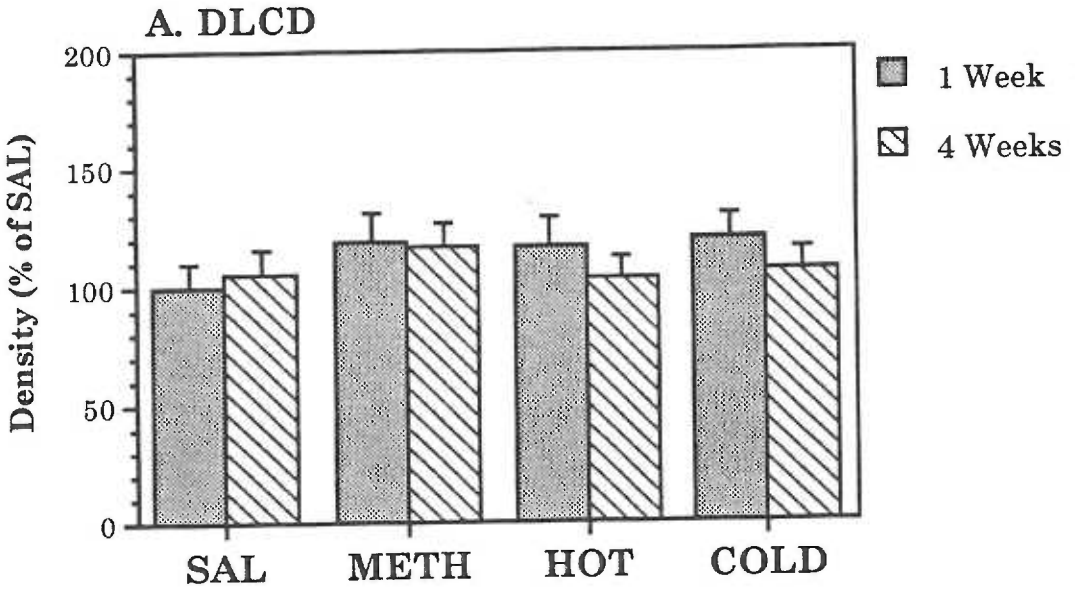

B. VLCD
Density (% of SAL)
150
100
50
0
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 15. Density of glutamate immunolabeling in mitochondria associated with immunolabeled nerve terminals, expressed as a percent of the SAL group (Mean ± SEM, n = 6-11 animals per group), in the DLCD (A), and VLCD (B). Labeling was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. No significant differences were found. Data are also presented in Table XI (Appendix B).


A. DLCD
Density (% of SAL)
200
150
100
50
0
SAL
METH
HOT
COLD
1 Week
4 Weeks

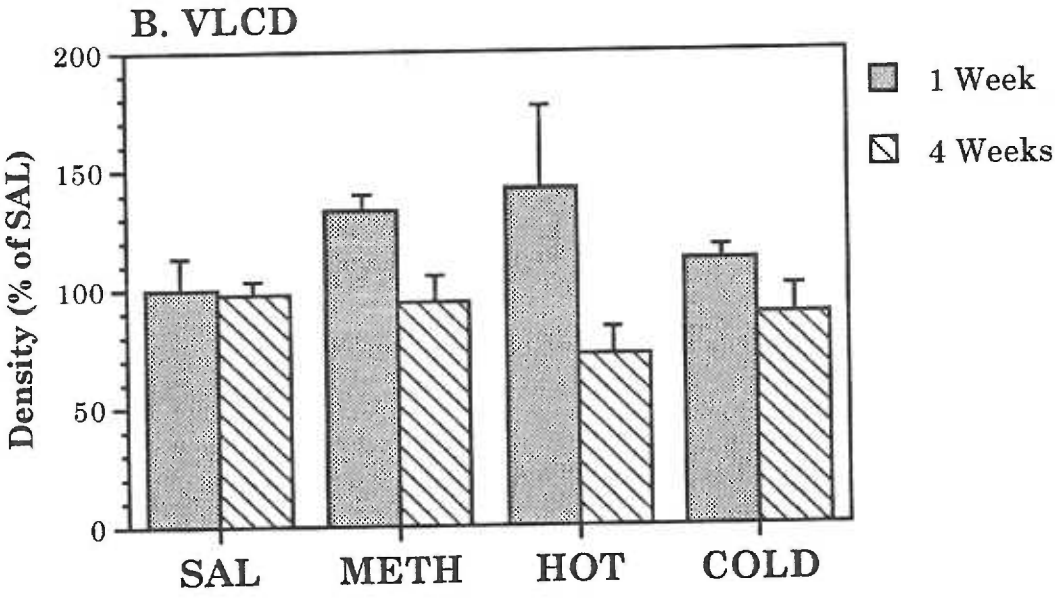

B. VLCD
Density (% of SAL)
200
150
100
50
0
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 16. Area of glutamate immuno-positive nerve terminals, (Mean ± SEM, n = 6-11 animals per group), in the DLCD **(A)**, and VLCD **(B)**. Area was measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. No significant differences were found. Data are also presented in Table X (Appendix B).

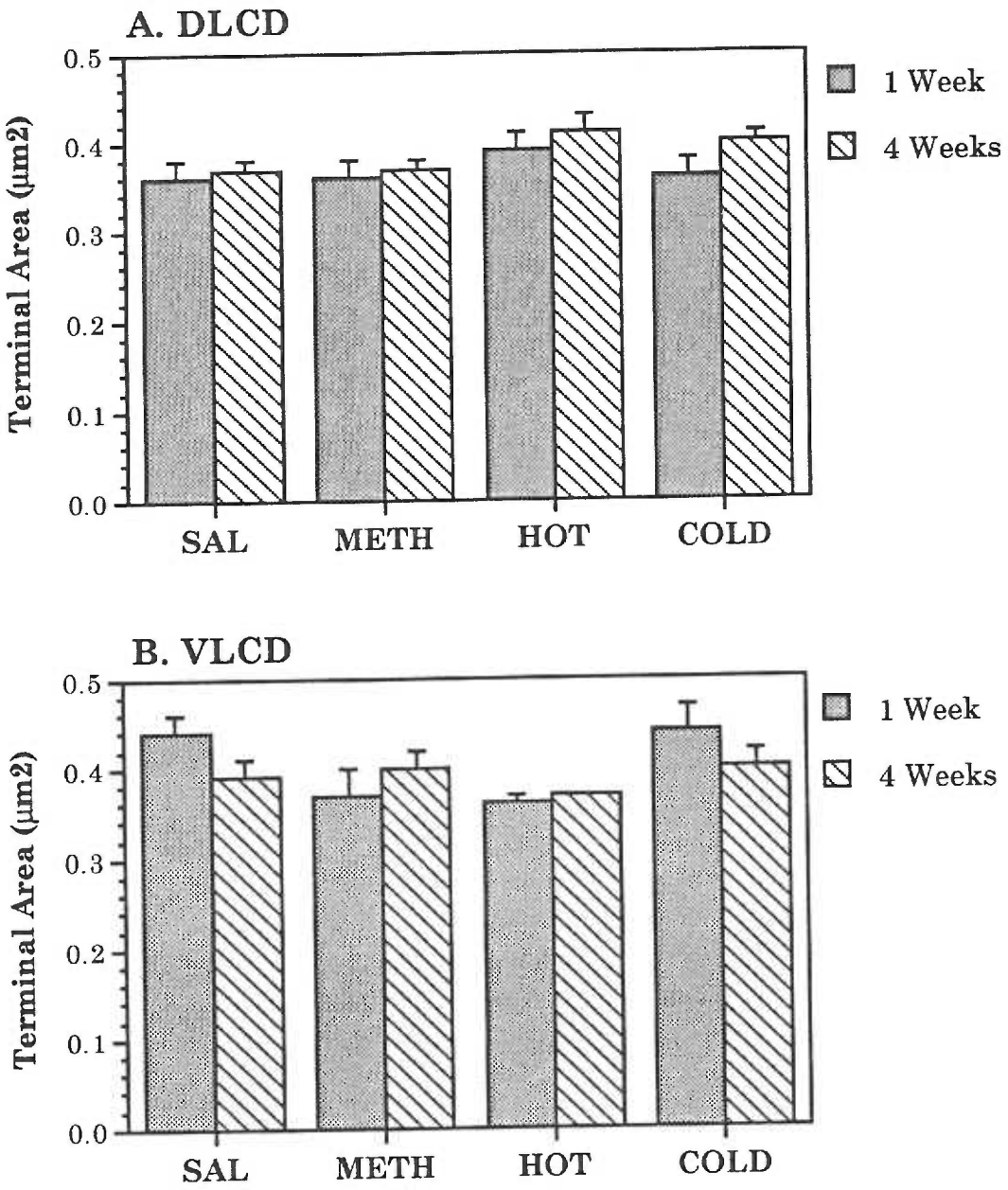
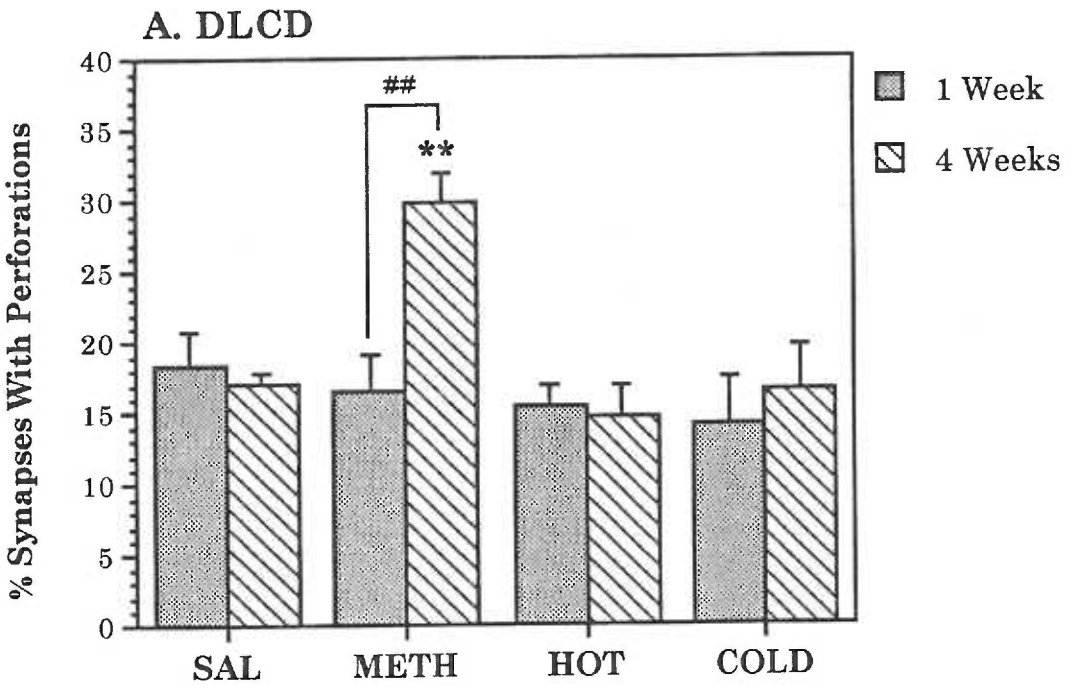

A. DLCD
Terminal Area (µm2)
0.5
0.4
0.3
0.2
0.1
0.0
SAL
METH
HOT
COLD
1 Week
4 Weeks
B. VLCD
Terminal Area (µm2)
0.5
0.4
0.3
0.2
0.1
0.0
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 17. The percent of glutamate-positive synapses containing a perforated postsynaptic density (Mean ± SEM, n = 6-11 animals per group), in the DLCD **(A)**, and VLCD **(B)**. Synapses with perforated postsynaptic densities were measured in tissue taken from rats killed 1 week (closed bars) or 4 weeks (striped bars) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test while significant interactions were characterized by simple main effects (* = $p < 0.05$ vs. SAL; ** = $p < 0.01$ vs. SAL; ## = $p < 0.01$ One vs. Four Weeks).


A. DLCD
% Synapses With Perforations
40
35
30
25
20
15
10
5
0
##
**
1 Week
4 Weeks
SAL
METH
HOT
COLD

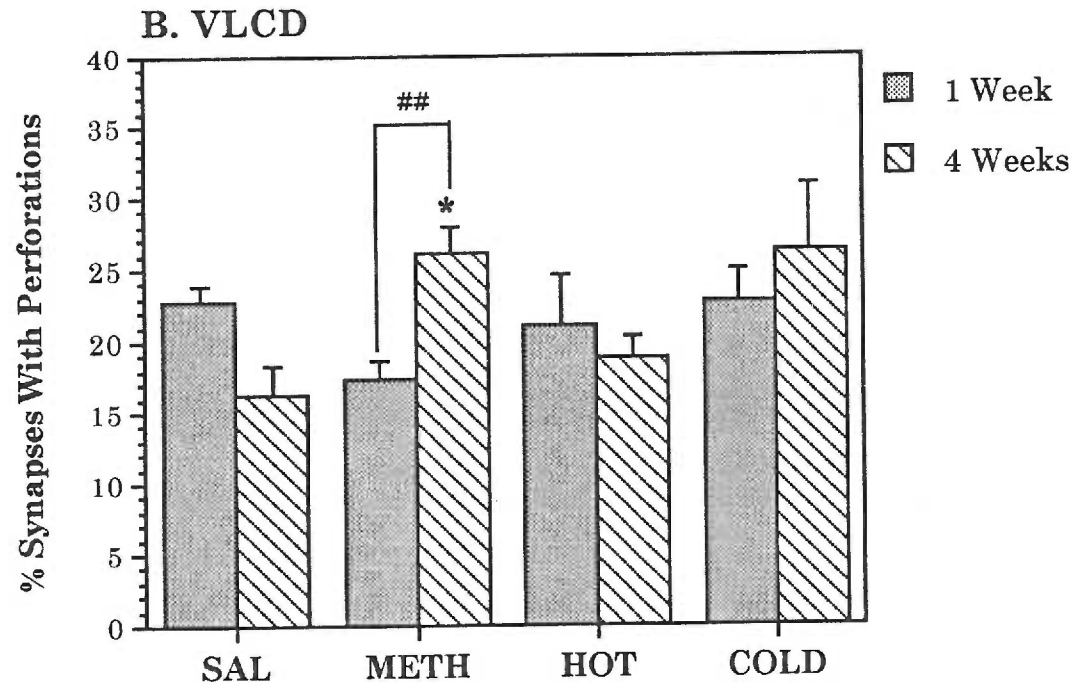

B. VLCD
% Synapses With Perforations
40
35
30
25
20
15
10
5
0
##
*
1 Week
4 Weeks
SAL
METH
HOT
COLD

Table VII. *Terminal Size and Immunoreactivity in Glutamate-positive Synapses with Perforated or Non-Perforated Post Synaptic Densities*

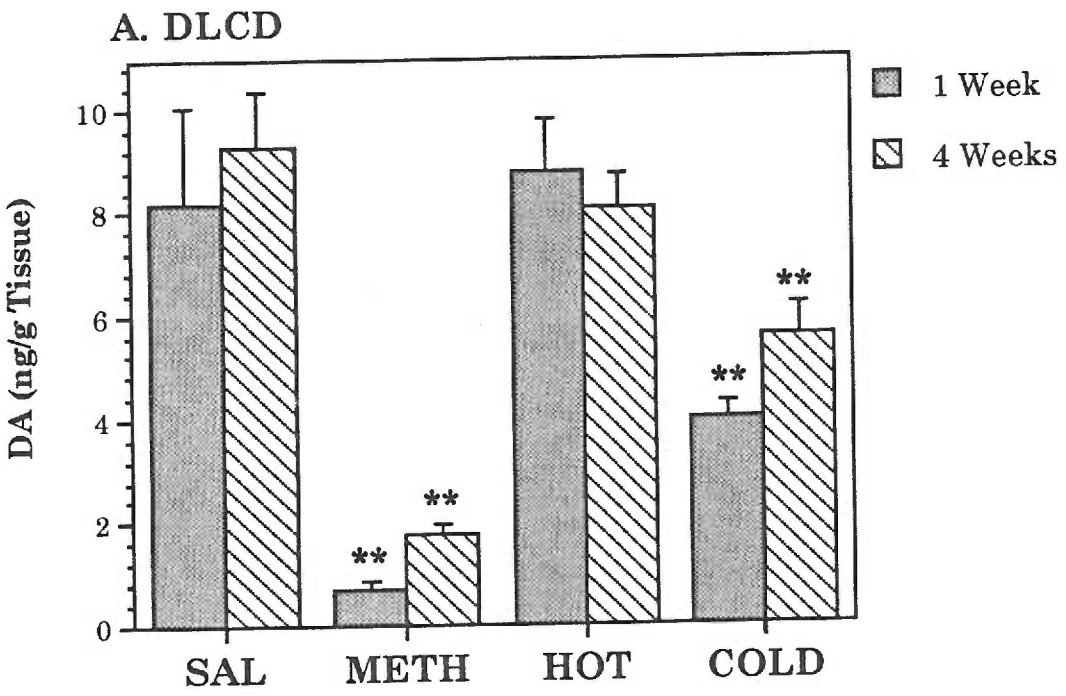
Region	Group	Terminal Area (μm^2)	Density of Immunolabeling (particles/μm^2)	% of Total Synapses
Perforated Synapses				
DLCD	SAL	0.487 ± 0.03 (7)	84.27 ± 6.72 (7)	17.13 ± 0.81 (7)
	METH	0.507 ± 0.03 (11)	88.27 ± 4.97 (11)	29.85 ± 2.07 (11)**
VLCD	SAL	0.550 ± 0.05 (7)	74.10 ± 4.51 (7)	16.26 ± 1.97 (7)
	METH	0.555 ± 0.02 (11)	76.03 ± 4.18 (11)	26.16 ± 1.83 (11)*
Non-Perforated Synapses				
DLCD	SAL	0.331 ± 0.02 (7)	85.49 ± 4.13 (7)	
	METH	0.319 ± 0.02 (11)	87.35 ± 4.62 (11)	
VLCD	SAL	0.340 ± 0.02 (7)	73.67 ± 3.29 (7)	
	METH	0.342 ± 0.02 (11)	76.48 ± 4.79 (11)	

Terminal area, glutamate immunolabeling, and percent of synapses (Mean ± SEM, n per group) in the striatum. Animals were killed 4 weeks after repeated MA (15 mg/kg every 6 hrs for 4 injections) or saline administration. Data were analyzed by ANOVA followed by the Newman-Keul's test. (* $p < 0.05$ vs. SAL; ** $p < 0.01$ vs. SAL).

administration depleted DA content (DLCD: $F_{3,56} = 71.8$, $p < 0.0001$; VLCD: $F_{3,54} = 95.2$, $p < 0.0001$). One week following MA administration, DA content in the METH group was decreased by >90% in both the VLCD ($p < 0.01$) and DLCD ($p < 0.01$) (Figure 18). DA levels did not recover and remained more than 80% depleted in the METH group after 4 weeks ($p < 0.01$). Although rats in the COLD group did show DA loss ($p < 0.01$), depletion was 55-70% at 1 week, and only 35-45% after 4 weeks. DA content in the DLCD and VLCD of the COLD group was significantly greater compared with DA levels in the METH group at both time points ($p < 0.05$), demonstrating a partial protective effect of cooling. Although DA content within treatment group varied across time within the VLCD ($F_{3,54} = 4.5$, $p < 0.01$), the source of the interaction was not found. A slight recovery of DA content in the METH ($p = 0.067$) and COLD ($p = 0.063$) groups may have been responsible.

Drug administration significantly depleted DOPAC levels in both striatal subregions examined (DLCD: $F_{3,56} = 12.8$, $p < 0.0001$; VLCD: $F_{3,54} = 13.9$, $p < 0.0001$). DOPAC levels were significantly decreased in the METH group after 1 week ($p < 0.01$) but recovered by 4 weeks (Figure 19). In the COLD and HOT groups, DOPAC levels were decreased within the VLCD 1 week post-drug ($p < 0.05$). The ratio of DOPAC/DA, a measure of DA metabolism, was also altered in both striatal subregions (DLCD: $F_{3,56} = 26.5$, $p < 0.0001$; VLCD: $F_{3,54} = 28.2$, $p < 0.0001$). Increases in the ratio of DOPAC/DA were seen only in the METH group 1 and 4 weeks post-drug ($p < 0.01$), suggesting that DA turnover was enhanced only in the rats with the greatest loss of DA content (Figure 20). The interaction between treatment group and time was not significant for either DOPAC or the ratio of DOPAC/DA.

Figure 18. DA content (Mean ± SEM, n = 4-15 animals per group) in the DLCD (A), and VLCD (B). DA was measured either 1 week (closed bar) or 4 weeks (striped bar) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test (** = $p < 0.01$ vs. SAL). Data are also presented in Table VIII (Appendix B).


A. DLCD
DA (ng/g Tissue)
0
2
4
6
8
10
**
**
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks

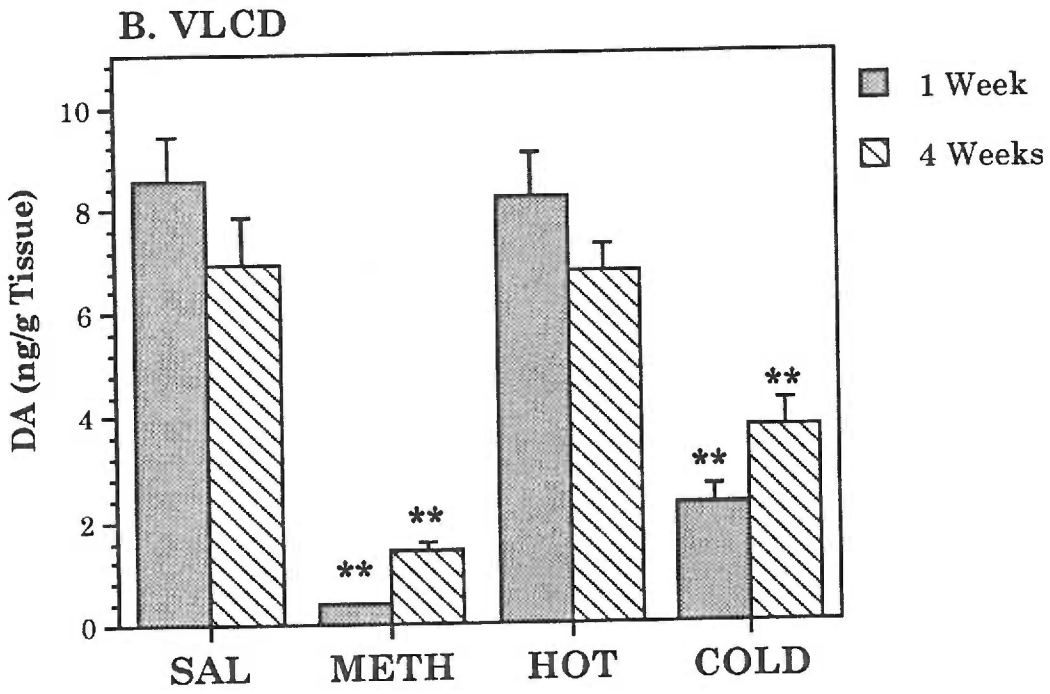

B. VLCD
DA (ng/g Tissue)
0
2
4
6
8
10
**
**
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 19. DOPAC content (Mean ± SEM, n = 4-15 animals per group) in the DLCD **(A)**, and VLCD **(B)**. DA was measured either 1 week (closed bar) or 4 weeks (striped bar) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test (* = $p < 0.05$ vs. SAL, ** = $p < 0.01$ vs. SAL). Data are also presented in Table VIII (Appendix B).

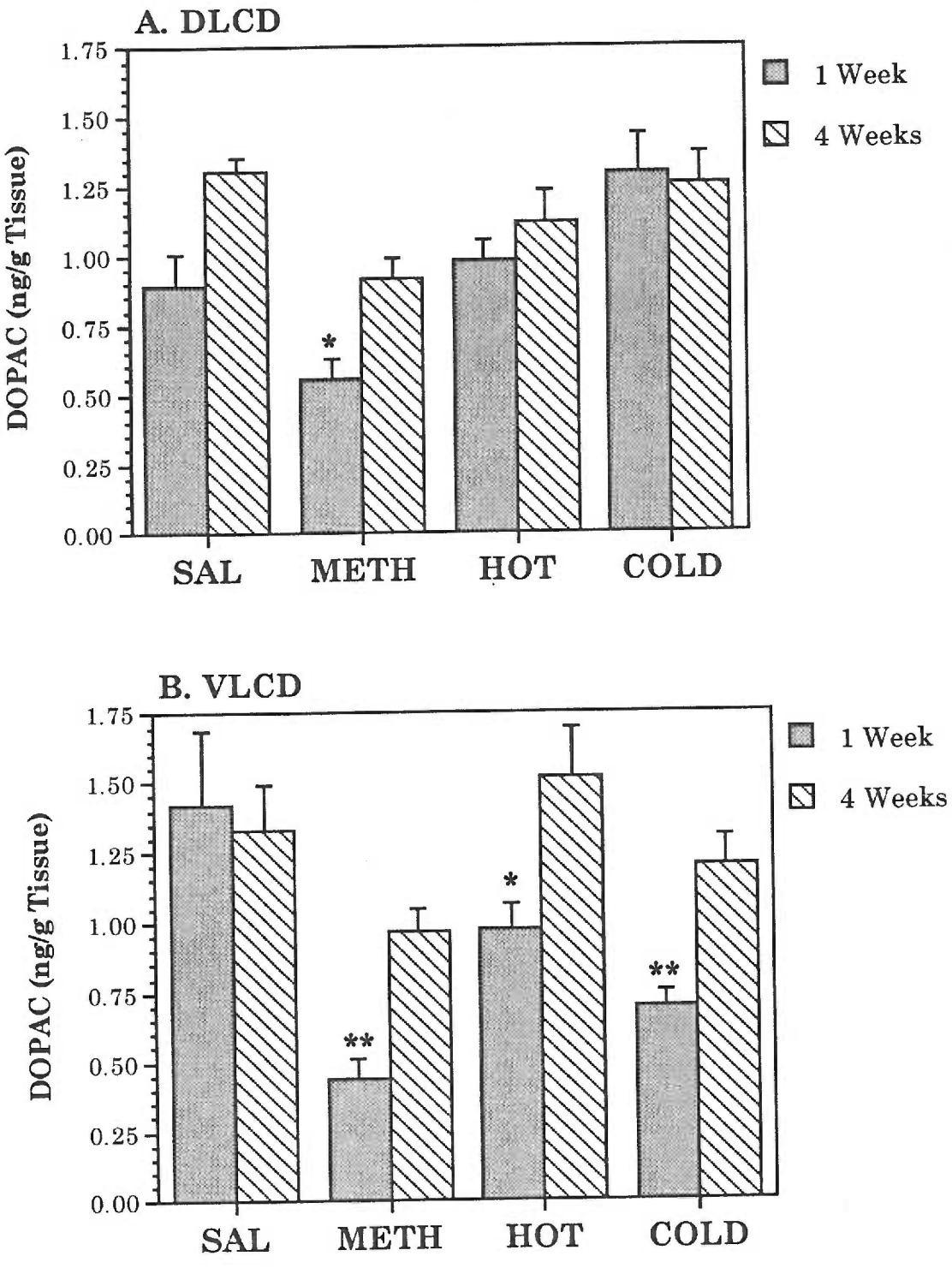
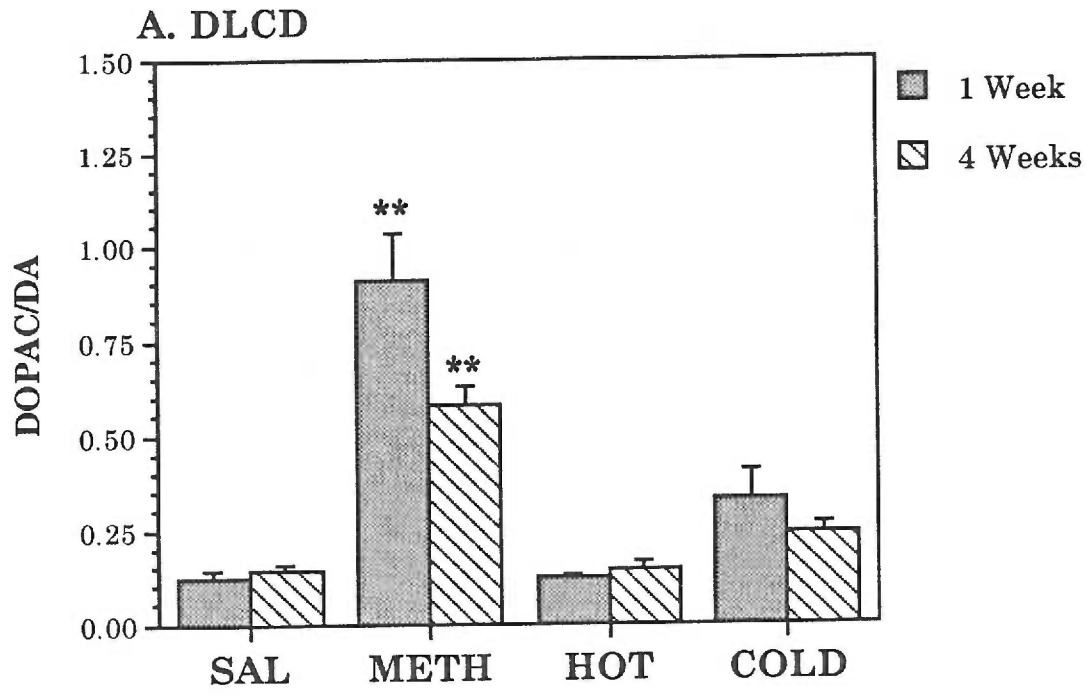
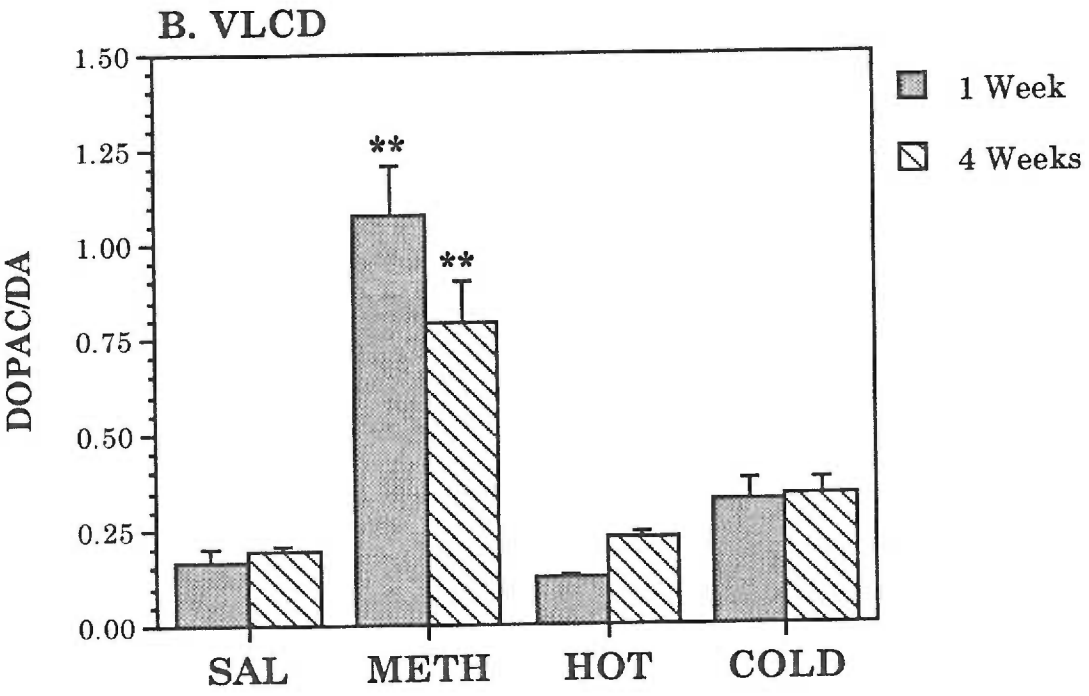

A. DLCD
DOPAC (ng/g Tissue)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
SAL
METH
HOT
COLD
1 Week
4 Weeks
B. VLCD
DOPAC (ng/g Tissue)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
*
**
SAL
METH
HOT
COLD
1 Week
4 Weeks

Figure 20. The ratio of DOPAC/DA (Mean ± SEM, n = 4-15 animals per group), in the DLCD **(A)**, and VLCD **(B)**. The ratio was determined either 1 week (closed bar) or 4 weeks (striped bar) following treatment. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test (** = $p < 0.01$ vs. SAL). Data are also presented in Table VIII (Appendix B).


A. DLCD
DOPAC/DA
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks


B. VLCD
DOPAC/DA
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
**
SAL
METH
HOT
COLD
1 Week
4 Weeks

DISCUSSION

A. Ultrastructural Immunocytochemistry: GABA

In the present study, dynamic changes in presynaptic GABA immunoreactivity were accompanied by altered terminal size in the METH group. Nigrostriatal DA loss mediated by 6-OHDA increases the activity of GABAergic striatal neurons (Schultz and Ungerstedt, 1978; Nisenbaum *et al.*, 1986; Calabresi *et al.*, 1993; Chang *et al.*, 1997). Such an increase in GABAergic activity can be seen as early as 3 days following 6-OHDA-induced lesioning (Schultz and Ungerstedt, 1978), while increases in GABA and ENK synthesis and content may take several weeks to develop (Thal *et al.*, 1983; Vernier *et al.*, 1988; Ingham *et al.*, 1991).

GABA labeling was decreased in all three brain regions 1 week after MA treatment in the METH group, suggesting early and dynamic effects following a toxic dose of MA. Several studies have found that decreased presynaptic immunoreactivity correlates with evidence for increased neurotransmitter release. For example, Meshul *et al.* (1997) reported that 6-OHDA-induced decreases in striatal glutamate immunoreactivity were accompanied by enhanced basal extracellular glutamate levels as measured by *in vivo* microdialysis. Similarly, transient decreases in glutamate immunoreactivity that occur following MA administration (Burrows and Meshul, 1997) correlate temporally with MA and amphetamine-induced glutamate release (Nash and Yamamoto, 1993; Stephans and Yamamoto, 1994; Yamamoto *et al.*, 1997). Thus, the early decrease in presynaptic GABA immunoreactivity found in the present study may reflect depleted stores due to increased activity of the striatal output neurons.

Compared to 1 week post-drug, GABA immunolabeling was increased in all three brain regions 4 weeks following MA-induced DA depletion. Although immunoreactivity returned to basal levels in the DLCD, a significant increase in GABA labeling was found in both the GP and VLCD. GABA-positive terminals in the VLCD were also found to be significantly larger 4 weeks after MA treatment in the METH group, and a similar trend was found in the GP of these animals. Such long-lasting alterations in GABAergic synapses are similar to those found 4 weeks following a 6-OHDA lesion of the nigrostriatal pathway (Ingham *et al.*, 1993; Ingham *et al.*, 1997; Meshul *et al.*, 1997), suggesting that the delayed changes in GABA synapses found in the current study could reflect a delayed increase in the synthesis and content of GABA.

Ingham *et al.* (1991, 1997) have found a 50-60% increase in the size of ENK-positive striatal and pallidal terminals one month after a 6-OHDA induced lesion. These changes were significantly larger compared with the 13-20% increase in terminal size found in the VLCD and GP of the METH group. It is likely that the differences between these studies reflects the fact that all GABA-positive terminals in the CD and GP were analyzed in the current study, as opposed to analysis of only terminals known to be associated with the striatopallidal output neurons. It has been reported that when all GABA-positive boutons in the GP or CD were analyzed, regardless of origin, no changes in terminal size were found 1 month following 6-OHDA treatment (Ingham *et al.*, 1997; Meshul *et al.*, 1997). In contrast, analysis of a subset of GABA-positive terminals in the GP that were known to have a striatal origin were significantly (57%) larger following 6-OHDA-induced lesions (Ingham *et al.*, 1997). Therefore, since it is likely that not all GABA terminals examined

in the current study contained ENK or were derived from striatopallidal output neurons, it is not surprising that a large increase in terminal size was not found. However, the small but significant increase seen within the VLCD, coupled with a similar trend in the GP, is consistent with the hypothesis that striatopallidal output is increased following a neurotoxic dose of MA. Furthermore, the late onset of these changes (4 weeks post-MA) is in agreement with the known timecourse of DA-depletion mediated morphological changes (Ingham *et al.*, 1993; Ingham *et al.*, 1997; Meshul *et al.*, 1997).

In the current study, the source of GABA-positive terminals analyzed in the CD and GP is not known. Within the CD, GABA labeled terminals could arise from local interneurons or from the striatal output neurons (Figure 2). Similarly, GABA terminals in the GP could arise from striatopallidal afferents, or collaterals from the pallidal output neurons that project to the subthalamic nucleus (Figure 1). Alterations in GABA terminals were found to occur similarly in both the VLCD and GP, suggesting that MA does affect the striatopallidal pathway. We cannot, however, conclude that interneurons or striatonigral neurons were unaffected. One way to identify the source of these GABA-positive terminals would be to double label tissue using an additional antibody against ENK or substance P. However, immunogold double labeling often decreases the amount and specificity of labeling (Buckman & Meshul, unpublished observation). For example, Ingham *et al.* (1997) found that double labeling decreased presynaptic GABA-immunoreactivity from seven times background levels to only two times background levels. If identification of terminals is the goal, then diminished immunoreactivity should not greatly affect the results. However, if

quantitative analysis of particle density is required, double labeling could severely alter the findings. Since the goal of the current study was to identify MA-induced alterations in the presynaptic density of GABA, and the degree of such changes, the ability to quantify GABA immunoreactivity was essential. Additional studies will be needed to determine the specificity of MA's effects on striatopallidal GABA neurons. Nevertheless, dynamic changes in presynaptic GABA labeling, and delayed increases in terminal size, were nearly identical in the VLCD and GP of the METH group, providing strong evidence for altered striatopallidal activity.

Increased terminal size and presynaptic GABA immunoreactivity have both been found to occur following 6-OHDA lesions (Ingham *et al.*, 1993; Ingham *et al.*, 1997; Meshul *et al.*, 1997) and have been interpreted as measures of increased synaptic activity and neurotransmitter content. Since both were found to occur in the VLCD 4 weeks following MA in the METH group, but neither occurred within the DLCD, this suggests that alterations in GABA activity are regionally heterogeneous within the striatum. Marshall and Navarrete (1990) reported a significant correlation between endogenous DA levels and vulnerability to MA toxicity. They found that, compared with other striatal subregions, the ventral and lateral regions of the CD had both the greatest striatal DA content and had a higher degree of DA depletion following MA treatment (Marshall and Navarrete, 1990). Others have reported that the VLCD was more vulnerable to the loss of DA uptake sites (Eisch *et al.*, 1992), decrease in NMDA receptor binding sites (Eisch *et al.*, 1996b), and the loss of tyrosine hydroxylase immunoreactivity (Pu *et al.*, 1994) following MA administration. In addition, astrogliosis and the swelling of DA-fluorescent axons were localized within the VLCD (Ellison *et al.*, 1978;

Pu *et al.*, 1994). Although a difference in the content of endogenous DA may mediate the degree of MA-toxicity (Marshall and Navarrete, 1990), the heterogeneity of MA's effects within the striatum has not been thoroughly explained. There is some evidence that 6-OHDA damage also results in heterogeneous changes within the striatum. Voorn *et al.* (1987) reported that 6-OHDA-induced increases in ENK immunoreactivity were greatest in the VLCD, and least within the DLCD. These data are consistent with the results seen in the current study, and when taken together, suggest that the VLCD is inherently more sensitive to the effects of neurotoxins that damage the nigrostriatal DA system.

One possible explanation for regional differences in the striatum could lie in the topographical organization of the output neurons. If the VLCD contained a higher percentage of striatopallidal neurons compared to the DLCD, then loss of DA would be expected to increase GABA activity in the VLCD to a greater extent. Although no thorough analysis of the distribution of output neurons within the DLCD and VLCD has been reported, several lines of evidence argue against this suggestion. If the D2 receptor mediated striatopallidal neurons were clustered in the VLCD, then the expectation would be to find a greater number of D2 receptors in this region. However, the highest concentration of D2 receptors can be found in the DLCD (Altar *et al.*, 1985). In addition, constitutive expression of preproenkephalin mRNA is ubiquitously distributed within the CD, not concentrated within the VLCD, while preprodynorphin mRNA is primarily localized within the VLCD (Wang and McGinty, 1996). Thus, the long-lasting GABAergic changes seen selectively in the VLCD do not appear to be related to the organization of striatal output neurons.

A second explanation for regional differences in MA susceptibility relates to differences in cortical input. We have recently reported that MA administration results in a transient decrease in glutamate immunoreactivity within the VLCD, while a similar decrease in the DLCD did not reach significance (Burrows and Meshul, 1997). Although both the DLCD and VLCD receive excitatory inputs from the primary motor cortex, the VLCD receives additional input from the hippocampus and somatosensory cortex (McGeorge and Faull, 1989). There is evidence indicating that these cortical regions are also differentially sensitive to MA toxicity. High-dose MA treatment increases binding to NMDA receptors only in the somatosensory cortex (Eisch *et al.*, 1996b), suggesting a loss of glutamate activity in this region. In addition, degenerating neurons and reactive gliosis have selectively been found within the somatosensory cortex (Commins and Seiden, 1986; Eisch *et al.*, 1996a; Pu *et al.*, 1996) and structures associated with the hippocampus (Schmued and Bowyer, 1997). This suggests that corticostriatal input to the DLCD and VLCD may be differentially altered following MA treatment. Selective damage to afferents innervating the VLCD may result in a different pattern of MA toxicity. We recently reported that transient changes in presynaptic glutamate immunoreactivity occur to a greater extent within the VLCD compared to the DLCD (Burrows and Meshul, 1997). Glutamate immunolabeling was decreased 12 hrs following MA administration in the VLCD, and returned to basal levels within 24 hrs. This study provides evidence indicating that initial regional differences in the glutamate response to MA may predict the degree of toxicity that occurs. Although no differences between corticostriatal terminals in the DLCD and

VLCD were found in the current study, early and transient alterations in glutamate immunoreactivity may have been overlooked.

In addition to decreases in the vesicular pool of GABA, labeling within mitochondria was also decreased 1 week within the VLCD after treatment. Since the GABA antibody is known to crossreact with several Krebs cycle intermediates, including succinate (Phend *et al.*, 1992), this decrease in mitochondrial labeling likely reflects a change in metabolism. In contrast to terminal labeling, these changes were seen in both the METH and HOT groups, suggesting a fairly long-lasting effect of prolonged hyperthermia. 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), the tie between hyperthermia and MA-induced DA depletion is not yet clear. Bowyer *et al.* (1992) have shown that the degree of DA depletion induced by MA can be altered by manipulation of the environmental temperature. In addition, many drugs which attenuate the neurotoxic effects of amphetamines are themselves hypothermic-inducing agents. Temperatures ranging from 40.6-42.6°C are known to induce ischemia, seizures, and peripheral hypoxia, and both MA and hypoxia are known to deplete energy stores (ATP levels) (Knochel, 1989; Callaway and Clark, 1994; Chan *et al.*, 1994; Farooqui *et al.*, 1994; Kao *et al.*, 1994). MA-induced depletion of ATP levels could be a nonspecific effect of increased metabolic stress resulting from increased energy consumption following prolonged DA and glutamate release (Chan *et al.*, 1994; Stephans and Yamamoto, 1994; Yamamoto *et al.*, 1997). MA may also directly deplete energy stores by disruption of the sodium gradient and reversal of the ATP

driven DA transporter (Yamamoto *et al.*, 1997). Regardless of the source of energy depletion, the current study suggests that severe hyperthermia could play role in MA's ability to decrease mitochondrial function. Furthermore, since these changes were found in the VLCD, but not the DLCD, it suggests that hyperthermia may also have regional effects within the striatum. If the VLCD is more susceptible to the metabolic stress resulting from prolonged hyperthermia, then perhaps this vulnerability can influence the degree of toxicity and subsequent morphological changes that occur in this region.

B. Ultrastructural Immunocytochemistry: Glutamate

In the current study, no changes in glutamate immunolabeling or terminal size were found in any of the groups (Figures 14 and 16). A decrease in presynaptic glutamate labeling was predicted in METH rats based, in part, on a study by Meshul *et al.* (1997) demonstrating lowered striatal glutamate immunolabeling 1 month following a 6-OHDA lesion, and other findings suggesting a possible increase in corticostriatal activity following DA deafferentation (Table IV). There was, however, an increase in the percent of synapses with a perforated, or discontinuous, postsynaptic density 4 weeks following MA administration in the METH group (Figure 17). Increases in such asymmetrical synapses have been found in rat striatum following 6-OHDA-induced lesions (Meshul *et al.*, 1997) and in the caudate of Parkinson's patients (Anglade *et al.*, 1996). Such synaptic changes are believed to correlate with increases in synaptic activity (Buchs and Muller, 1996; Meshul *et al.*, 1996; Meshul *et al.*, 1997). In the current study however, changes in synaptic morphology were not accompanied by altered terminal size or glutamate immunoreactivity.

The dissociation between glutamate immunoreactivity and the mean percent of synapses with perforated postsynaptic densities is puzzling. No other studies have reported both an increase in synapses with perforated postsynaptic densities and a lack of an effect on glutamate immunoreactivity. Altered glutamate synthesis or uptake following MA-induced DA loss could negate any presynaptic changes. For example, an increase in glutamate release accompanied by an increase in glutamate synthesis could result in a density of presynaptic glutamate immunolabeling which did not differ from control levels. However, differing consequences of MA or 6-OHDA administration may be related to the fact that MA is administered systemically and is distributed throughout the brain where it is likely to have more global actions. For example, although damage to DA terminals is primarily localized within the striatum, DA loss can also occur in the nucleus accumbens and frontal cortex (Seiden *et al.*, 1988; Burrows and Meshul, 1997). In contrast, 6-OHDA is generally administered locally in the substantia nigra or medial forebrain bundle to specifically damage the nigrostriatal pathway. In addition, 6-OHDA and MA have differing effects on non-dopaminergic systems. Although 6-OHDA is not a serotonergic neurotoxin, MA does deplete serotonin levels in the neocortex, striatum, septum, amygdala, hypothalamus, substantia nigra, olfactory tubercle, nucleus accumbens, hippocampus, and brain stem (Ricaurte *et al.*, 1980; Seiden *et al.*, 1988). The consequences of combined DA and serotonin loss may be important in the regulation of post-drug changes seen in the current study. It appears that DA damage in the striatum resulting from MA or 6-OHDA administration has similar consequences on GABAergic systems, but

there appear to be differences in the regulatory changes within striatal glutamate systems.

C. Analysis of DA and DOPAC Levels

In the current study, MA administration in the METH group resulted in immunocytochemical and morphological alterations in glutamate- and GABA-positive terminals. In contrast, the same dose of MA had no effects on rats that were not allowed to become hyperthermic (COLD group). Since blockade of hyperthermia is known to be neuroprotective (Bowyer *et al.*, 1992; Bowyer *et al.*, 1994), DA content was analyzed in a separate group of rats to determine if immunocytochemical and morphological differences were correlated with the degree of DA loss. In the METH group, initial striatal DA loss was profound (>90%), and remained more than 80% depleted even 4 weeks following drug treatment. In contrast, DA levels in the COLD group were decreased by only 35-45% after 4 weeks, providing further evidence for the partial protective effects of hyperthermic prevention. Similar to previous studies (Bowyer *et al.*, 1994), exposure to hyperthermia comparable to that induced by MA did not induce DA or DOPAC loss in the HOT group, nor did it alter the ratio of DOPAC/DA.

An increased ratio of DOPAC/DA is an indication accelerated metabolism (or turnover) of DA within surviving terminals. Such an increase in DA metabolism could contribute to behavioral and/or neurochemical recovery by increasing DA availability to DA receptors. Increased DOPAC levels in relation to DA can result from increases in the activity of remaining DA neurons, extracellular metabolism of DA by monoamine oxidase following release, or intracellular metabolism by monoamine oxidase following

reuptake. For these reasons striatal DOPAC concentrations are believed to reflect changes in DA metabolism, rather than just the extent of DA release (Altar *et al.*, 1987). In the current study, there was a 4-5 fold increase in the ratio of DOPAC/DA in the METH group both 1 and 4 weeks post-MA (Figure 20). Although the ratio of DOPAC/DA did increase by 1.5-2.5 fold in the COLD group, this change did not reach significance. These results are similar to a study by Hefti *et al.* (1980) which reported that increases in DA metabolism were not found in rats where 6-OHDA mediated DA loss did not exceed 60%. In addition, they found a progressive increase in DA turnover in animals with a 60-80%, 80-90%, or 90-100% loss of DA (Hefti *et al.*, 1980). In the present study, no evidence for compensatory changes in DA, GABA, or glutamate systems were found in rats with only partial DA depletions (COLD group), while changes in all three neurotransmitter systems were present in the METH group. Together, this evidence indicates that a majority of DA needs to be lost before compensatory changes in non-dopaminergic systems occur. Thus, it is the extent of DA loss, rather than the mechanism of DA deafferentation, that appears to mediate shifts in neurotransmitter function.

Marshall and Navarrete (1990) reported that MA-induced DA depletion was found to be correlated with the level of endogenous DA content within striatal subregions, suggesting that the degree of toxicity was related to the amount of DA release following MA administration. Although DA depletion in the COLD group did appear to be more severe within the VLCD at both 1 and 4 weeks post MA (Figure 18), the extent of DA loss and subsequent increase in DA metabolism in the METH group appeared to occur equally within these striatal subregions. In contrast, immunocytochemical differences were found between these striatal subregions in the METH group,

suggesting that the degree of DA depletion alone cannot account for the heterogeneity found in the present study. We have previously reported that transient decreases in glutamate immunoreactivity are greater in the VLCD, suggesting that the degree of MA-induced glutamate release may play a role in the subsequent level of toxicity. Since the DLCD and VLCD have differing cortical inputs, perhaps differential activation of these inputs plays a role in the heterogeneous effects of MA. In addition, to examine regional differences in MA toxicity, Eisch *et al.* (1992) divided the CD into 12 subregions prior to HPLC analysis of DA content, while in the current study the CD was quartered. By pooling these subregions, subtle differences in the regional specificity of MA-induced DA depletion may have been masked.

Administration of a neurotoxic regimen of MA typically results in approximately a 50% loss of striatal DA content (Table I). Surprisingly, DA depletions exceeding 90% in the METH group were consistently found in this study. Although DA depletions greater than 90% have been reported in mice (Sonsalla *et al.*, 1996), they have not been previously found in rats. A severe depletion in catecholamine levels is consistent with the behavioral changes present in the METH group. More than half of these animals were lean and became dehydrated for several days following MA treatment, and at least 10% demonstrated vacuous chewing movements similar to those seen after 6-OHDA lesions (Singer and Armstrong, 1976; Jicha and Salamone, 1991).

There could be several reasons why this particular drug regimen resulted in such severe DA loss. First, it was noted that animals in both the METH and COLD groups remained stereotypic throughout the entire experiment, indicating that drug was most likely present for at least 24 hours. Secondly, rats in the METH treatment group experienced extreme

hyperthermia (41.8°C) which is known to correlate with DA loss. Studies in mice have shown that 80% of animals with peak body temperatures exceeding 41.6°C die following MA administration (Askew, 1962). Recent evidence indicates that animals which become extremely hyperthermic can be rescued by cooling, resulting in a significantly decreased mortality rate while maintaining DA toxicity. Bowyer *et al.* (1994) reported that repeated administration of 5 mg/kg MA (every 2 hrs for 4 doses) resulted in only a 37% depletion of DA levels in animals that did not experience extreme hyperthermia, but levels were decreased by 70% in animals that were allowed to become hyperthermic then rescued by rapid cooling (Bowyer *et al.*, 1994). In addition, we have found that more than 50% of animals that collapse and become adipsic or aphasic die after 2-3 days unless given fluids and nutrients (Burrows, unpublished observation). In the current study, all animals receiving post experimental care survived. Mortality rates and rescuing efforts are usually not reported in studies examining MA toxicity. Thus, in many studies it is likely that animals with the greatest toxicity did not survive drug treatment. The high dose of MA used and the prolonged time course employed in the current study, coupled with the rescuing of animals in the METH group by cooling and post-experimental care likely contributed to the survival of animals that would otherwise have died. Together, these factors likely account for the extreme and long lasting DA depletions found. Such large DA depletions allowed for the detection of changes in the immunocytochemistry and synaptic morphology of GABAergic and glutamatergic nerve terminals.

SUMMARY AND CONCLUSIONS

In the current study, manipulation of core body temperature in the HOT and COLD groups allowed for both the separation of drug versus temperature effects and the examination of animals with severe or partial loss of striatal DA content. Presynaptic GABA immunoreactivity was decreased in all three brain regions 1 week following MA treatment in the METH group. This decrease in GABA immunolabeling may reflect depleted stores due to increased activity of the striatal output neurons. Similarly, an increase in both GABA immunolabeling and terminal size was found after 4 weeks in the METH group, and could indicate increased synthesis and content of GABA known to occur following DA loss induced by 6-OHDA. It is likely that changes in striatopallidal neurons are primarily responsible for the dynamic effects of MA on GABA systems, although it cannot be concluded that interneurons or striatonigral neurons were unaffected. Additional studies are needed to determine the exact source and specificity of presynaptic fluctuations in GABA immunoreactivity. Long term increases in GABA immunolabeling were found in the VLCD but not in the DLCD. Together with data collected from 6-OHDA (Voorn *et al.*, 1987) and other MA studies (Marshall and Navarrete, 1990), these results suggest that the VLCD is inherently more sensitive to the effects of DA depleting drugs. The heterogeneous effects of DA neurotoxins within the striatum could be due to regional variations in endogenous DA levels (Marshall and Navarrete, 1990), differences in the distribution of striatal output neurons (Voorn *et al.*, 1987; Wang and McGinty, 1996), differences in corticostriatal inputs (McGeorge and Faull,

1989; Eisch *et al.*, 1996a; Pu *et al.*, 1996; Schmued and Bowyer, 1997), or to other unidentified differences within these regions.

No changes in presynaptic glutamate immunoreactivity or terminal size were found in any of the groups. There was, however, an increase in the percent of synapses with a perforated postsynaptic density 4 weeks following MA administration in the METH group. Although increases in such synapses are hypothesized to correlate with increases in synaptic activity (Greenough *et al.*, 1978; Meshul *et al.*, 1992), analysis of synapses with perforated contacts did not reveal evidence for increased activity. The dissociation between glutamate immunoreactivity and the number of synapses containing a perforated postsynaptic density has not been previously reported, but the morphological change is consistent with studies suggesting increased corticostriatal activity following DA deafferentation (Weihmuller *et al.*, 1993; Meshul *et al.*, 1997).

To determine if immunocytochemical and morphological alterations were correlated with DA loss, catecholamine content was measured in a separate group of animals. In the METH group, initial striatal DA loss was profound (>90%), and remained more than 80% depleted even 4 weeks following drug treatment. In contrast, levels in the COLD group had partly recovered to 55-65% of control values, providing further evidence for the protective effects of hyperthermic prevention and allowing for comparison of immunocytochemical alterations following a partial versus severe loss of DA. In addition to DA loss, MA administration resulted in a 4-5 fold increase in the ratio of DOPAC/DA in the METH group, and a non-significant (1.5-2.5 fold) increase in the COLD group. These findings demonstrate that a severe, but not a partial, loss of DA following a toxic dose of MA significantly

increases DA metabolism in a manner similar to that found following 6-OHDA (Hefti *et al.*, 1980). This result provides further evidence that the extent of DA loss, rather than the mechanism of DA deafferentation, appears to mediate compensatory increases in DA metabolism. In addition, these results demonstrate that large DA depletions are required for alterations in non-dopaminergic systems to occur.

The immunocytochemical and morphological changes seen in the METH group resemble those found in animals with 6-OHDA-induced DA loss (Ingham *et al.*, 1997; Meshul *et al.*, 1997), and in patients with Parkinson's disease (Perry *et al.*, 1983; Tossman *et al.*, 1986; Segovia and Garcia-Munoz, 1987). Only one additional study has demonstrated changes in the striatal output pathways following MA administration. Melega *et al.* (1997a) recently reported that high-dose MA treatment in monkeys increases iron content and ferritin immunoreactivity in the GP and substantia nigra pars reticulata. Similar increases in pallidal and nigral iron content are known to occur in 6-OHDA lesioned animals (Oestreicher *et al.*, 1994; Sastry and Arendash, 1995) and in Parkinson's disease (Gerlach *et al.*, 1994). Since iron content is correlated with GABA utilization (Hill, 1985), increased iron content has been interpreted as an indication of enhanced GABA activity (Melega *et al.*, 1997a). Such findings add support to the results in the current study suggesting long-term increases in the activity of striatopallidal GABA neurons. Overactivity of the striatopallidal pathway under resting conditions is believed to underlie parkinsonian symptoms by interfering with the generation of movement (Miller and DeLong, 1987; Klockgether and Turski, 1989; Mitchell *et al.*, 1989). It is not known if MA abuse is a risk factor in Parkinson's disease, but it is possible that such damage to the nigrostriatal DA system could result in

an earlier onset of symptoms in individuals predisposed to develop Parkinson's. The current study adds support to recent evidence that MA administration can have long-lasting effects on the basal ganglia of both humans and animals. Further analysis of MA-induced alterations in the extrapyramidal motor circuit may have important clinical implications for humans with a history of psychostimulant abuse.

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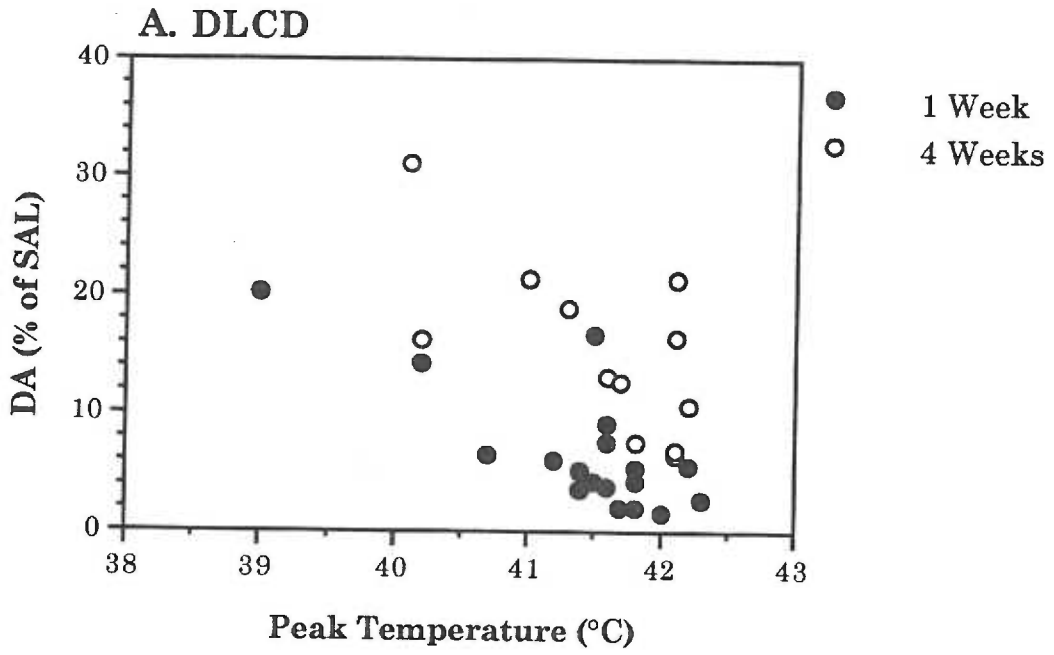
APPENDIX A:
Exclusion of METH Rats With Moderate Hyperthermia

ous studies have demonstrated that the degree of MA-induced DA
ed with peak body temperature (Bowyer *et al.*, 1992;
r results were obtained in the current study
the highest peak temperature also had the
oth 1 and 4 weeks post-MA. Preliminary
in the METH group whose maximal rectal
ed 41°C (n = 2) did not show changes in GABA
le those that displayed severe hyperthermia (n = 6) had
nunolabeling after 4 weeks (Figure 22). These changes
thin the VLCD ($F_{2,10} = 5.41$, $p < 0.05$) and GP ($F_{2,10} =$
In order to maximize DA depletion and increase the
possibi detecting changes in GABA and glutamate immunolabeling,
rats in the METH group whose peak body temperature did not exceed 41°C (n
= 9) were excluded from the study.

Figure 21. Scatter diagram showing the relationship between DA content and peak body temperature in the DLCD (A), and VLCD (B). DA content was analyzed 1 week (closed circles) or 4 weeks (open circles) following treatment. Note that the greatest and longest lasting DA depletions occurred in the animals with the greatest hyperthermia.


A. DLCD
DA (% of SAL)
Peak Temperature (°C)
1 Week
4 Weeks
0
10
20
30
40
38
39
40
41
42
43

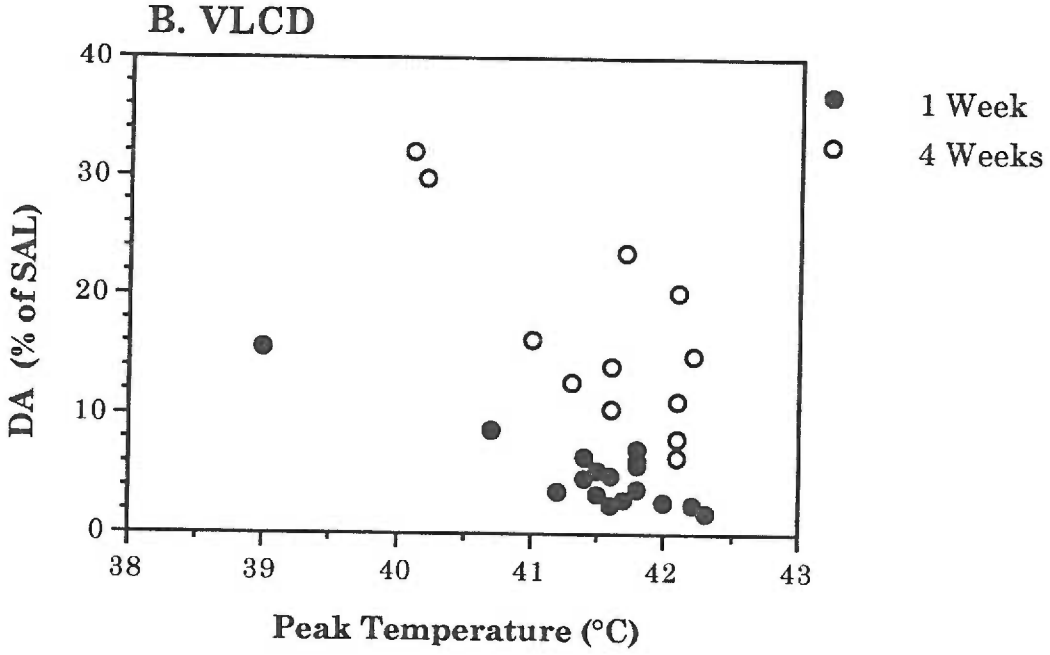
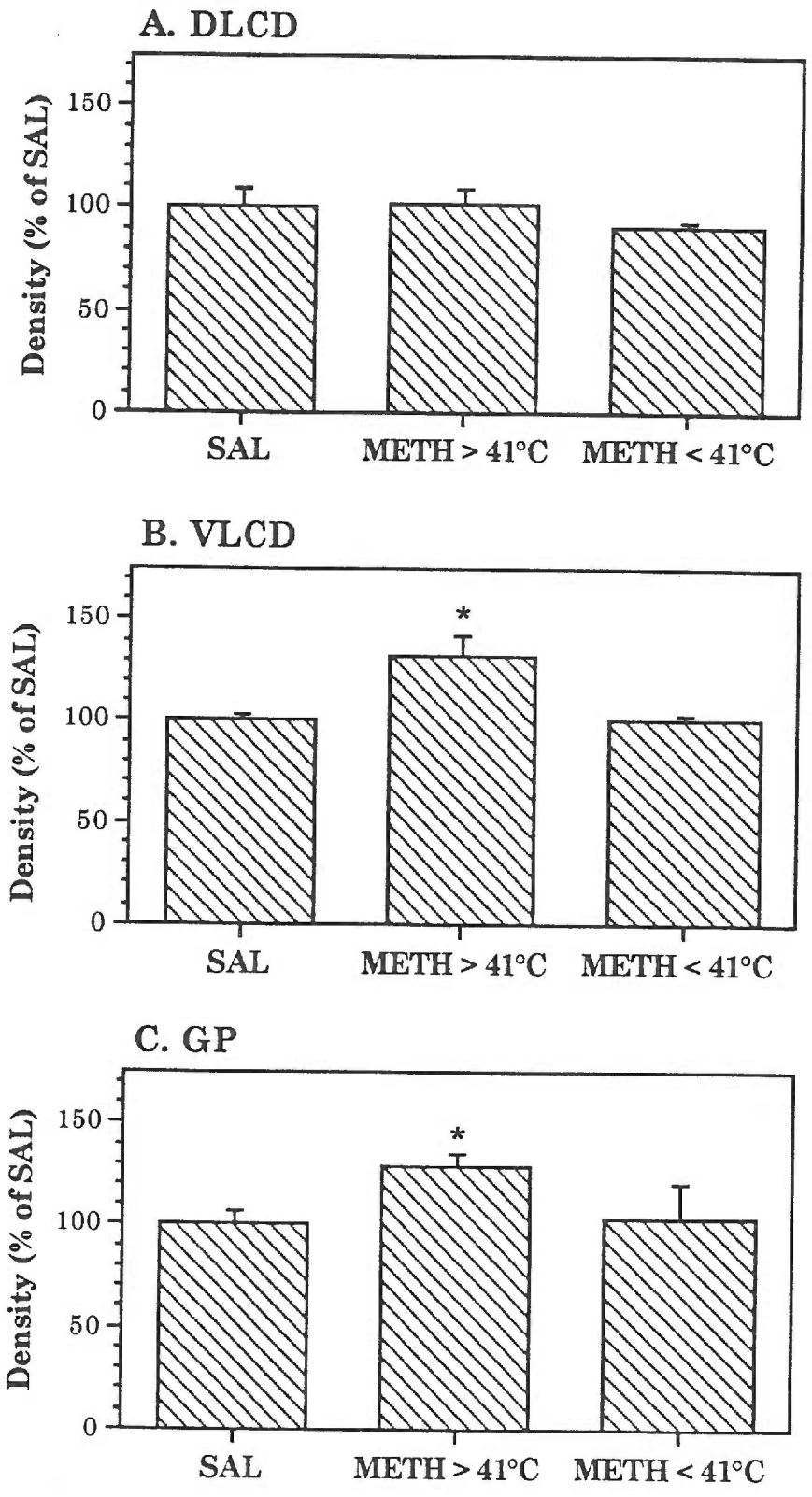

B. VLCD
DA (% of SAL)
Peak Temperature (°C)
1 Week
4 Weeks
0
10
20
30
40
38
39
40
41
42
43

Figure 22. Preliminary analysis of the density of presynaptic GABA immunolabeling, expressed as a percent of the SAL group (Mean ± SEM, n = 2-5 animals per group), in the DLCD (A), VLCD (B), and GP (C). Labeling was analyzed 4 weeks following treatment. Note that increased immunolabeling was seen only in rats whose peak temperature exceeded 41°C (METH>41°C) and not in rats whose peak temperature was less than 41°C (METH<41°C). Data were analyzed by one-way ANOVA followed by the Newman-Keul's test (* = $p < 0.05$ vs. SAL).


A. DLCD
Density (% of SAL)
150
100
50
0
SAL
METH > 41°C
METH < 41°C
B. VLCD
Density (% of SAL)
150
100
50
0
*
SAL
METH > 41°C
METH < 41°C
C. GP
Density (% of SAL)
150
100
50
0
*
SAL
METH > 41°C
METH < 41°C

APPENDIX B

Table VIII. Catecholamine Content Within the Striatum

A. DLCD

Group & Time	DA (ng/g tiss)	DOPAC (ng/g tiss)	RATIO DOPAC/DA
SAL 1 WK	8.15 ± 1.87 (4)	0.89 ± 0.11 (4)	0.12 ± 0.02 (4)
SAL 4 WKS	9.27 ± 1.08 (5)	1.31 ± 0.05 (5)	0.15 ± 0.01 (5)
METH 1 WK	0.71 ± 0.13 (15)**	0.55 ± 0.08 (15)*	0.91 ± 0.12 (15)**
METH 4 WKS	1.74 ± 0.22 (11)**	0.92 ± 0.07 (11)	0.58 ± 0.05 (11)**
HOT 1 WK	8.78 ± 1.04 (6)	0.98 ± 0.08 (6)	0.12 ± 0.01 (6)
HOT 4 WS	8.07 ± 0.64 (8)	1.12 ± 0.11 (8)	0.14 ± 0.01 (8)
COLD 1 WK	4.02 ± 0.30 (5)**	1.30 ± 0.14 (5)	0.33 ± 0.08 (5)
COLD 4 WKS	5.56 ± 0.62 (10)**	1.26 ± 0.11 (10)	0.24 ± 0.03 (10)

B. VLCD

Group & Time	DA (ng/g tiss)	DOPAC (ng/g tiss)	RATIO DOPAC/DA
SAL 1 WK	8.57 ± 0.86 (4)	1.42 ± 0.27 (4)	0.17 ± 0.03 (4)
SAL 4 WKS	6.91 ± 0.88 (6)	1.34 ± 0.17 (6)	0.20 ± 0.01 (6)
METH 1 WK	0.43 ± 0.05 (15)**	0.44 ± 0.07 (15)**	1.08 ± 0.13 (15)**
METH 4 WKS	1.41 ± 0.18 (11)**	0.97 ± 0.08 (11)	0.79 ± 0.11 (11)**
HOT 1 WK	8.20 ± 0.86 (6)	0.98 ± 0.09 (6)*	0.12 ± 0.01 (6)
HOT 4 WS	6.78 ± 0.82 (7)	1.52 ± 0.17 (7)	0.23 ± 0.05 (7)
COLD 1 WK	2.29 ± 0.36 (5)**	0.71 ± 0.06 (5)**	0.33 ± 0.05 (5)
COLD 4 WKS	3.78 ± 0.49 (8)**	1.20 ± 0.11 (8)	0.34 ± 0.04 (8)

Catecholamine content (Mean ± SEM, n per group), and the ratio of DOPAC/DA (mean ± SEM, n per group) in the DLCD **(A)**, or VLCD **(B)**. Animals were killed 1 or 4 weeks after repeated MA (15 mg/kg every 6 hrs for 4 injections) or saline administration. Data were analyzed by two-way ANOVA. Significant main effects were analyzed by the Newman-Keul's test. (* = $p < 0.05$ vs. SAL; ** = $p < 0.01$ vs. SAL).

Table IX. The Density of Presynaptic Labeling for GABA or Glutamate

Brain Region	Group	GABA 1 Week	GABA 4 Weeks	Glutamate 1 Week	Glutamate 4 Weeks
DLCD	SAL	100.00 ± 4.99 (7)	98.24 ± 6.20 (7)	100.00 ± 5.58 (7)	102.72 ± 4.81 (7)
	METH	67.71 ± 3.99 (7)**	106.01 ± 7.42 (11)	103.52 ± 5.19 (8)	101.05 ± 5.22 (11)
	HOT	84.63 ± 6.01 (6)	105.51 ± 6.02 (6)	103.77 ± 9.65 (6)	97.55 ± 3.35 (7)
	COLD	83.69 ± 4.67 (7)	110.84 ± 5.01 (7)	107.02 ± 6.08 (7)	102.45 ± 2.50 (7)
VLCD	SAL	100.00 ± 3.27 (6)	102.02 ± 6.17 (7)	100.00 ± 5.89 (6)	100.98 ± 5.99 (7)
	METH	55.51 ± 3.82 (7)**	139.00 ± 12.44 (11)**	100.40 ± 3.03 (7)	106.57 ± 4.64 (10)
	HOT	88.73 ± 6.35 (6)	93.08 ± 3.01 (7)	98.37 ± 8.53 (6)	92.70 ± 5.39 (7)
	COLD	106.57 ± 10.32 (7)	90.07 ± 1.85 (7)	88.29 ± 4.29 (7)	100.43 ± 5.73 (6)
GP	SAL	100.00 ± 5.32 (8)	96.06 ± 7.16 (7)		
	METH	61.89 ± 6.30 (8)**	132.96 ± 6.65 (10)**		
	HOT	86.09 ± 3.76 (6)	95.93 ± 6.42 (6)		
	COLD	90.44 ± 2.78 (6)	96.50 ± 7.15 (6)		

Particle density expressed as a percent of the SAL control group (Mean ± SEM, n per group) in presynaptic terminals analyzed for GABA or glutamate immunoreactivity. Animals were killed 1 or 4 weeks after repeated MA (15 mg/kg every 6 hrs for 4 injections) or saline administration. Data were analyzed by ANOVA followed by the Newman-Keul's test. (** $p < 0.01$ vs. SAL).

Table X. The Density of Mitochondrial Labeling in GABA or Glutamate Immuno-positive Nerve Terminals

Brain Region	Group	GABA 1 Week	GABA 4 Weeks	Glutamate 1 Week	Glutamate 4 Weeks
DLCD	SAL	100.00 ± 5.01 (7)	97.43 ± 12.43 (7)	100.00 ± 9.54 (7)	105.45 ± 10.00 (7)
	METH	105.14 ± 12.72 (7)	91.50 ± 8.86 (11)	118.29 ± 12.56 (8)	116.04 ± 10.98 (11)
	HOT	85.94 ± 13.61 (6)	82.28 ± 12.07 (6)	116.52 ± 11.78 (6)	103.41 ± 8.32 (7)
	COLD	80.17 ± 13.45 (7)	81.17 ± 9.42 (7)	120.33 ± 9.97 (7)	106.35 ± 9.34 (7)
VLCD	SAL	100.00 ± 7.73 (6)	95.02 ± 8.75 (7)	100.00 ± 13.82 (6)	98.44 ± 5.09 (7)
	METH	66.24 ± 12.86 (7)*	104.79 ± 10.62 (11)	134.06 ± 6.82 (7)	95.03 ± 11.38 (10)
	HOT	66.83 ± 6.64 (6)*	68.19 ± 10.35 (7)	142.22 ± 34.53 (6)	72.85 ± 10.80 (7)
	COLD	107.99 ± 4.81 (7)	80.09 ± 8.01 (7)	113.12 ± 5.44 (7)	88.96 ± 13.04 (6)
GP	SAL	100.00 ± 11.59 (8)	96.81 ± 5.43 (7)		
	METH	97.13 ± 13.32 (8)	94.89 ± 13.36 (10)		
	HOT	78.16 ± 9.37 (6)	93.34 ± 11.49 (6)		
	COLD	88.33 ± 10.43 (6)	76.69 ± 9.05 (6)		

Particle density expressed as a percent of the SAL control group (Mean ± SEM, n per group) in mitochondria associated with presynaptic terminals analyzed for GABA or glutamate immunoreactivity. Animals were killed 1 or 4 weeks after repeated MA (15 mg/kg every 6 hrs for 4 injections) or saline administration. Data were analyzed by ANOVA followed by the Newman-Keul's test. (* $p < 0.05$ vs. SAL).

Table XI. The Area of GABA or Glutamate Immuno-positive Nerve Terminals

Brain Region	Group	GABA 1 Week	GABA 4 Weeks	Glutamate 1 Week	Glutamate 4 Weeks
DLCD	SAL	0.48 ± 0.03 (7)	0.46 ± 0.02 (7)	0.36 ± 0.02 (7)	0.37 ± 0.01 (7)
	METH	0.51 ± 0.04 (7)	0.51 ± 0.05 (11)	0.36 ± 0.02 (8)	0.37 ± 0.01 (11)
	HOT	0.59 ± 0.03 (6)	0.48 ± 0.04 (6)	0.39 ± 0.02 (6)	0.41 ± 0.02 (7)
	COLD	0.50 ± 0.02 (7)	0.51 ± 0.03 (7)	0.36 ± 0.02 (7)	0.40 ± 0.01 (7)
VLCD	SAL	0.49 ± 0.03 (6)	0.44 ± 0.02 (7)	0.44 ± 0.02 (6)	0.39 ± 0.02 (7)
	METH	0.46 ± 0.03 (7)	0.53 ± 0.02 (11)**	0.37 ± 0.03 (7)	0.40 ± 0.02 (10)
	HOT	0.50 ± 0.04 (6)	0.45 ± 0.02 (7)	0.36 ± 0.01 (6)	0.37 ± 0.00 (7)
	COLD	0.52 ± 0.03 (7)	0.46 ± 0.02 (7)	0.44 ± 0.03 (7)	0.40 ± 0.02 (6)
GP	SAL	0.59 ± 0.02 (8)	0.60 ± 0.04 (7)		
	METH	0.60 ± 0.03 (8)	0.68 ± 0.03 (10)‡		
	HOT	0.60 ± 0.03 (6)	0.53 ± 0.03 (6)		
	COLD	0.58 ± 0.02 (6)	0.55 ± 0.03 (6)		

Areas (μm^2) (Mean ± SEM, n per group) of presynaptic terminals analyzed for GABA or glutamate immuno-reactivity. Animals were killed 1 or 4 weeks after MA (15 mg/kg every 6 hrs for 4 injections) or saline administration. Data were analyzed by ANOVA followed by the Newman-Keul's test. (** $p < 0.01$ vs. SAL; ‡ $p < 0.10$ vs. SAL).