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# Investigations into the Potential for 3,4-methylenedioxymethamphetamine to Induce Neurotoxic Terminal Damage to Serotonergic Neurons

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# INVESTIGATIONS INTO THE POTENTIAL FOR 3,4-METHYLENEDIOXYMETHAMPHETAMINE TO INDUCE NEUROTOXIC TERMINAL DAMAGE TO SEROTONERGIC NEURONS

A Dissertation Presented

by

DOMINIK BIEZONSKI

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# ABSTRACT

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High doses of 3,4-methylenedioxymethamphetamine (MDMA; “Ecstasy”) are known to reduce levels of various serotonergic markers outside of the raphe nuclei. To test the hypothesis that these deficits reflect a degeneration of distal axons/terminals, we investigated the effects of an MDMA binge (10mg/kg x 4) on the relative protein and genetic expression of several serotonergic markers in rats, as well as the effects of this compound on the quantity of serotonergic terminals in these animals. In experiment I, we examined whether MDMA alters serotonin transporter (SERT) levels as determined by lysate binding and immunoblotting analyses. Both methods of analysis revealed MDMA-induced reductions in regional SERT content. Experiment II investigated MDMA-induced changes in terminal-specific levels of SERT and the vesicular monoamine transporter 2 (VMAT-2) in the hippocampus, a region with sparse dopaminergic innervation, after lesioning noradrenergic input with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). Animals were administered 100 mg/kg DSP-4 or saline 1 week prior to MDMA (or saline). As determined by immunoblotting of synaptosomal tissue, the DSP-4/MDMA group showed little change in hippocampal VMAT-2 protein expression compared to DSP-4/Saline controls, despite large reductions in SERT levels in all regions examined in the MDMA-treated animals. Experiment III examined whether MDMA alters genetic expression of SERT and VMAT-2. When compared to saline-treated controls, animals given MDMA showed a striking decrease in SERT gene expression (and a lesser effect on VMAT-2) in dorsal/median raphe as assessed by quantitative RT-PCR. Experiment IV(a) investigated the effects of MDMA on gene and protein expression of tryptophan hydroxylase (TPH) in the hippocampus. Levels of TPH protein were unchanged between treatment groups, while transcript levels were decreased 15-fold in the dorsal/median raphe. In experiment IV(b), flow cytometry was used to measure whether MDMA alters the quantity of serotonergic terminals in the hippocampus. MDMA-treated animals showed an increase in the number of serotonergic synaptosomes identified by co-labeling for synaptosome-associated protein of 25 kDa (SNAP-25) and TPH. These results demonstrate that MDMA causes substantial regulatory changes in the expression of serotonergic markers with no evidence for synaptic loss, questioning the need to invoke distal axotomy as an explanation of MDMA-related serotonergic deficits.

Keywords: MDMA, neurotoxicity, neurodegeneration, serotonin transporter, vesicular monoamine transporter 2, tryptophan hydroxylase, serotonin, immunoblotting, gene expression, flow cytometry

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| | |
|---|---|
| 5,7-DHT | 5,7-dihydroxytryptamine |
| 5-HIAA | 5-hydroxyindolacetic acid |
| 5-HT | 5-hydroxytryptamine or serotonin |
| ADME | absorption, distribution, metabolism, excretion |
| AMPH | amphetamine |
| AUC | area under the curve |
| BGG | bovine gamma globulin |
| Cmax | maximum concentration |
| CNS | central nervous system |
| DA | dopamine |
| DβH | dopamine-beta-hydroxylase |
| DNTP | deoxynucleotidetriphosphate |
| DRN | dorsal raphe nuclei |
| DSP-4 | N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine |
| FACS | fluorescence-activated cell sorting |
| FITC | fluorescein-5-isothiocyanate |
| GABA | gamma-aminobutyric acid |
| GAP-43 | growth-associated protein of 43 kilodaltons |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| GFP | green fluorescent protein |
| HPLC | high-pressure liquid chromatography |
| HRP | horseradish peroxidase |
| HSP27 | heat-shock protein of 27 kilodaltons |
| HSP32 | heat-shock protein of 32 kilodaltons |
| i.c. | intracisternal |
| i.c.v. | intracereberoventricular |

| | |
|---|---|
| i.p. | intraperitoneal |
| IHC | immunohistochemistry |
| KRP | Kreb's-Ringer phosphate buffer |
| MDA | 3,4-methylenedioxyamphetamine |
| MDMA | 3,4-methylenedioxymethamphetamine |
| METH | methamphetamine |
| MRN | median raphe nuclei |
| NE | norepinephrine |
| NET | norepinephrine transporter |
| PAGE | polyacrylamide-gel electrophoresis |
| PBR | peripheral benzodiazepine receptor |
| PCPA | *para*-chlorphenylalanine |
| PD | postnatal day |
| PE | phycoerythrin |
| PET | positron emission tomography |
| PNS | peripheral nervous system |
| PVDF | polyvinylidine difluoride |
| qRT-PCR | quantitative real-time polymerase chain reaction |
| RIPA | radioimmunoprecipitation assay |
| RT | reverse transcriptase |
| s.c. | subcutaneous |
| SDS | sodium dodecyl sulfate |
| SERT | serotonin transporter |
| SERT-KO | serotonin transporter knockout |
| SNAP-25 | synaptosome-associated protein of 25 kilodaltons |
| $t_{1/2}$ | elimination half-life |
| $t_{max}$ | time to maximum plasma concentration |
| TPH | tryptophan hydroxylase |
| VMAT-2 | vesicular monoamine transporter 2 |

# CHAPTER 1

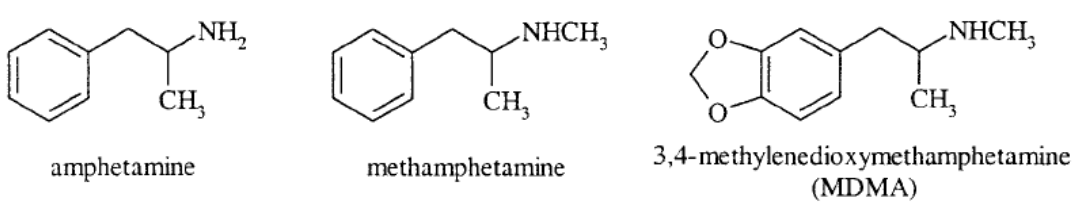
# INTRODUCTION

The illicit compound 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") is a ring-substituted amphetamine in the phenylisopropylamine family of substances (Lyles and Cadet, 2003). It is categorized as an "entactogen" primarily due to its empathogenic effects in human users, and given its ability to facilitate closeness and emotional insight was originally used clinically from the late 1960's through late 1970's as an adjunct to psychotherapy (Nichols, 1986). The use of this substance soon spread to the general population, and despite its reported utility in the therapeutic setting, MDMA (and 3,4-methylenedioxyamphetamine (MDA); parent compound) was criminalized in 1985 as a Schedule I substance due to its high abuse potential, lack of assessed safety for use under medical supervision, and emerging evidence suggesting MDMA and its derivatives as having "neurotoxic" effects in animals, particularly on the serotonergic neuromodulatory system in the brain (Ricaurte et al., 1985; Green et al., 2003). This seminal finding has subsequently given rise to a plethora of research examining the neurotoxic potential of MDMA, almost ineluctably leading to emergence of debate throughout the drug-abuse research community. Perhaps the most critical issues have dealt with how MDMA-neurotoxicity can or should be defined, determination of the mechanism(s) responsible for observed neurochemical changes following MDMA exposure, and concern surrounding the use of certain biochemical techniques as appropriate tools for assessing the capacity of this compound to cause neurodegeneration. More than twenty years later, these issues remain unresolved and further novel approaches are necessary to better our understanding of the adverse effects this compound may have on the serotonergic system.

## Chemical Structure and Mechanisms of Action

As its chemical name implies, MDMA bears the intrinsic structure of amphetamine (AMPH) with an *N*-methyl group and a methylenedioxy-ring substitution on the third and fourth carbon of the phenyl ring (see Figure 1 below). It is thought that this variation in structure is responsible for dichotomous effects on brain neurochemistry exerted by these two substances. More specifically, AMPH (and its derivative, methamphetamine (METH)) has potent, lasting effects on the dopamine (DA) neuromodulatory system while, in the long-term, MDMA affects mainly the serotonergic system. Additionally, MDMA more subtly affects the dopamine, norepinephrine (NE), gamma-aminobutyric acid (GABA), glutamate, and other systems as well (Green et al., 2003).

Figure 1. Chemical structure comparison of amphetamine, methamphetamine, and MDMA. Adapted from Green et al. (2003).




Acutely, MDMA causes a rapid, calcium-influx independent, transporter-mediated efflux of serotonin (5-hydroxytryptamine; 5-HT), NE, and DA from respective monoaminergic terminals. In terms of serotonergic release, this effect is mediated by the drug's interaction with both the plasmalemmal serotonin transporter (SERT) as well as with the intracellular vesicular monoamine transporter 2 (VMAT-2), both proteins being involved in the selective transport of 5-HT across phospholipid bilayers. More specifically, MDMA is a substrate for both SERT and VMAT-2, allowing it to enter the terminal and subsequently vesicles bearing 5-HT, respectively (Rudnick and Wall, 1992). Once inside the vesicles, the slightly alkaline nature of MDMA causes dissipation of the proton gradient between the vesicle and the cytosol necessary for proper functioning of VMAT-2, and in this respect, it inhibits VMAT-2-mediated influx and proper storage of 5-HT in the terminal (Sulzer and Rayport, 1990). Coupled with its ability to cause functional reversal of both VMAT-2 and SERT, MDMA allows 5-HT to passively efflux from terminal vesicles and subsequently from the neuron itself, ultimately leading to a global increase in extracellular 5-HT throughout brain regions bearing raphe afferents (Rudnick and Wall, 1992). This effect is further potentiated by MDMA-induced inhibition of 5-HT reuptake, a consequence of competition for SERT-binding by both 5-HT and MDMA.

## Evidence Supporting the Neurodegeneration Hypothesis

One of the most researched and controversial issues surrounding MDMA has been the empirical establishment of its potential to induce damage to serotonergic axons and terminals. Some of the most important studies targeting this question were performed by O'Hearn et al. and Battaglia et al. in the late 1980's and early 1990's, and have provided the basis for biochemical indexing of MDMA effects on the brain. These groups administered MDMA to rats according to what they considered a neurotoxic dosing regimen (20mg/kg, one dose every 12h; 4 consecutive days), and measured levels of 5-HT, DA, NE, and their respective major metabolites using high-pressure liquid chromatography (HPLC) two weeks after the final administration. This approach has commonly been used in the literature, and has revealed major decrements in 5-HT and 5-hydoxyindolacetic acid (5-HIAA; major metabolite) levels following MDMA exposure, with very modest, if not negligible, effects on levels of other neurotransmitters (Battaglia et al., 1987). The length and extent of these depletions following MDMA exposure is dependent on a variety of factors, ranging from differences in dosing regimens, to which enantiomer or derivative of MDMA is administered, routes of administration, inter-species differences in ADME (absorption, distribution, metabolism, and excretion) profiles, and many other factors (Ricaurte et al. 1988; Green et al. 2003). The effect of ambient temperature is also of particular importance, in that exposure to the drug in higher temperatures (>21-23°C) tends to cause hyperthermia and an exacerbation of neurotoxic effects while exposure in lower temperatures (<19-21°C) tends to yield a hypothermic response and an attenuation of these effects (Green et al., 2005). At around 21°C, previous findings from our laboratory indicate that MDMA causes temperature dysregulation wherein animals have an equal chance of becoming either hyper- or hypothermic (Piper and Meyer, 2006).

Battaglia et al. (1987) also performed assays targeting the integrity of 5-HT neurons, such as the assessment of binding potential of specific radioligands to the SERT protein in brain-lysate, plasma-membrane preparations. The idea behind analyzing SERT as a measure of neurotoxicity lies in the fact that this protein is specific to 5-HT axons and terminals, so disparities in its levels after MDMA exposure could be indicative of altered terminal integrity. In fact, this group, among many others thereafter, has demonstrated dramatic decreases in SERT-binding following various MDMA dosing regimens and post-administration periods of analysis. Importantly, these analyses have also demonstrated the effect of MDMA on serotonergic neuron 5-HT depletion and SERT levels to be region specific. For example, regions such as the striatum, hippocampus, and cortex seem to be affected more strongly than other regions such as the hypothalamus or certain thalamic nuclei (Battaglia et al., 1991).

Other studies have used brain sections for immunohistochemical (IHC) as well as autoradiographical analyses of anatomically-specific 5-HT axon/terminal fiber density following various MDMA regimens. Almost all IHC studies have used antibodies against 5-HT itself, though a few have stained for the SERT or tryptophan hydroxylase (TPH), the rate-limiting enzyme in the biosynthesis of 5-HT (Xie et al., 2006; Kovacs et al., 2007; Meyer et al., 2008). Irrespective of the marker used, analyses of forebrain immunoreactive raphe afferents following MDMA exposure have revealed evidence of transient drug-induced fiber “swelling” (particularly when stained for 5-HT) followed by long-term “degeneration”, especially of fine-caliber axons emanating from the dorsal raphe nuclei (DRN) (O’Hearn et al., 1988; Molliver et al., 1990). Large-varicose axons of passage originating from the median raphe nuclei (MRN) seem more resistant to these “neurodegenerative” effects, though it is difficult to discern whether the large varicosities visualized in these studies are indeed inherent to unaffected axons of passage or exist as a pathological effect of MDMA on fine-caliber axons (O’Hearn et al., 1988). Importantly, these analyses have failed to find any significant effect of MDMA on serotonergic cell bodies contained within the raphe nuclei, indicating that MDMA perhaps causes terminal-pruning rather than cellular death. Autoradiographical analyses of radioactively-labeled SERT fibers have shown similar results (Battaglia et al., 1991).

Studies focusing on long-term effects of MDMA have also used these techniques to demonstrate gradual post-MDMA recovery of 5-HT fiber density, which ostensibly may occur anywhere from 8 weeks to a full year, again depending on choice of dosing regimen and species used in the particular study, and brain region examined (Scanzello et al., 1993). Importantly, at least two research groups examining the long-term effects of MDMA have noted a hyperinnervation of 5-HT fibers in certain brain regions, a phenomenon perhaps resulting from initial terminal pruning or axonal damage (Ricaurte et al., 1992; Meyer et al., 2004).

## Evidence Against the Neurodegeneration Hypothesis

Some evidence also exists in opposition to the current presumption that MDMA causes distal axotomy of 5-HT neurons, particularly from the laboratories of O'Callaghan and Miller (1993), Pubill et al. (2003), as well as from Rothman et al. (Wang et al., 2004, 2005). O'Callaghan and Miller were the first researchers to measure glial responses to MDMA insult. In their original 1993 study, they compared glial responses following either d-MDMA or METH administration to mice by quantifying the levels of glial fibrillary acidic protein (GFAP), a structural protein intrinsic to astroglial cells. Their argument for GFAP expression as being indicative of neural degeneration rests on the fact that most insults upon the central nervous system (CNS) are usually followed by both degradative as well as neuroprotective events resulting from astroglial and microglial involvement. In particular, microglia tend to proliferate and migrate quickly to the site of injury following CNS insult, while astroglial cells typically hypertrophy locally over a longer time span (Pubill et al., 2003). Since astroglial hypertrophy is argued as a standard response to neural injury, and because this growth causes an increase in GFAP expression, the quantification of this protein has been used to assess different purported mediators of neural damage. Importantly, both the labs of O'Callaghan and Miller as well as Pubill et al. have found a disparity in the expression of neurotoxicity as measured by GFAP levels following either METH or MDMA exposure. More specifically, METH caused profound terminal degeneration (as measured by dopamine transporter binding) as well as significant increases in GFAP, while MDMA failed to invoke this increase despite causing a decrease in SERT-binding. Another measure of astrogliosis, the expression of heat shock protein 27 (HSP27), followed the same trend. Additionally, when microglial activation was measured by binding of [$^3$H]PK-11195 to the peripheral benzodiazepine receptor (PBR) as well as OX-6 immunoreactivity, MDMA failed to cause an increase in either of these measures (Pubill et al., 2003). As an important control to assess whether 5-HT neurons, following neurotoxic insult, are acted upon in the same manner by glia as other neurons in the CNS, Wang et al. (2004, 2005) demonstrated that damage to 5-HT neurons following the administration of 5,7-dihydroxytryptamine (5,7-DHT), an established serotonergic neurotoxin, lead to a significant increase in GFAP and heat shock protein 32 (HSP32) protein expression. This increase was not seen following MDMA administration and thus corroborated previous findings.

## The Neurotoxicity Controversy

Although much research has been devoted to demonstrating MDMA-induced neurotoxicity and elucidating its causal mechanisms, there exists some debate in the field as to whether or not MDMA is truly neurotoxic. Much of the problem lies in how researchers have defined what toxicity entails, as well as in the constant failure of many researchers to recognize alternative, rather than purely dogmatic interpretations of MDMA-induced serotonergic deficits as assessed by certain biochemical techniques. While many researchers have argued MDMA effects as being potentially neurodegenerative as evidenced by immunohistochemical and binding analyses, other researchers, particularly after the major Battaglia et al. studies, have resorted to quantitative analysis of 5-HT and its metabolites as sole indices of neurotoxicity. It has, until recently, become almost a formality to perform a certain study using a particular MDMA dosing regimen and to argue for "neurotoxic effects" when evidenced by merely a decrement in 5-HT or 5-HIAA. Although, in a broad sense, neurotoxicity may be viewed as a change in the neurochemical properties of a neuron (even in the absence of damage), others have strongly argued for neurodegeneration as a requisite part of its definition. Signs of neurodegeneration resulting from MDMA exposure have been assessed and documented in various ways, and include increases in silver staining, increases in Fluoro-Jade B staining, and, as mentioned, decreases in regional 5-HT-immunoreactive fiber density (Green et al, 2003). It should be noted that the first two methods are non-specific and thus their outcomes cannot be ascribed to MDMA effects on 5-HT neurons alone. For instance, silver staining analysis has long been used to visualize degenerating neurons which tend to develop an increased affinity for silver following various insults to the CNS (Jensen et al., 1993). Fluoro-Jade B is a novel, anionic fluorescent dye which seems to exclusively stain degenerating neurons irrespective of the origin of toxicity, although the exact mechanism for how this occurs is unclear (Schmued et al., 1997). Consequently, since these methods do not allow attribution of damage to a specific type of neuron, it cannot be ruled out that MDMA may be causing non-serotonergic neural degeneration.

Additionally, although SERT-binding as well as SERT-, TPH-, and 5-HT-immunoreactive fiber density is indeed reduced by MDMA, it is important to underscore that such analyses depend on the binding of radioligands and antibodies, respectively, to proteins that are liable to regulation. For instance, as discussed, MDMA causes depletion of both TPH and 5-HT, perhaps reducing levels of these antigens in IHC studies to below thresholds for detection, thus only giving the appearance of missing fibers. In the long-term, some evidence suggests that SERT gene expression may be negatively regulated by MDMA (Kirilly et al., 2007), which could lead to reductions in SERT-binding and immunoreactive fiber density in the absence of physical damage. Additionally, since binding assays typically make use of plasma-membrane preparations, it is possible that MDMA-induced enhancement of SERT trafficking by endocytosis (Carneiro and Blakely, 2006; Ramamoorthy and Blakely, 1999) may lead to decreases in plasmalemmal SERT-binding irrespective of altered terminal integrity, although this phenomenon has never directly been shown to be regulated by MDMA. Given these considerations, although many researchers argue that the depleting effects of MDMA on serotonergic markers reflect neurodegeneration, it is important to appreciate the possibility that most of these drug-induced serotonergic deficits can also be explained by other factors not necessarily dependent on axonal damage.

Recently, Wang et al. (2004, 2005) have suggested that MDMA, as well as other presumed 5-HT neurotoxins such as fenfluramine and *para*-chloroamphetamine, might not cause neurodegeneration since SERT protein expression in drug-treated animals, when measured by means of immunoblotting, did not change from levels expressed in control animals. After this initial 2004 finding, it was hypothesized that SERT-binding assays show decreases in SERT due to internalization (increased trafficking) of the protein rather than as an effect of terminal pruning, a process that might reduce the binding of radioligands to membrane-bound SERT proteins. In their 2005 follow-up, the group used differential centrifugation to further analyze SERT content from various subcellular compartments, including SERT embedded in plasma membranes as well as SERT compartamentalized in endosomes. Surprisingly, they found no differences in transporter protein expression (again, using immunoblotting analysis) between neural membranes or endosomes, suggesting that an increase in SERT trafficking following MDMA exposure probably does not occur and is therefore not a likely explanation for why binding assays show reductions in SERT. They concluded that perhaps MDMA causes SERT inactivation in some other way, possibly through interference between SERT and lipid rafts within the plasma membrane bilayer, structures recently suggested as being vital to SERT functionality (Magnani et al., 2004).

It is important to note that although the above Wang et al. studies are intriguing, upon closer scrutiny of their methods, one begins to question whether the group made careful use of appropriate controls necessary to correctly quantify SERT, identified initially as a 70 kDa (2004) and later a 50 kDa (2005) band. The importance behind this arises from the fact that SERT may exist in several different forms depending on glycosylation and oligomerization patterns, and since, as recent findings suggest, it undergoes proteolytic cleavage *in vivo* (Yamamoto et al., 1998; Dmitriev et al., 2005). Thus, it may appear as several protein bands of different molecular weight following immunoblotting, many (or all) of which may or may not correspond to intact, membrane-bound SERT. The appearance of non-specific bands near the hypothesized molecular weight of glycosylated, plasmalemmal SERT (~76 kDa; Qian et al., 1995) is also a problem which may occur with the currently available anti-SERT antibodies. Consequently, it is possible that the null effect of MDMA on SERT levels obtained in these two studies may have resulted from the inadvertent quantification of a non-SERT band. Given this, when immunoblotting for this protein, it has become crucial to initially use a set of specific controls in order to safeguard quantification of the correct band, or bands corresponding to membrane-bound SERT. Once this determination is made, only then can one apply an experimental manipulation and make confident claims regarding its effects on the expression of this serotonergic marker.

In this vein, Xie et al. (2006) used immunoblotting in conjunction with several validation measures to identify a genuine SERT band prior to determining the effects of MDMA on expression of this protein. In their preliminary validation analyses, the group measured SERT content from several brain tissue sources known to contain different amounts of the protein, including tissue from SERT-knockout mice (no SERT), tissue from rats treated with the 5-HT neurotoxin 5,7-DHT (low SERT), and tissues from various brain regions heterogeneous in SERT expression (for example, the parietal cortex contains high levels of SERT, while the cerebellum contains low levels of SERT (Kish et al., 2005)). The purpose of this analysis was to distinguish protein bands which changed densitometrically in predicted directions from static non-specific bands as a function of tissue source, the former presumably SERT in origin. Following identification of a genuine SERT “band”, major decrements were found in levels of this protein following

MDMA exposure, further corroborated by dramatic decreases in both SERT- and TPH-immunoreactive fiber densities. Although these findings have essentially contradicted those of Wang et al., several problems have limited the conclusiveness and possibly the validity of this study. First of all, whereas most SERT-blotting procedures use less than 60ug of protein per well (Wang et al., 2004, 2005), up to 200ug of protein was loaded in this study, creating a significant amount of background staining. Strikingly, the SERT "band" was defined as a "smear" spanning 5 kDa (63-68 kDa), much fainter than the surrounding, crisp protein bands, and which was inexplicably still somewhat present in the SERT-knockout tissue. Furthermore, the molecular weight range of this smear did not include the 76 kDa protein presumed to correspond to membrane-bound SERT. Lastly, the study suffered from inaccuracies in data presentation, though an erratum was offered in 2008 (Xie et al., 2008).

These findings, in conjunction with previously mentioned evidence, have made the question of whether or not MDMA is neurotoxic difficult to reconcile. Can 5-HT/5-HIAA depletions following MDMA exposure be considered definitive evidence for neurotoxicity, or should neurodegeneration be included? If neurodegeneration is requisite to the definition of neurotoxicity, can MDMA be considered neurotoxic in the absence of astrogliosis? Does SERT protein expression change following MDMA when measured by immunoblotting? If MDMA is indeed benign, how can it cause long-term hyperinnervation of certain brain regions?

## Overall Rationale for the Current Investigations

Given the number of studies demonstrating the capacity of MDMA to reduce various markers of 5-HT axons and terminals, the aim of this proposal was to test the hypothesis that these depletions occur as a consequence of MDMA-induced neurotoxic damage (i.e., axon and terminal loss) rather than as an effect of the compound on the expression of these markers in the absence of axotomy (i.e., biochemical down-regulation). To this end, we focused more specifically on the following four aims:

Specific Aim 1: The goal of the first experiment was to resolve the current dispute over the capacity of MDMA to alter protein expression of the SERT. Initially, by way of immunoblotting, we aimed to characterize a genuine SERT protein band(s) using several positive controls, allowing distinction of SERT from non-specific bands. These measures included analyzing SERT content in brain tissues processed from SERT-knockout mice (and wild-types), from rats treated with the selective 5-HT neurotoxin 5,7-DHT (or saline), and from various brain regions known to exhibit differential SERT expression. Following the establishment of an authentic SERT band(s), we aimed to measure the levels of SERT protein two weeks following an MDMA regimen known to significantly decrease SERT-binding in our laboratory. Since we hypothesize that the long-term effects of MDMA on 5-HT neurons are neurodegenerative in nature, it was expected that SERT levels would be reduced to a similar extent when measured by immunoblotting. In separate analyses, the convergence of these two methods in their ability to measure levels of this protein was also explored.

Specific Aim 2: The aim of the second experiment was to further establish the potential for MDMA to damage 5-HT terminals. Using immunoblot analysis of pinched-off terminals (synaptosomes), MDMA-induced changes in SERT levels were compared to levels of the vesicular monoamine transporter 2 (VMAT-2), a vesicular membrane protein expressed by serotonergic, dopaminergic, and noradrenergic neurons. Importantly, VMAT-2 is an established marker of terminal loss in such monoamine-specific lesioning diseases as Parkinson's disease, and thus comparing its levels to that of SERT helped distinguish whether or not MDMA may be causing distal axotomy. To develop a preparation in which VMAT-2 expression was limited mainly to serotonergic sources, we focused on the hippocampus because (1) this structure is only sparsely innervated by dopaminergic afferents, and (2) noradrenergic fibers and terminals could be selectively lesioned with the compound N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). One week following DSP-4 treatment, animals were given the same MDMA binge as in the previous study, and sacrificed two weeks later for analysis. Immunoblotting for SERT and VMAT-2 in the hippocampus was expected to reveal significant decrements in levels of both proteins, further suggesting that MDMA may damage 5-HT fibers.

Specific Aim 3: The aim of the third experiment was to address the major limitation of the previous experiment outlined in Specific Aim 2. Since uncertainty exists regarding the potential of MDMA to cause 5-HT terminal pruning, it is also possible that ascertained changes in post-MDMA levels of SERT or VMAT-2 may have in part been due to drug-induced alterations in the genetic expression of either protein. Thus, in order to gain a more unequivocal understanding of the effects of this compound on 5-HT terminals, it was necessary to investigate changes in not only protein levels but also the genetic expression of these serotonergic markers following MDMA. Only a few studies exist demonstrating the capacity for MDMA to alter SERT gene expression, and none have ever been performed to examine the compound's effects on VMAT-2. This study thus employed the use of quantitative, real-time PCR (qRT-PCR) techniques to measure SERT and VMAT-2 transcript levels two weeks following the same regimen of MDMA as in the previous two experiments. To ensure anatomical specificity, cryostat sectioning using specific landmarks allowed the identification of both the dorsal and median raphe nuclei, both of which contribute 5-HT fibers to the hippocampus. A tissue punch encompassing only these nuclei was then collected and processed for SERT/VMAT-2 qRT-PCR.

Specific Aim 4: In the first part of the fourth experiment, we aimed to determine whether other serotonergic markers known to be depleted following MDMA exposure were subject to similar neuroregulatory changes as were documented for SERT and VMAT-2 in our studies. To this end, we determined the effects of MDMA on the protein and genetic expression of the marker tryptophan hydroxylase. Importantly, to date, no study has ever used immunoblotting or qRT-PCR to measure changes in TPH protein and transcript levels after exposure to this compound. Thus, we analyzed the effects of MDMA on TPH protein expression by immunoblotting synaptosomal tissue (Sal/Sal and Sal/MDMA groups) from experiment II, while MDMA-induced alterations to TPH transcript levels were assessed by qRT-PCR analysis of dorsal/median raphe punches from experiment III. Given the potential for MDMA to reduce TPH activity and TPH-immunoreactive fiber density, levels of this marker as assessed by immunoblotting were expected to be similarly reduced, further supporting the notion that this compound may cause loss of serotonergic terminals.

Additionally, since very little direct evidence exists supporting the contention that MDMA causes physical damage to 5-HT axons and terminals, a final study was performed to examine the direct effects of this compound on quantity of serotonergic terminals. To this end, we made novel use of flow cytometry, a technique which is typically used to decipher and count cells based on a number of parameters, such as size, granularity, and the coincident detection of various fluorescent markers. Importantly, this technique can discriminate cells as small as fractions of a micron, thus allowing the analysis of synaptosomes. Two weeks following the same binge-regimen of MDMA as before, crude synaptosomal preparations were double-labeled with antibodies against SNAP-25 and TPH, and relative, drug-induced changes in the number of presynaptic serotonergic terminals (SNAP-25/TPH positive) were examined. Again, given the potential for MDMA to damage 5-HT projections, it was expected that binge administration of the compound would reduce the number of 5-HT-specific synaptosomes in drug-treated animals compared to controls.

# CHAPTER 2

# EXPERIMENT I: EFFECTS OF MDMA ON AXONAL/TERMINAL SERT PROTEIN EXPRESSION

## SERT-Immunoblotting Validation Measures: Identification of Genuine SERT Protein Band(s)

### Rationale

Since it is well established that MDMA causes long-term decreases in regional SERT-binding, this first study targeted the validity of using immunoblotting analysis to quantify SERT as an index of MDMA-induced neurotoxicity. To this end, three validation measures were performed in order to determine the correct band(s) for SERT quantification following SERT-immunoblotting. Since commercially available SERT antibodies seem to yield different band patterns and various extents of non-specific binding, each of these studies utilized a panel of different antibodies (see Appendix B) in order to determine which distributor or epitope variant yields the cleanest blots with an identifiable protein band(s) corresponding to SERT.

### SERT-Knockout Analysis

#### Background and Rationale

The first validation measure used B6.J mice with a targeted mutation in the gene encoding the SERT protein. These SERT-knockout (SERT-KO) mice are devoid of SERT in brain regions which normally express the protein, and thus were instrumental in determining which protein band(s) following SERT-immunoblotting were SERT in origin.  More specifically, SERT blots from the hippocampus and parietal cortex of wild type B6.J mice were compared to SERT blots from the same brain regions of SERT-KO mice, and the same molecular weight bands which appeared absent in the SERT-KO blots were considered corresponding to SERT.  Thus, this preliminary analysis allowed determination of which band(s) of particular molecular weight should be regarded as SERT, and therefore which should be quantified as a measure of SERT protein levels following experimentation with MDMA.

Experimental Design

Briefly, BJ.6 wild type (n=5) and BJ.6 SERT-KO mice (n=5) were sacrificed and brain tissue dissected as discussed in Appendix B.  The parietal cortex and hippocampus were collected and analyzed for levels of SERT by immunoblotting.

Results

Following immunoblot analysis of SERT content in the parietal cortex (Figure 2) and hippocampus (Figure 3), a broad band of predicted molecular weight (~76 kDa) present in lanes containing tissue from wild type animals was absent in lanes with protein from animals lacking the transporter.  It is noteworthy that this band occasionally resolved into a doublet.  Since the SERT-KO's are presumably devoid of their SERT protein, we inferred the missing band as SERT in origin.

Figure 2. Representative SERT blot comparing protein band patterns in the parietal cortex between wild-type ("WT") and SERT-KO ("KO") mice.

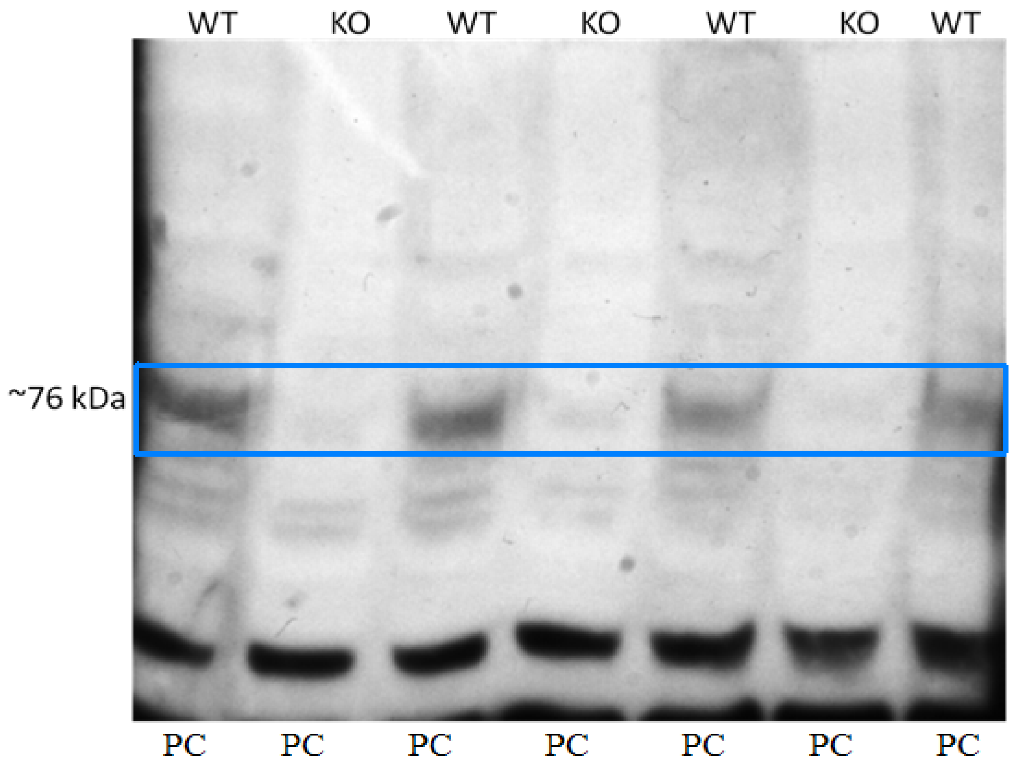
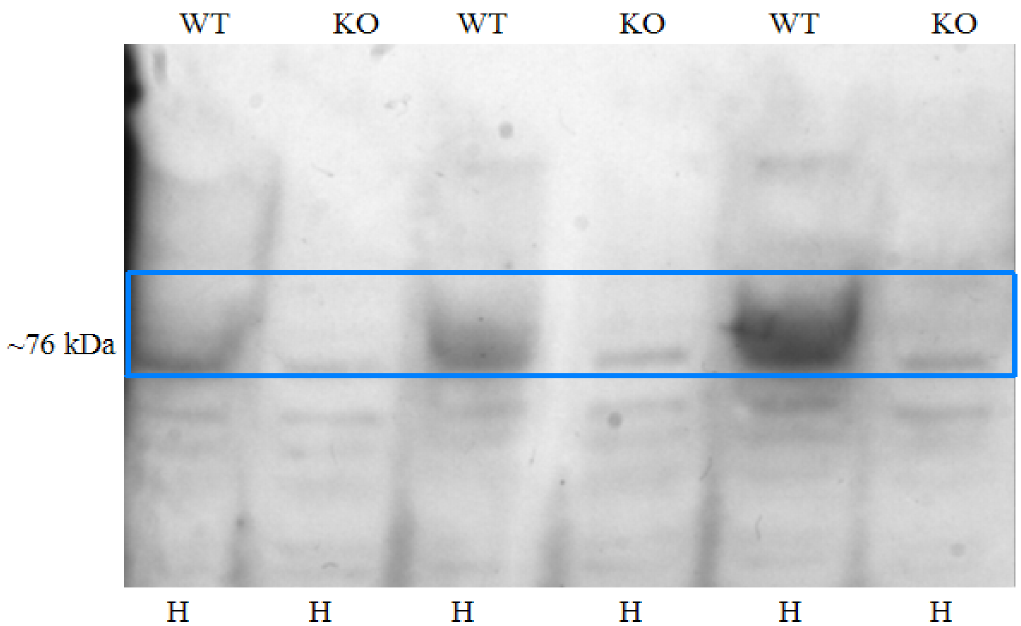



Figure 3. Representative SERT blot comparing protein band patterns in the hippocampus between wild-type ("WT") and SERT-KO ("KO") mice.



## 5,7-DHT Treatment Analysis

### Background and Rationale

Although the first validation measure was crucial in distinguishing SERT bands from non-specific antibody binding, it utilized mice rather than rats for comparison, bringing into question whether SERT and its resulting protein bands are in any way inherently different between these species. Because no strain of similar commercially available genetic knockouts for SERT exists in rat, the second validation measure used the compound 5,7-DHT, an established serotonergic neurotoxin, to substantially reduce SERT expression in rats relative to saline-treated controls, so as to compare and perhaps corroborate the SERT-immunoblotting results from the previous study. It was of particular interest to administer this compound to neonatal rats and quantify the resulting changes in SERT expression several weeks following exposure. This is important since, in contrast to administration in adults, 5,7-DHT administration to neonates causes permanent reductions in all previously mentioned markers of serotonergic integrity (including perikarya) with little potential for long-term compensatory regenerational sprouting (Lytle et al., 1973; Breese et al., 1975, 1978; Towle et al., 1984; Pranzatelli et al., 1989, 1992). This compound also has some neurotoxic effects on the NE system which can be prevented by pretreatment with the norepinephrine transporter (NET) inhibitor desipramine prior to 5,7-DHT administration, thus allowing selective lesioning of only the 5-HT system (Breese et al., 1978). Four weeks following this manipulation, we predicted SERT-immunoblotting to reveal decreased density of a SERT-specific protein band in lanes containing tissue from 5,7-DHT-treated animals relative to controls. Importantly, we expected this band to be at a similar molecular weight range as the band deemed SERT-specific in the mouse study, since the amino acid sequence is 97% homologous between both species (Chang et al., 1996).

Experimental Design

To circumvent intracereberoventricular (i.c.v.) infusion of the compound to adults, we administered the compound to neonatal rats since they lack an efficient blood-brain-barrier allowing peripheral administration (Towle et al., 1984; Pranzatelli et al., 1989). Though most of the previously discussed studies on 5,7-DHT have utilized the intracisternal (i.c.) or i.c.v. route of administration, evidence suggests that delivering the drug intraperitoneally (i.p.) leads to similar serotonergic neurotoxicity, particularly in the hippocampus (Pranzatelli et al., 1989, 1992). Importantly, i.p. administration seems to avoid convulsive effects in animals seen following i.c. or i.c.v. infusions of the compound (Pranzatelli et al., 1989). We thus administered 5,7-DHT to pups via this route.

Briefly, on day of parturition (postnatal day 0; PD0), litters were culled to 8 pups, and individual litters were randomly assigned into treatment or control groups (n=1 litter per group). On PD2, pups in the treatment group were administered 20mg/kg desipramine (i.p.) 60 min. prior to a 100mg/kg i.p. administration of 5,7-DHT. This treatment was repeated on PD5. Pups in control groups were given i.p. injections of saline vehicle on both days. All pups were housed with respective mothers until weaning, and then pair-housed until PD 35, at which point they were sacrificed as described in Appendix B. The hippocampus was dissected out from each animal, and the tissue from one side of the brain underwent immunoblotting for SERT, whereas the other hippocampal sample was processed for SERT-binding analysis to confirm efficacy of 5,7-DHT lesions.

## Statistical Analysis

The density of protein bands (% control) corresponding to SERT were compared between control and 5,7-DHT treated rats by means of Student's t-tests. SERT-binding results were analyzed in the same way. A $p<0.05$ was used to determine statistical significance.

## Results

Four weeks following 5,7-DHT treatment in neonates, we found significant SERT reductions in the hippocampus as measured by SERT binding ($t(13) = 6.463$, $p<0.0001$) (Figure 4). Following immunoblot analysis of SERT in this region, we identified two protein bands in lanes containing tissue from 5,7-DHT-treated animals which were significantly reduced in density compared to bands from saline-treated controls ($t(8) = 5.2204$, $p=0.001$) (Figure 5). Importantly, these two bands resolved at the same molecular weight range (~76 kDa) as the SERT-positive band identified in the previous measure, and thus were presumed SERT in origin.

Figure 4. SERT binding in hippocampus four weeks following 5,7-DHT (or saline) treatment.

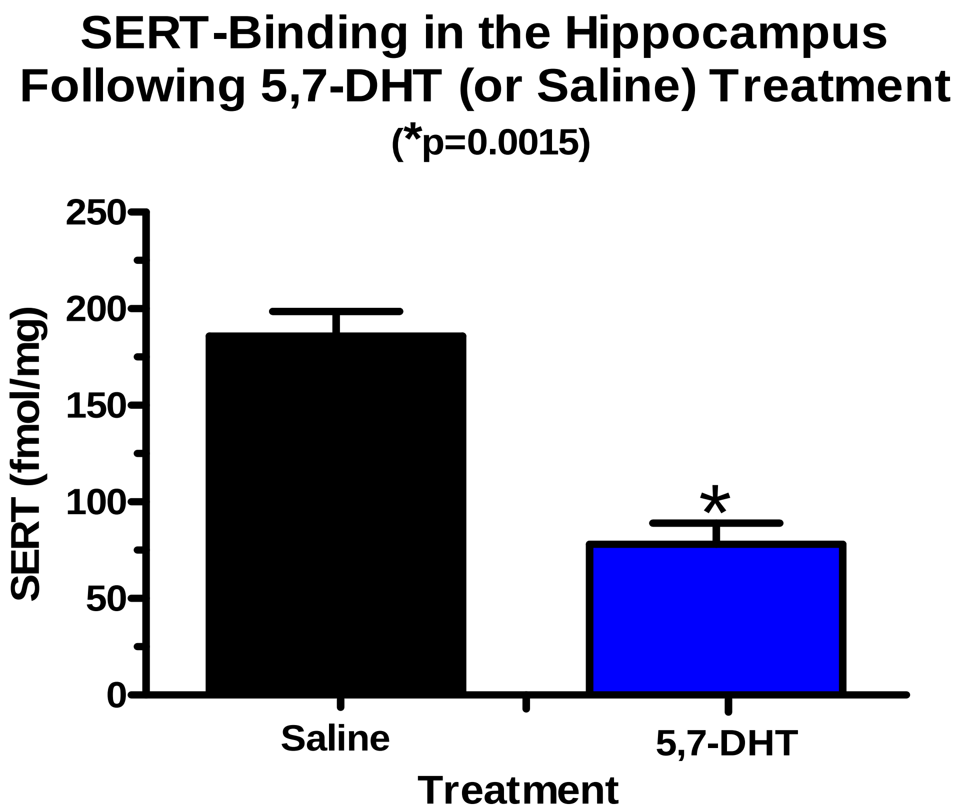
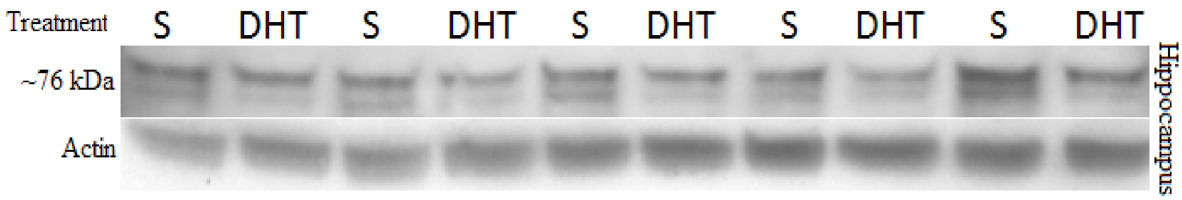
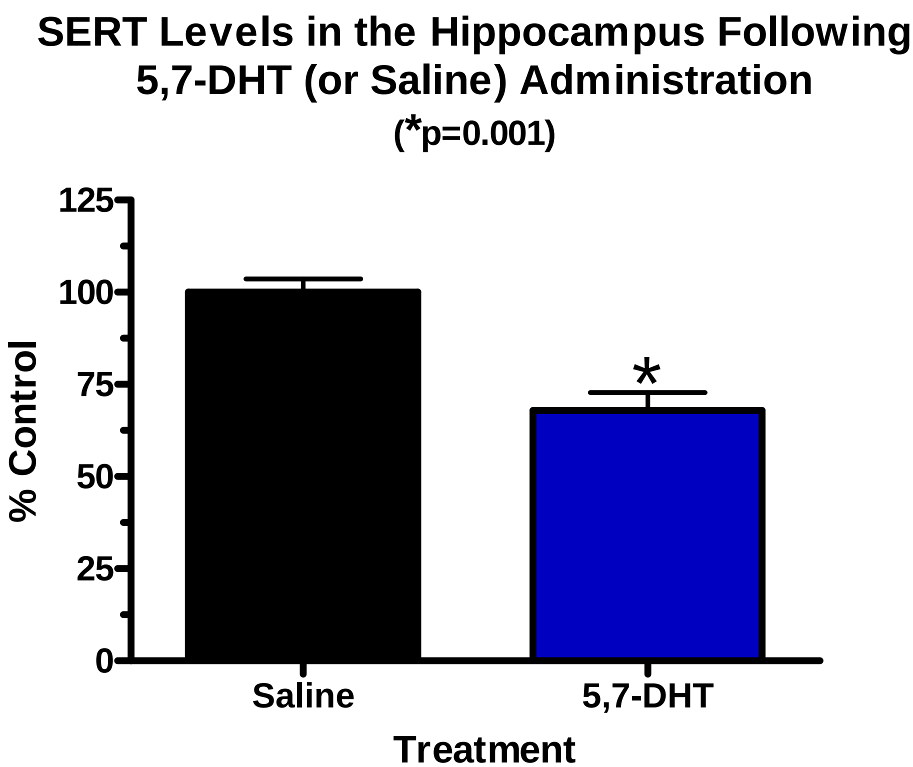


Figure 5. Representative SERT blot (a) and corresponding graphical comparison (b) comparing protein band patterns in the hippocampus between 5,7-DHT ("DHT") or saline ("S") treated rats.

(a)




(b)



## Regional SERT Distribution Analysis

### Background and Rationale

The final control study exploited the fact that serotonergic fibers exhibit regional differences in distribution, allowing analysis of which protein band(s) following SERT-immunoblotting change in optical density as a function of brain region and thus correspond to SERT. For example, serotonergic afferents are much more abundant in the parietal cortex than they are in the cerebellum, and thus we expected the SERT band from the cortex to have a much higher optical density than the band corresponding to SERT in the cerebellum (Kish et al., 2005). Other brain areas such as the striatum, hippocampus, and occipital cortex were similarly compared. Additionally, we compared regional expression of SERT by immunoblotting to regional differences in SERT as measured by radioligand binding assays. This allowed us to determine the convergence of these two methods in quantifying SERT.

### Experimental Design

Briefly, adult rats (n=4) were sacrificed and brain tissue was extracted as discussed in Appendix B. Unilateral parietal cortex, hippocampus, striatum, occipital cortex, and cerebellum were then collected and analyzed for levels of SERT by immunoblotting as well as SERT-binding analysis.

Statistical Analysis

Regional SERT expression as measured by SERT-immunoblotting and SERT-binding was analyzed for degree of methodological convergence by way of Pearson's product-moment correlation analysis. A $p<0.05$ was used to determine statistical significance.

Results

In this final validation measure, we found predicted changes in the density of a broad protein band following immunoblotting analysis of regional SERT content in adult animals (Figure 6), though a doublet was occasionally observed. This band differed in density (relative to other, non-specific bands) across regions and was relatively faint in the cerebellum, a region known to contain sparse 5-HT innervation. Importantly, this band was of the same molecular weight (~76 kDa) as those identified in the previous two validation measures, and taken together, presumably corresponded to genuine SERT. Additionally, we found a high, significant correlation ($\underline{r}(20) = 0.79$, $p<0.0001$) in regional SERT expression as assessed by SERT-binding and immunoblotting analyses (Figure 7).

Figure 6. Regional SERT expression as measured by SERT-immunoblotting. ST = striatum; PC = parietal cortex; HIP = hippocampus; OC = occipital cortex; CE = cerebellum

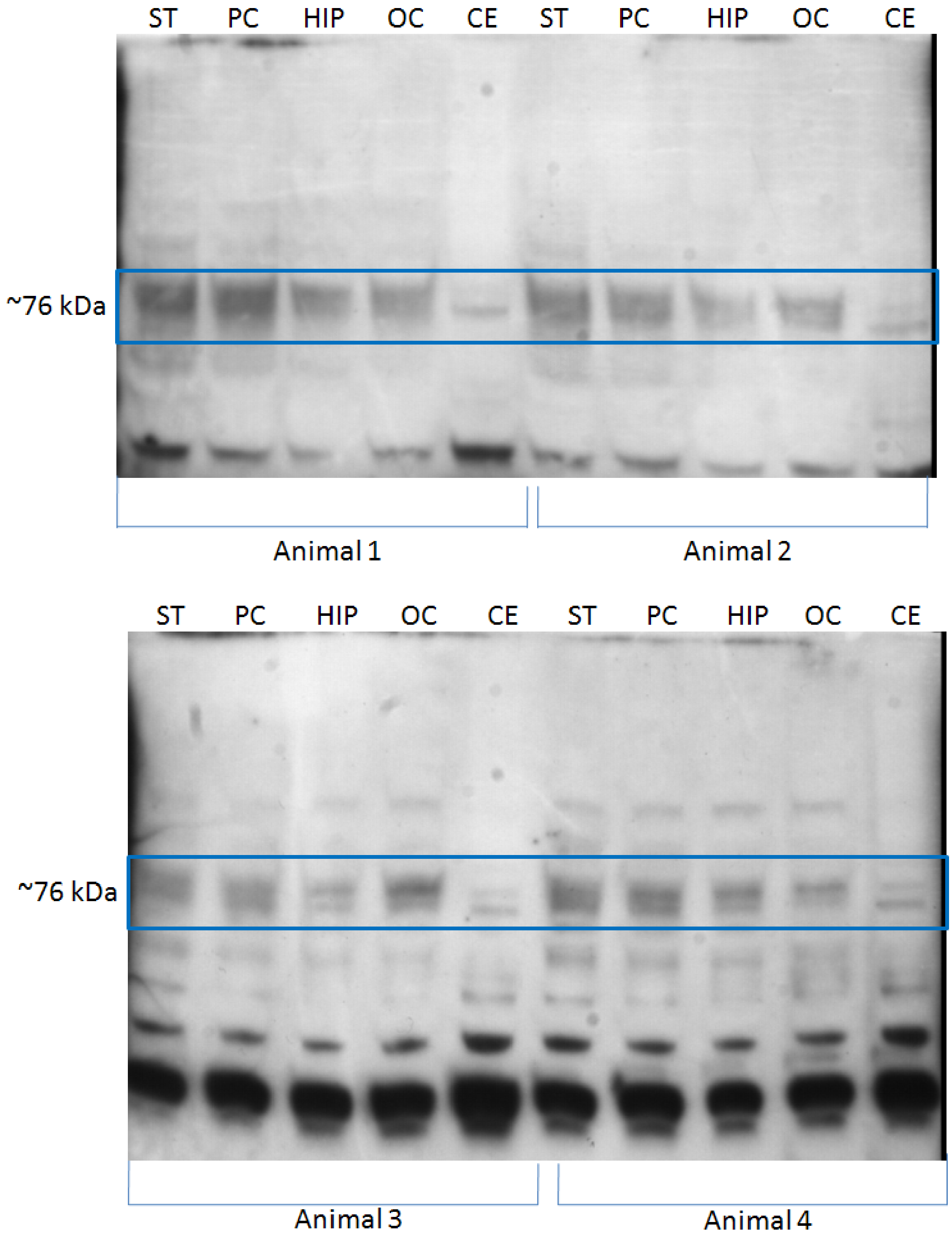
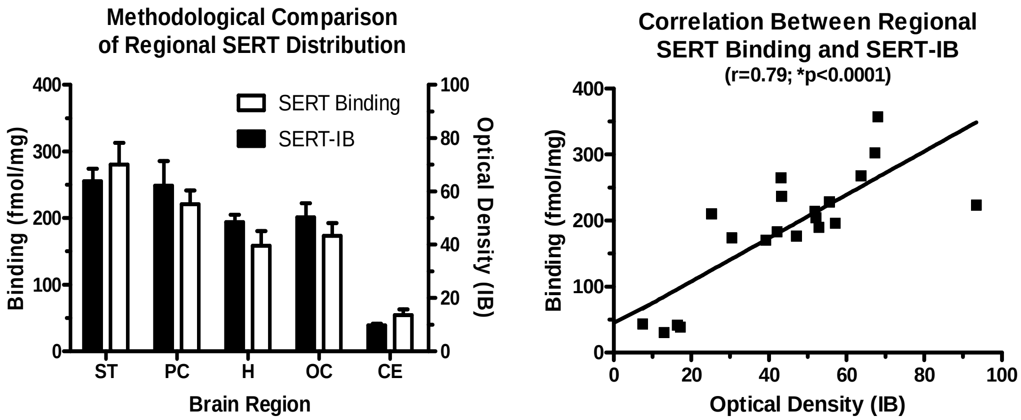


Figure 7. Comparison of regional SERT expression as assessed by SERT binding and immunoblotting.




## Overall Discussion

As described in Xie et al. (2006), it is crucial when using immunoblotting to validate the choice of band(s) selected for measurement to ensure that it corresponds to the authentic protein of interest. Based on comparison of protein band patterns from whole-tissue lysates derived from SERT-KO mice (vs. wild type), 5,7-DHT treated rats (vs. saline), and brain regions known to differentially express SERT in normal rats, we confirmed the identity of an authentic, broad SERT band near the predicted molecular weight of the protein (~76 kDa; Qian et al., 1995), though this band occasionally resolved into a doublet in our blots. The distinction between the two bands in the doublet is unknown but may be related to different phosphorylation states of the protein (Jayanthi et al., 2005). The ~76 kDa SERT band(s) was thus used to measure levels of the transporter in all subsequent experiments. Strikingly, only one antibody variant allowed establishment of a genuine SERT protein band (Table 1) in our study, indicating that some commercially available antibodies are seemingly not SERT-specific and should not be used when performing immunoblots for this protein. Given this, caution should be taken in interpreting the significance of SERT changes in publications utilizing antibodies that have failed to detect genuine SERT in our studies.

Additionally, we demonstrated a high correlation between regional SERT levels as measured by SERT-immunoblotting and SERT binding, indicating that both methods similarly measure levels of this serotonergic marker. This finding is important as it further reinforces the notion that either method can be used to accurately measure levels of genuine SERT.

Table 1. Comparison of SERT antibody distributors and epitope variants in their capacity to yield authentic SERT bands.

| Company/Product Name | Epitope | Yielded Authentic SERT bands? |
|---|---|---|
| Calbiochem | 579-599 (r) | Yes |
| Santa-Cruz (H-115) | 516-630 (h) | No |
| Santa-Cruz (C-20) | C-terminus (h) | No |
| Santa-Cruz (N-14) | N-terminus (h) | No |
| Santa-Cruz (24A5; mono) | C-terminus (r) | No |

<u>Effects of MDMA on Axonal/Terminal SERT Protein Expression</u>

Background and Rationale

By measuring the ~76 kDa band(s) previously identified as SERT, this study established whether levels of this serotonergic axon/terminal marker, as determined by immunoblotting, are indeed altered following doses of binge-MDMA known to significantly reduce SERT-binding. Two weeks following a treatment regimen frequently used in literature to analyze different parameters of MDMA-induced toxicity, levels of SERT were quantified from various brain regions including the parietal cortex, striatum, hippocampus, and occipital cortex, and were compared to levels expressed in control animals.

## Experimental Design

Briefly, adult rats (n=8) were treated with four subcutaneous (s.c.) injections of 10mg/kg ±MDMA (interdose interval of 1h), while control rats (n=8) received the saline vehicle only. MDMA dosing and rationale are further discussed in Appendix A. Temperature measurements were taken as described in Appendix B. Two weeks following drug administration, all animals were sacrificed, and their striatum, parietal cortex, hippocampus, and occipital cortex dissected free-hand for SERT-immunoblotting as well as binding analyses. These regions are commonly investigated in studies of MDMA neurotoxicity as they bear substantial serotonergic input and are highly susceptible to MDMA-induced serotonergic deficits (Green et al., 2003).

## Statistical Analysis

For every brain region, density of SERT bands (% control) as well as levels of SERT-binding between groups were analyzed by means of Student's t-tests. A $p<0.05$ was used to determine statistical significance.

## Results

Following repeated high-dose MDMA administration, the expected hyperthermic effect of MDMA was observed (for time 30, $t(14) = -2.395$, $p=0.031$; for time 240 (exemplary), t (14) = 7.343, p<0.0001) (Figure 8). MDMA treatment caused significant decreases in SERT binding in the striatum (t (12) = -3.214, p=0.007), hippocampus (t (12) = -6.093, p<0.0001), and the occipital cortex (t (12) = -11.232, p<0.0001) (Figure 9), as well as reduced SERT protein expression in these regions when measured by immunoblotting (see Figure 10 for striatum (t (12) = -3.322, p=0.0061) and hippocampus (t (12) = -4.117, p=0.0014); see Figure 11 for occipital cortex (t (12) = -5.645, p=0.0001); all results are graphically represented in Figure 12).

Figure 8. Body temperature during, and following MDMA (or saline) administration in experiment I(b).

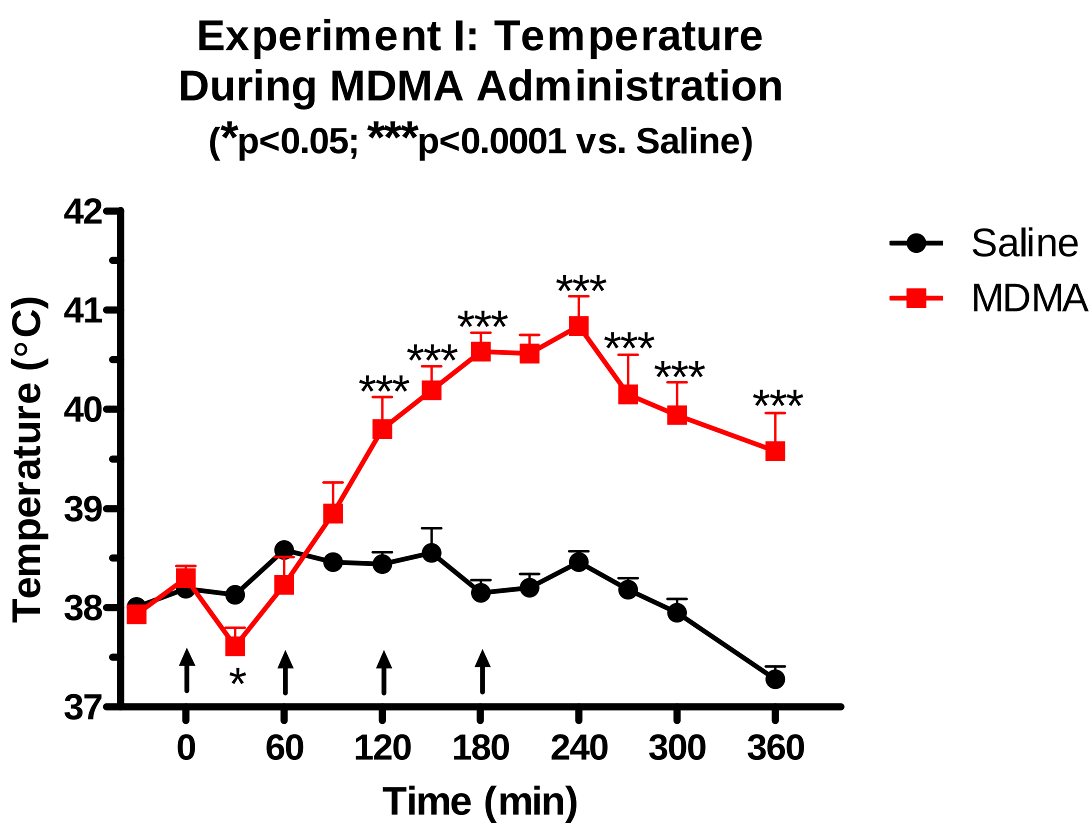


Figure 9. Regional SERT binding two weeks following MDMA (or saline) treatment.

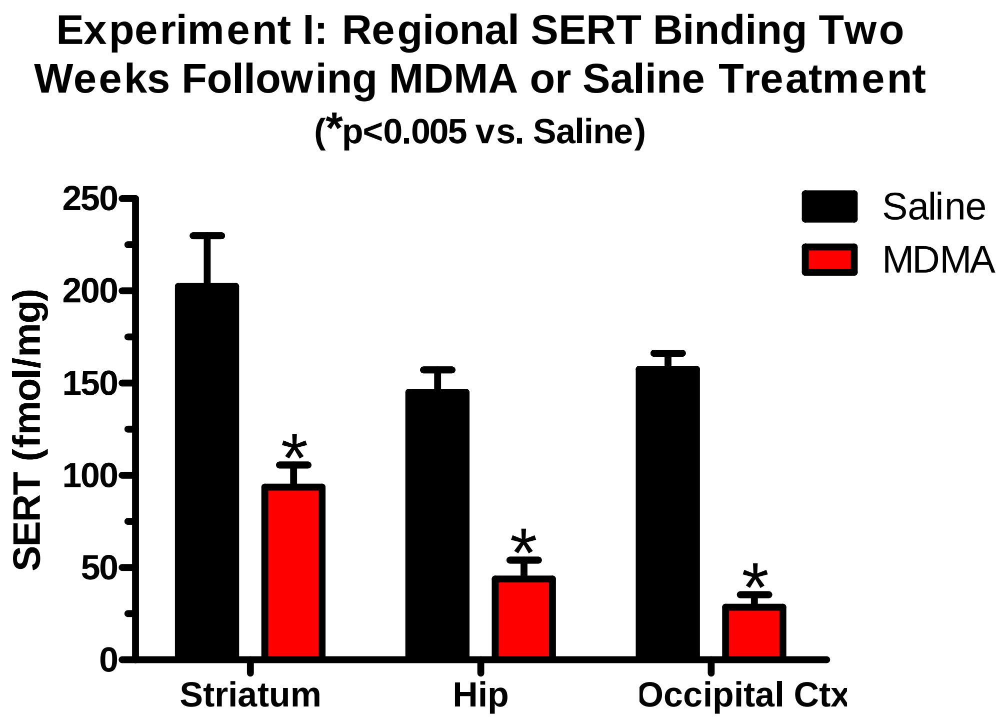



Figure 10. Representative SERT immunoblots from striatum and hippocampus of MDMA (or saline) treated animals.

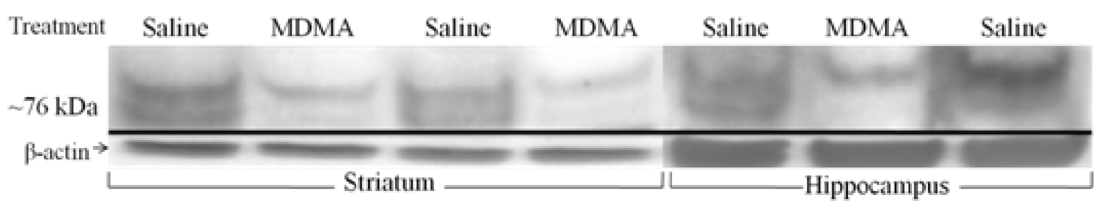



Figure 11. Representative SERT immunoblots from occipital cortex of MDMA (or saline) treated animals.

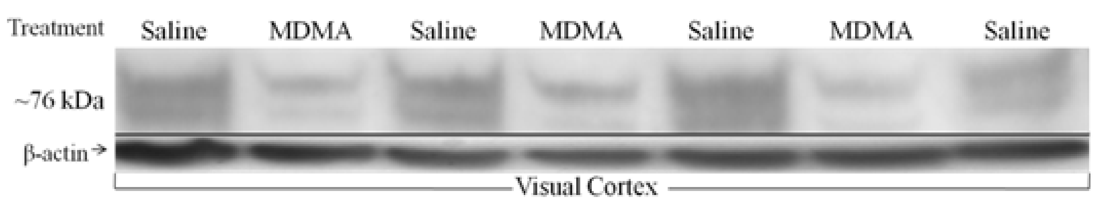
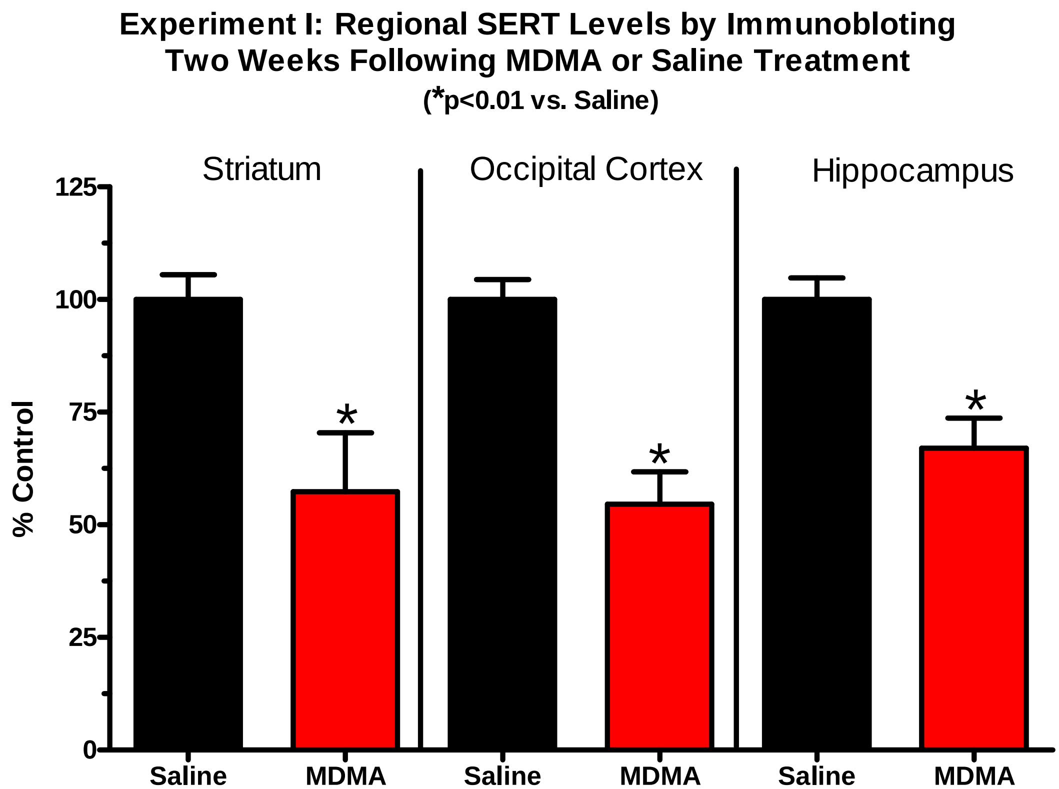


Figure 12. Graphical comparison of regional SERT levels in MDMA (or saline) treated animals as measured by SERT-immunoblotting.




Discussion

In contrast to the findings of Wang et al. (2004, 2005), MDMA administration to rats in our study lead to severe decrements in regional SERT levels when measured by either radioligand binding or previously validated immunoblotting methods. It is noteworthy that the effect of MDMA on SERT protein expression was smaller when measured by immunoblotting, perhaps indicating a lower sensitivity of this method to detect such changes. Notwithstanding, these results confirm that MDMA can produce deficits in this marker of serotonergic axons and terminals, though the question of whether these reductions occur in the presence of axotomy was further explored in experiments II, III, and IV.

Although serotonergic deficits following MDMA are well established in many brain areas (Green et al., 2003), only a few laboratories have investigated the effects of this compound on 5-HT neurons within the occipital cortex. Interestingly, it has been shown that MDMA can dramatically activate this brain region, perhaps underlying the enhancements in visual perception experienced by human users of the compound (Brevard et al., 2006; Meyer et al., 2006). Additionally, at least one group has demonstrated that this area can be particularly vulnerable to 5-HT neurotoxicity as evidenced by reduced SERT levels in humans when measured by positron emission tomography (PET) (Buchert et al., 2004). In support of the rat as a propitious translational model for the human effects of MDMA, we corroborated the capacity of this compound to reduce SERT levels in the occipital cortex of drug-treated animals. Given the paucity of research concerning 5-HT modulation of the visual system, it is difficult to surmise the consequences of SERT depletion in this region, underscoring the need for further studies to investigate the effects of this compound on visual processing.

# CHAPTER 3

# EXPERIMENT II: EFFECTS OF MDMA ON TERMINAL-SPECIFIC SERT AND VMAT-2 PROTEIN EXPRESSION

## Background and Rationale

The view that MDMA causes a distal axotomy of serotonergic neurons is based not only drug-induced reductions in biochemically-assessed serotonergic markers (i.e., 5-HT and SERT) but also on an apparent loss of serotonergic fibers visualized by means of 5-HT or SERT immunohistochemistry. Nevertheless, critics of this "neurodegeneration" interpretation argue that MDMA-related decreases in 5-HT or SERT (whether assessed biochemically or histochemically) could be due to a persistent down-regulation in these markers rather than a loss of serotonergic fibers. This argument is difficult to counter using only markers that are selective to serotonergic neurons. Consequently, in this experiment, we used a novel approach in which we investigated the effects of repeated MDMA treatment not only on SERT protein expression, but also on regional VMAT-2 expression as determined by immunoblotting. VMAT-2 is a vesicular transporter found in all monoamine neurons, notably those using 5-HT, NE, and DA. In these neurons, the protein is responsible for loading synaptic vesicles with the neurotransmitter synthesized by each cell type. As a vesicle-specific protein, VMAT2 is highly concentrated in nerve terminals and is known to be severely diminished by various monoamine neurotoxins and in neurological diseases (e.g., Parkinson's disease) that are associated with a loss of specific monoamine (in this case, DA) neurons (Miller et al., 1999). Thus, quantification of VMAT-2 allowed us to determine whether MDMA-induced changes in SERT levels were accompanied by changes in an established marker of terminal integrity.

It was of particular importance to measure levels of these proteins in the hippocampus, since it is known that this particular brain region is significantly affected by MDMA treatment (Battaglia et al., 1987), and more importantly, that it bears a specific distribution of monoaminergic afferents allowing the expression of VMAT-2 to be limited mainly to serotonergic sources. More specifically, it has been demonstrated that the hippocampus bears a high number of 5-HT and NE terminals, with a much lower abundance of DA terminals (~20-fold lower) (Hortnagl et al., 1991; Ciliax et al., 1995; Freed et al., 1995; Delis et al., 2004; Kitahama et al., 2007). Thus, introducing a selective lesion to NE neurons prior to administering binge-MDMA enabled determination of how this compound may affect the integrity of 5-HT terminals using an alternative marker to SERT.

The best studied method for achieving a selective NE lesion is with the compound N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). Importantly, DSP-4 can be administered peripherally to lesion NE terminals in the CNS without affecting NE neurons in the peripheral nervous system . Upon crossing the blood-brain-barrier, this substance acts as a strong alkylating agent which targets the norephinephrine transporter as well as structures within NE terminals, causing pruning of locus coeruleus afferents throughout the brain, an effect which peaks at 2-4 weeks following administration (Jaim-Etcheverry and Zieher, 1980; Fritschy and Grzanna, 1990, 1992; Schuerger and Balaban, 1995; Zhang et al., 1996). The inference that DSP-4 is indeed neurotoxic is based on consistent findings that it causes severe depletions in NE, *in-vivo* inhibition of [$^3$H]-NE uptake, and most importantly, lasting reductions in dopamine-β-hydroxylase (DβH; enzyme which synthesizes NE from DA) immunoreactive fiber density. Ultimately, the introduction of a selective NE-lesion using this compound in turn allowed quantification of VMAT-2 (and SERT) following MDMA administration, and attribution of changes in levels of this protein in the hippocampus as being mainly serotonergic in origin.

As part of this experiment, we initially aimed to validate immunoblotting for VMAT-2 since preliminary analyses in our laboratory yielded several protein bands that may or may not have corresponded to the actual VMAT-2 protein. Furthermore, whereas the manufacturer of the typically used anti-VMAT-2 antibody (Chemicon, CA) recommends quantification of a ~88 kDa band, all published studies to date have quantified a ~70 kDa band (Sandoval et al., 2002; Eyerman and Yamamoto, 2005), reported as the only protein band to change in parallel with VMAT-2 binding following exposure to substances known to reduce the levels of this protein, such as methamphetamine. Because of these ambiguities, as in the previous experiment, we performed a control study to determine which band(s) following VMAT-2 immunoblotting corresponds to genuine VMAT-2 protein so as to prevent erroneous quantification of a non-specific band. We reasoned that this could be achieved by comparing protein band patterns from animals lacking or having reduced levels of the transporter to normal animals, and identifying protein bands that change in density accordingly. However, since neither genetic knockouts (in rat) nor specific neurotoxins for VMAT-2 exist, an alternative approach was used. It is well known that the striatum bears dense DA innervation, and that afferents containing 5-HT or NE are comparatively sparse. Consequently, the majority of VMAT-2 in the striatum is harbored in DA terminals, which can be selectively lesioned by treatment with methamphetamine, a drug known to destroy DA terminals in this brain region (Kita et al., 2003). It was thus expected that administration of this compound would dramatically reduce levels of striatal VMAT-2 protein, allowing the identification of a genuine VMAT-2 band by immunoblotting as discussed above.

## Experimental Design

For VMAT-2 immunoblot validation, adult rats (n=5 per group) were administered s.c. injections of either ±methamphetamine HCl (Sigma Chemical Co., St. Louis MO) (4 x 10mg/kg, 2h interdose interval) or 0.9% saline vehicle. One week after the treatment all animals were sacrificed and striatal samples were obtained for analysis of VMAT-2 by immunoblotting.

In the general experiment, adult rats were administered either 100 mg/kg DSP-4 (i.p.) or saline 1 week prior to either a binge dose of MDMA or saline, thus forming four groups: Saline/Saline (n=8), DSP-4/Saline (n=6), Saline/MDMA (n=8), and DSP-4/MDMA group (n=6). During MDMA administration, temperature measurements were taken as described in Appendix B. All animals were sacrificed 2 weeks following the MDMA or saline binge, and the hippocampus (unilateral), striatum, and parietal cortex were dissected and processed for synaptosomal VMAT-2 and SERT immunoblotting (see Appendix B). The remaining hippocampal tissue underwent NET binding to confirm DSP-4 lesions.

## Statistical Analysis

Temperature measurements during MDMA administration were quantified by determining the area under the curve (AUC) of each temperature reading from 30min before and up to 3h following dosing. AUC measurements were then used to compare body temperature changes between treatment groups using Student's t-tests. Per brain region, the density of protein bands (% control) corresponding to SERT and VMAT-2 was also compared between groups by way of Student's t-tests. NET-binding was analyzed the same way. A $p<0.05$ was used to determine statistical significance.

## Results

Analysis of synaptosomal VMAT-2 levels in the striatum 1 week following the METH binge confirmed the identity of a single, ~70 kDa protein band corresponding to the transporter (Figure 13). This band was at the same molecular weight range as reported in other studies (Sandoval et al., 2002; Eyerman and Yamamoto, 2005) and was used to quantify VMAT-2 protein levels in this experiment.

During the MDMA binge, all MDMA-treated animals experienced hyperthermia compared to the saline-treated controls (Sal/MDMA group: $t(14) = 8.138$, $p<0.0001$; DSP-4/MDMA group: $t(13) = 2.671$, $p=0.0192$) (Figure 14, Figure 15), with a trend towards reduction in the extent of MDMA-induced hyperthermia seen in DSP-4 pretreated animals ($t(13) = 1.989$, $p=0.0682$) (Figure 14, Figure 15). The efficacy of DSP-4 in lesioning the noradrenergic innervation of the hippocampus was confirmed by a significant reduction in NET binding (~68%) in the DSP-4/MDMA group compared to saline controls ($t(12) = 12.99$, $p<0.0001$; for DSP-4/Sal vs. Sal/Sal, $t(11) = 11.02$, p<0.0001) (Figure 16). Immunoblotting revealed that hippocampal levels of synaptosomal VMAT-2 remained relatively unchanged in the DSP-4/MDMA group compared to DSP-4/Saline controls (see Figure 17(a) for representative blots, Figure 17(b) for graphical comparisons), despite a large reduction in regional SERT levels in all groups given MDMA (see Figure 18(a) for representative blots, Figure 18(b) for graphical comparisons; for statistical outcomes see Table 2).

Figure 13. Immunoblot comparing VMAT-2 levels in the striatum between METH- and saline- treated animals.

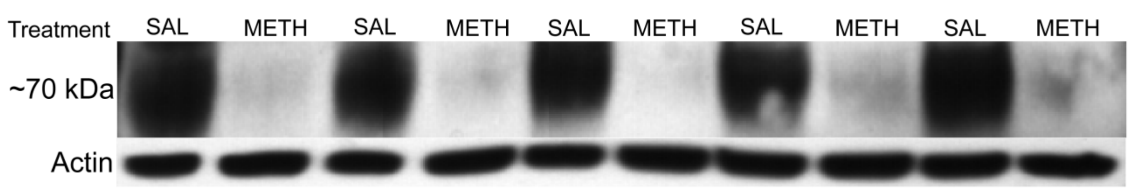



Figure 14. Body temperature during, and following MDMA (or saline) administration in experiment II.

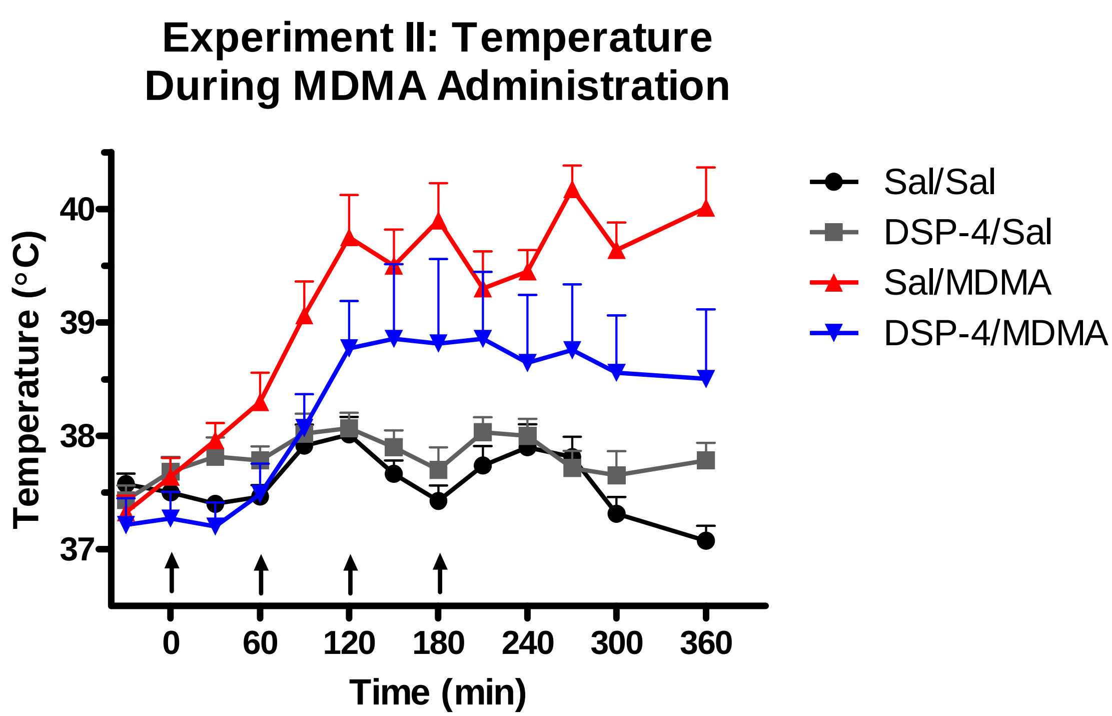


Figure 15. Area under the curve analysis of body temperature during MDMA (or saline) administration in experiment II.

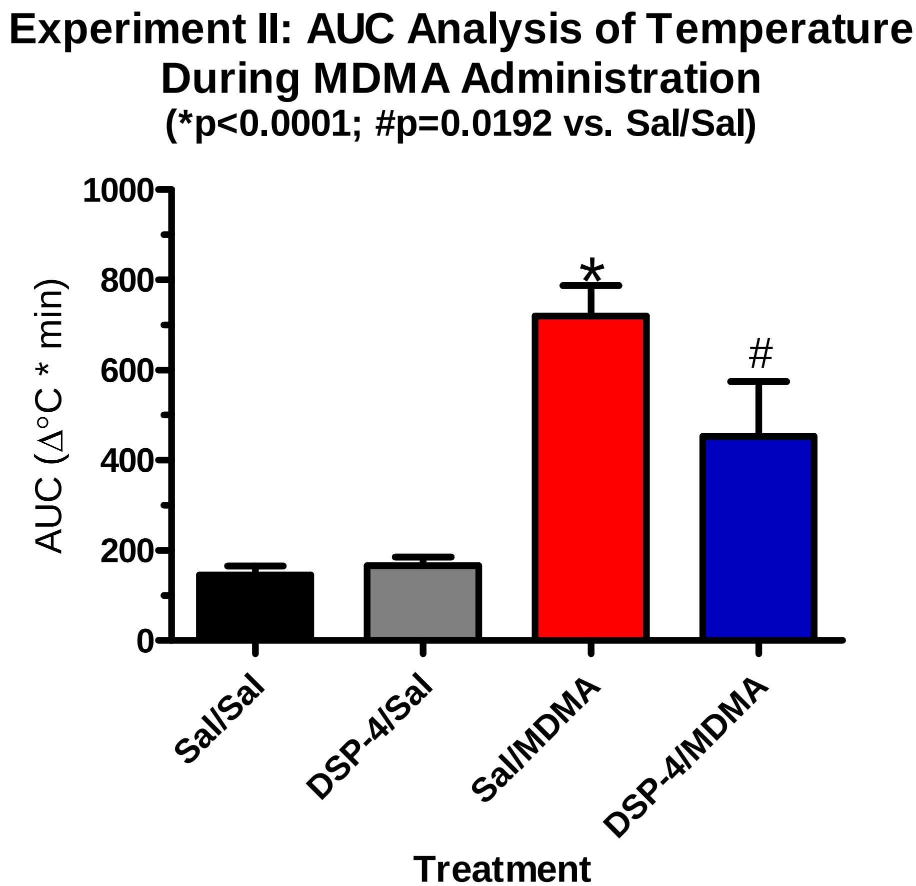


Figure 16. Norepinephrine transporter binding three weeks following DSP-4 (or saline) pretreatment.

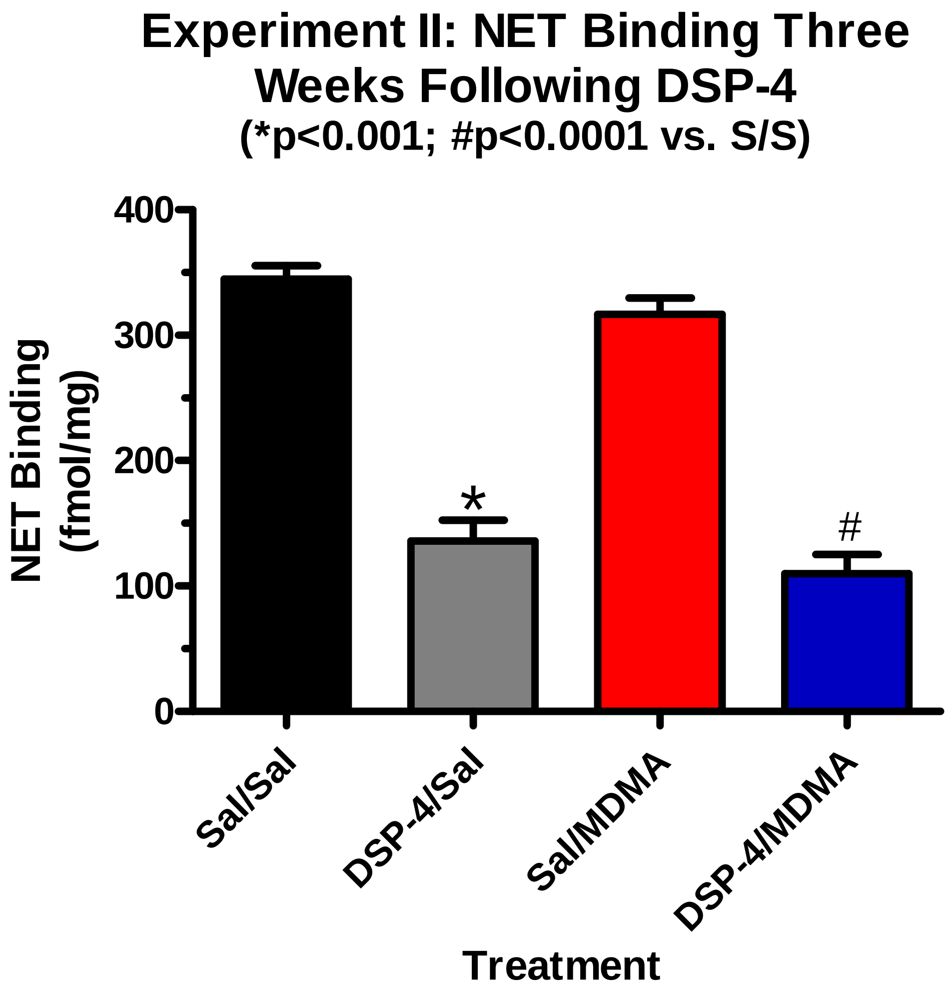


Figure 17. Representative blots (a) and corresponding graphical comparison (b) of VMAT-2 levels in striatum, parietal cortex, and hippocampus two weeks following all drug treatments.

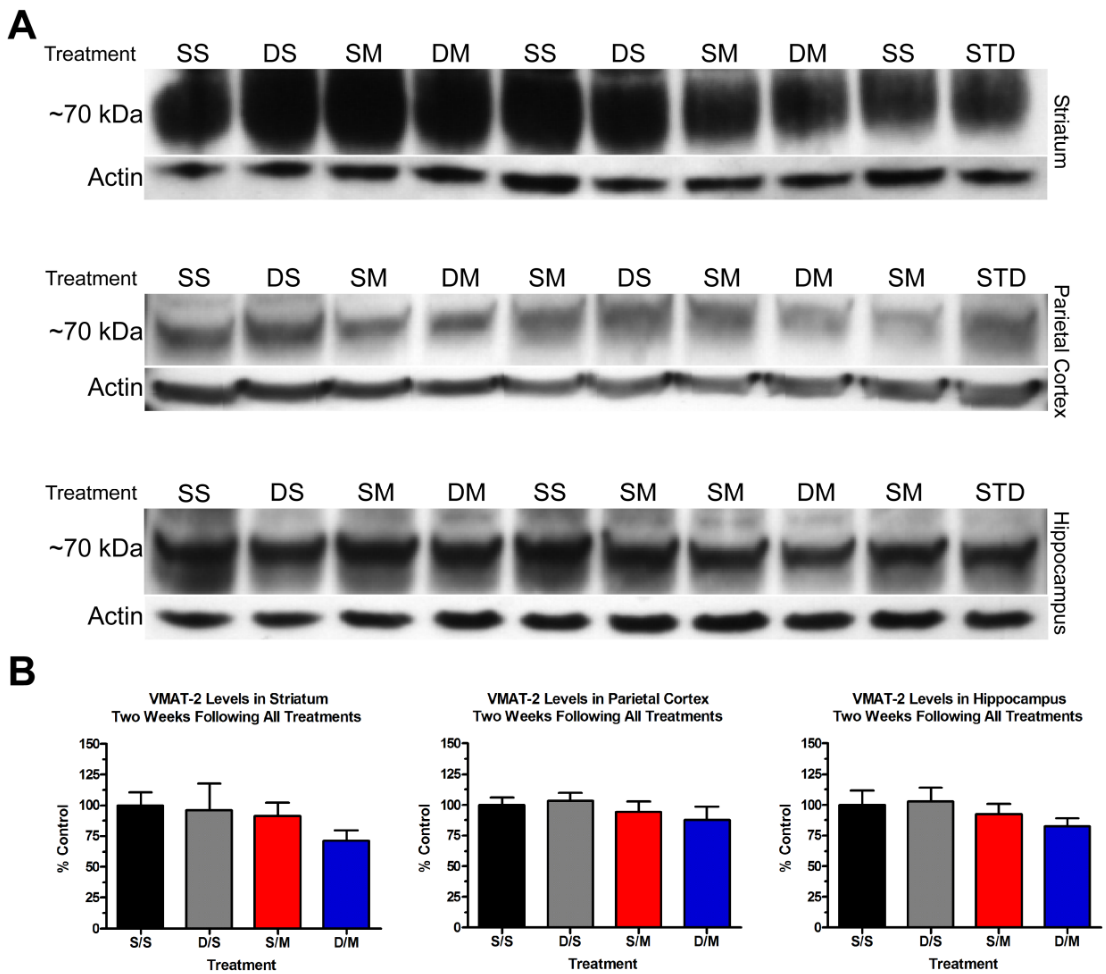


Figure 18. Representative blots (a) and corresponding graphical comparison (b) of SERT levels in striatum, parietal cortex, and hippocampus two weeks following all drug treatments.

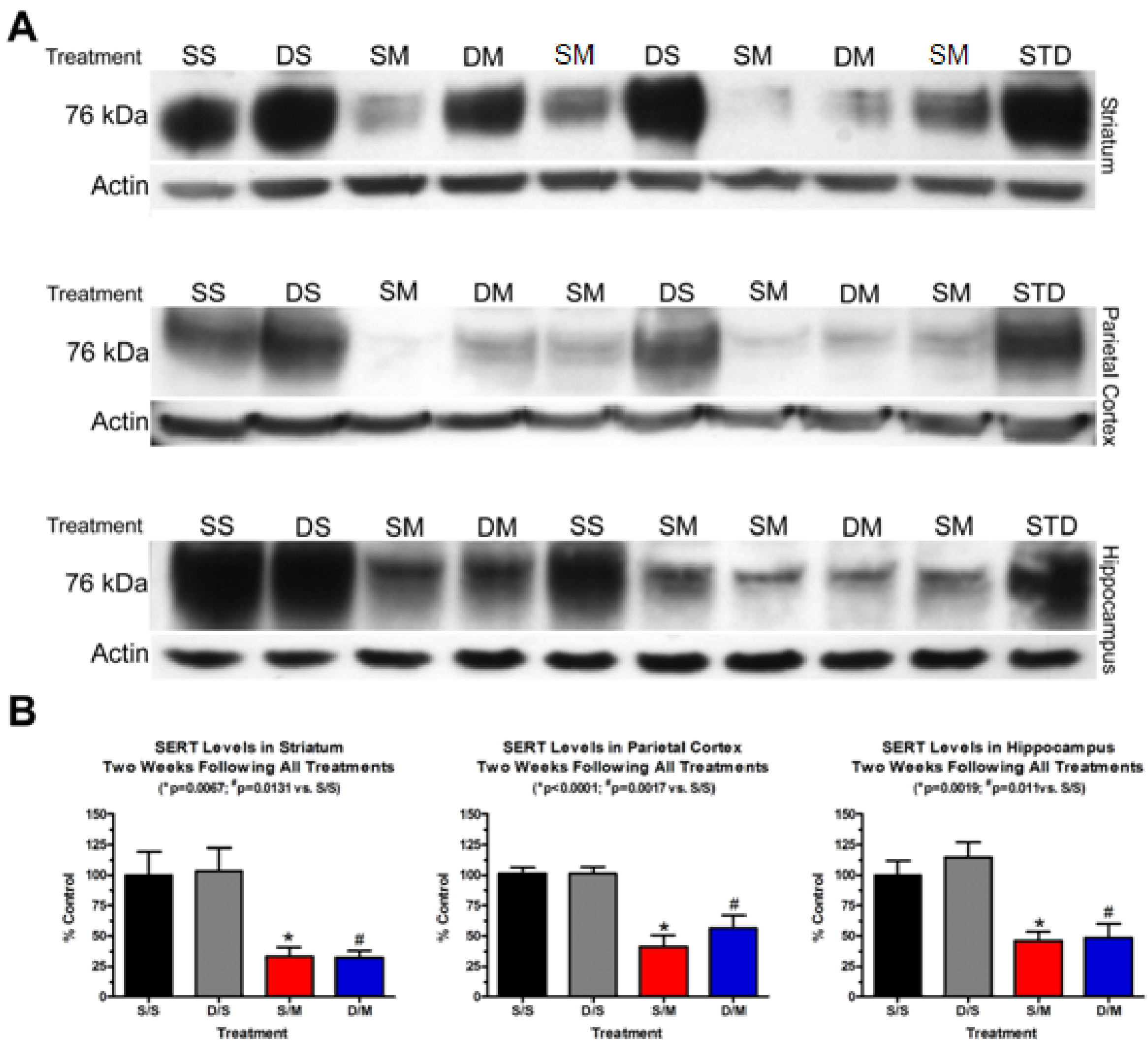


Table 2. Statistical comparison of regional SERT levels between drug-treated groups and saline-only controls.

| Brain Region | Group vs. Sal/Sal | df | t-value | **p-value** |
|---|---|---|---|---|
| Striatum | Sal/MDMA | 14 | 3.180 | **0.0067** |
| Striatum | DSP-4/MDMA | 12 | 2.910 | **0.0131** |
| Parietal Ctx | Sal/MDMA | 13 | 5.743 | **<0.0001** |
| Parietal Ctx | DSP-4/MDMA | 11 | 4.126 | **0.0017** |
| Hippocampus | Sal/MDMA | 14 | 3.817 | **0.0019** |
| Hippocampus | DSP-4/MDMA | 12 | 3.001 | **0.0110** |

## Discussion

As was previously documented in experiment I(b), MDMA treatment caused a significant rise in body temperature when compared to saline controls. Importantly, DSP-4 pretreatment only modestly reduced the extent of MDMA-induced hyperthermia, an important variable to consider given that this effect can undermine the neurotoxic potential of MDMA (Green et al., 2003). Nevertheless, it should be noted that the magnitude of reduction in SERT levels resulting from MDMA exposure was similar among all MDMA-treated groups irrespective of pretreatment conditions, indicating that the DSP-4-induced reduction in MDMA hyperthermia did not modify the degree of MDMA-induced neurotoxicity.

Three weeks following DSP-4 pretreatment, NET binding in the hippocampus of drug-treated animals was reduced substantially (~65-68%) compared to saline-treated controls. Assuming this decrease was reflective of DSP-4-induced distal axotomy of NE terminals, this analysis confirmed that the majority of VMAT-2 content in the hippocampus could be attributable to mainly serotonergic sources in our study. Importantly, however, as is the case with SERT and VMAT-2, NET is also a neural marker which may be liable to regulation following drug (in this case, DSP-4) exposure. This may also be true for DβH, which has been used as to stain NE neurons in IHC studies demonstrating the capacity for DSP-4 to presumably lesion noradrenergic projections from the locus coeruleus. Taken together, akin to ambiguities in interpreting the long-term sequelae of MDMA, it is also difficult to delineate whether the effects of DSP-4 are neurodegenerative or neuroregulatory. This is an important consideration given that we found no change in hippocampal levels of VMAT-2 in our DSP-4/Sal-treated animals, which would be expected to decrease given the potentially equal 5-HT:NE contributions of VMAT-2 in this region (Oleskevich et al., 1989; Oleskevich and Descarries, 1990). This in turn may be either reflective of the inability of DSP-4 to lesion NE fibers, or an upregulation of VMAT-2 protein in spared NE-terminals following DSP-4-induced axotomy, a possibility which may have normalized VMAT-2 levels in our hippocampal blots. The implications of this and other pertinent considerations are discussed further in Chapter 6.

Irrespective of the success of DSP-4 in lesioning NE input to the hippocampus, we found striking reductions in regional SERT levels in all MDMA-treated groups, with little relative change in the amount of VMAT-2 protein. With respect to the latter finding, this was expected to occur in the striatum and perhaps the parietal cortex, given that the extent of catecholaminergic contributions to VMAT-2 content in these regions (Reader et al., 1989; Kabani et al., 1990) may have provided enough background noise to preclude discernible reductions occurring in 5-HT neurons. In the hippocampus, however, all groups given MDMA experienced very little change in VMAT-2 protein levels, particularly in the DSP-4/MDMA group relative to saline-treated controls. Importantly, VMAT-2 levels were almost completely unchanged in the Sal/MDMA group, where modest reductions (~30% assuming equal 5-HT:NE contribution to global VMAT-2 content) would have been expected if serotonergic terminals were damaged by an order of magnitude relative to the extent of SERT depletions in this region (60%). This finding is in agreement with Fantegrossi et al. (2004) where VMAT-2 levels, measured by PET and radioligand binding analysis, were found to be unaltered in midline structures (thalamic/hypothalamic nuclei) and basal ganglia following MDMA self-administration in rhesus monkeys, indicating that terminal integrity may be unaltered following exposure to this compound. However, it should be noted that two other studies have examined the effects of MDMA on VMAT-2 levels, found to be decreased in the caudate/putamen and frontal cortex two weeks following repeated administration of the compound to baboons (Ricaurte et al., 2000), and in the striatum 24h following binge administration of the compound to Sprague-Dawley rats (Hansen et al., 2002). Though these studies suggest that MDMA may reduce VMAT-2 expression in monoaminergic neurons, it is important to emphasize that the identity of the affected neurons was unknown, and given the extensive VMAT-2 depletions in the DA-rich striatum (in both studies), it is likely that this effect may have occurred in non-serotonergic terminals (i.e. dopamine). Taken together, our data suggest that MDMA may cause substantial alterations in levels of SERT (and other serotonergic markers) but not necessarily in the presence of damage. In order to investigate a potential mechanism for this possibility, we further tested the effects of this compound on genetic expression of the SERT in experiment III.

As is the case with all serotonergic markers, it is important to note that VMAT-2 is yet another protein which may be liable to regulation following MDMA exposure, bringing into question whether this compound in any way modulated the expression of this terminal marker in our study. This is an important consideration since several possibilities exist to explain why VMAT-2 levels were only marginally altered in the hippocampus following DSP-4/MDMA exposure. For instance, it is possible that MDMA indeed caused terminal pruning, but that upregulation of VMAT-2 in remaining terminals (to enhance 5-HT sequestration and thus function) could have normalized the global VMAT-2 signal in our immunoblots. One mechanism by which this could occur is through either direct or indirect transcriptional upregulation of the VMAT-2 gene, which could in turn increase production of this protein. Conversely, is it just as likely that MDMA has little influence on VMAT-2 gene expression, suggesting that the lack of effect of MDMA on VMAT-2 protein levels may indeed be indicative of unaltered terminal integrity. Given these possibilities, we additionally measured the transcript levels of VMAT-2 in experiment III to examine whether MDMA exposure modulates the genetic expression of this marker.

# CHAPTER 4

# EXPERIMENT III: EFFECTS OF MDMA ON GENETIC EXPRESSION OF SERT AND VMAT-2

## Background and Rationale

Despite much research on the neurochemical effects of MDMA on 5-HT axons and terminals, very little research exists regarding MDMA-induced genetic changes in the expression of proteins necessary for the proper functioning of the 5-HT system. There has been only one comprehensive study on MDMA effects on the genetic expression of various 5-HT receptors (Kindlundh-Högberg et al., 2006), while only two efforts have described such changes in the expression of SERT (Kirilly et al., 2007; Li et al., 2006) and TPH (Bonkale and Austin, 2008; Garcia-Osta et al., 2004). Interestingly, whereas SERT gene expression seems to ultimately decline two weeks (Kirilly et al., 2007) and up to several months following MDMA (Li et al., 2006), TPH gene expression may be upregulated (Bonkale and Austin, 2008), presumably as a compensatory response to MDMA-induced perturbations to the 5-HT system (although see Garcia-Osta et al., 2004). Given the possibility that similar changes may occur in response to MDMA treatment in our animals, we investigated whether this compound alters gene expression of 5-HT markers utilized in our previous experiments so as to potentially elucidate whether the observed changes in their protein levels following MDMA result from neurodegeneration or neuroregulation. In particular, we compared changes in transcript levels of both SERT and VMAT-2 to respective post-MDMA alterations in hippocampal expression of these proteins as obtained in the previous study. To this end, real-time

PCR methods were used to quantify levels of SERT and VMAT-2 mRNA in combined tissue punches containing the dorsal and median raphe nuclei following binge-administration of the compound, since both regions contain serotonergic perikarya which project extensively to the hippocampus (Piñeyro and Blier, 1999). Importantly, these nuclei contain very few other monoaminergic neurons with the exception of a small population of DA-containing cells (Descarries et al., 1986), thus ensuring that VMAT-2 gene expression in the tissue punches would almost exclusively reflect activity in the serotonergic neurons (Piñeyro and Blier, 1999; Vertesa and Linley, 2007).

## Experimental Design

Briefly, adult rats (n=12) were treated with repeated doses of MDMA as before, while control rats (n=10) received the saline vehicle only. Temperature measurements were taken as detailed in Appendix B. Two weeks following drug administration, all animals were lightly anesthetized with $CO_2$ and decapitated. Brains were quickly removed, frozen, and processed for real-time PCR as discussed in Appendix B.

## Statistical Analysis

Body temperature during MDMA administration was quantified as detailed in Experiment II. SERT and VMAT-2 transcript levels (expressed as fold-difference in gene expression) in pooled dorsal and median raphe were compared across treatment groups by means of Student's t-tests. One outlier in the Saline/SERT group was detected using the extreme studentized deviation (Grubb's) method and was removed from all subsequent analyses. A $p<0.05$ was used to determine statistical significance.

## Results

During treatment, animals given MDMA experienced significant elevations in body temperature compared to saline-treated controls ($t(18) = 7.181$, $p<0.0001$) (Figures 19 and 20). Two weeks following drug administration, animals in the MDMA group exhibited a striking reduction (~50-fold) in SERT transcript levels ($t(16) = 2.619$, $p=0.0186$) with a smaller (~10-fold) but still significant reduction in VMAT-2 mRNA compared to saline-treated controls ($t(17) = 2.944$, $p=0.0091$) (Figure 21).

Figure 19. Body temperature during, and following MDMA (or saline) administration in experiment III.

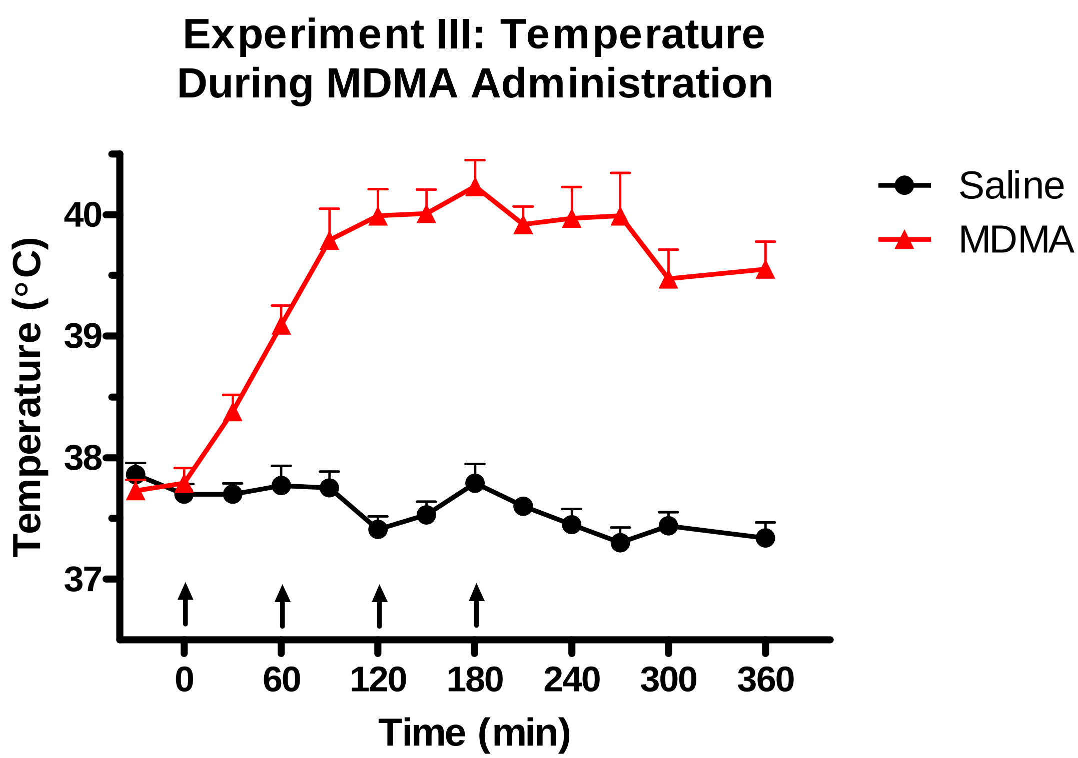


Figure 20.  Area under the curve analysis of body temperature during MDMA (or saline) administration in experiment III.

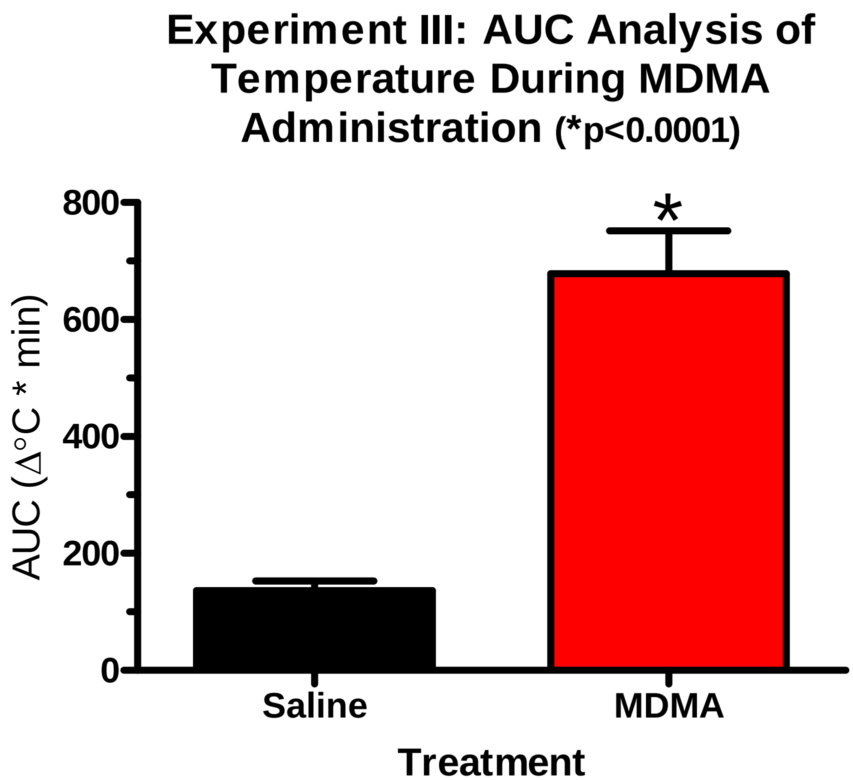
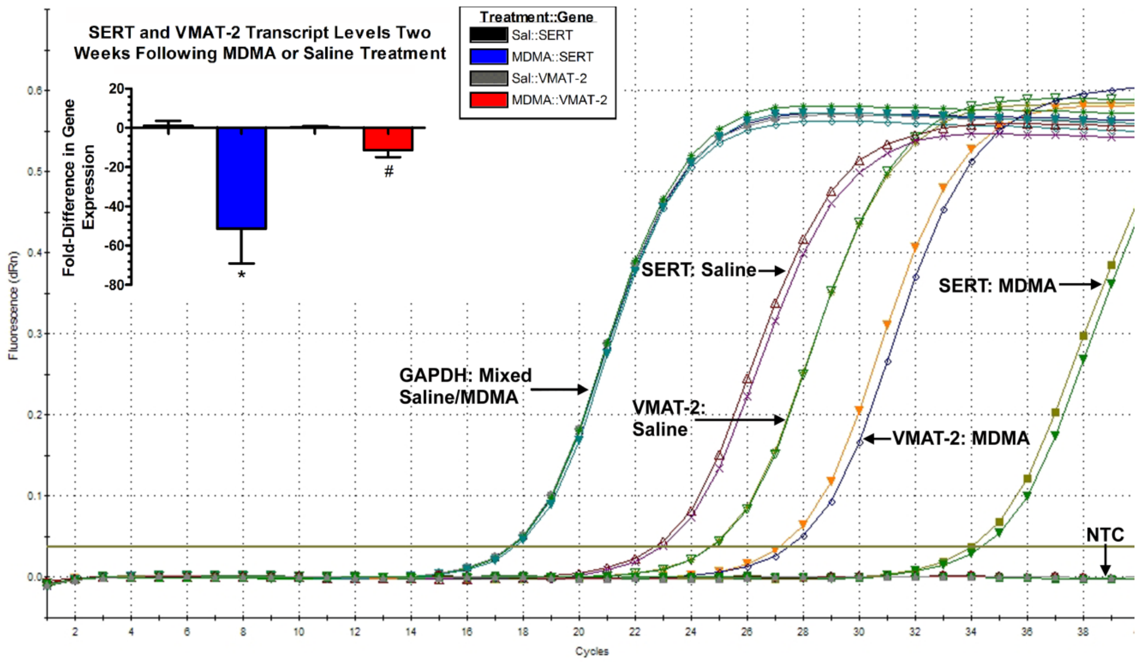


Figure 21. SERT and VMAT-2 transcript levels two weeks following MDMA (or saline) treatment. The plot shows representative amplification curves (n=2 per group) for SERT and VMAT-2 cDNA as a function of treatment, as well as curves for housekeeping-gene loading controls (GAPDH) and no template controls (NTC).




## Discussion

Two weeks following repeated administration of MDMA to our animals, levels of the SERT transcript measured by qRT-PCR were found to be tremendously depleted, corroborating a recent study which found reductions in SERT mRNA by in-situ hybridization 21 days following a single, high-dose of MDMA in Dark-Agouti rats (Kirilly et al., 2007). To our knowledge, this is the first study to measure mRNA levels of serotonergic markers by qRT-PCR, a technique far more sensitive to changes in gene expression than “qualitative” in-situ hybridization. This is reflected in our finding of a much greater deficit in SERT transcript levels following MDMA (50-fold) than was documented in the Kirilly et al. (2007) effort (25% in the median raphe), though it should be noted that our study employed binge treatment in Sprague-Dawley rats, whereas in the latter study Dark-Agouti rats (more sensitive strain) were treated with only one 15mg/kg dose of the compound. Given the magnitude of this effect (50-fold reduction), it is clear that exposure to MDMA can have devastating effects on the genetic regulation of key proteins necessary for the proper functioning of the serotonergic system. Assuming that the production of SERT protein is in turn severely compromised, this may at least in part account for MDMA-induced depletions of this marker as documented in experiments I and II, a scenario which could occur independent of distal axotomy.

It is interesting that the extent of SERT protein reductions (up to 65%) two weeks after drug exposure is disproportionate to the effect of this compound on SERT transcript levels (50-fold), indicating that a disconnect exists between transcript and protein levels of the transporter at this timepoint. A similar phenomenon has previously been documented in rats following *para*-chlorphenylalanine (PCPA: irreversible inhibitor of TPH)-induced serotonin depletion (Rattray et al., 1996). In this study, a single administration of PCPA lead to depletion in SERT mRNA 1 and 2 days following dosing, with no concomitant change in regional SERT binding 7 days later (though a decrease was documented on day 14). In both our study and that of Rattray et al., this apparent dichotomy may reflect a slow turnover rate of the SERT protein or perhaps an increase in the efficiency of SERT mRNA translation following drug exposure, though neither of these possibilities have ever been investigated for SERT in the long-term (see Vicentic et al. (1999) for information regarding acute SERT degradation rates; Kumer and Vrana (1996) for a similar example of the aforementioned phenomenon; Bolognani and Perrone-Bizzozero (2008) for general review). It is likewise possible that SERT-protein turnover rates may have been reduced in response to both MDMA and PCPA, possibly as an effect of altered post-translational modification of the SERT protein (Steiner et al., 2008). Additionally, since we assessed SERT transcript levels only at one time point following MDMA administration (2 weeks), it is also possible that post-MDMA deficits in SERT transcription may have commenced too close to this particular time-point to have more dramatic effects on SERT protein levels. Given this, investigating the effects of MDMA on SERT protein expression at a later time point (e.g. 4 weeks following dosing) may have revealed even more pronounced decreases in levels of this marker, assuming sustained reduction in SERT genetic expression during this timeframe.

In conjunction with dramatic effects on SERT gene expression, MDMA also caused a lower, albeit significant, reduction in levels of VMAT-2 mRNA (10-fold) two weeks following dosing. To our knowledge, this is the first study to document such changes in this terminal marker in response to MDMA treatment, and again attests to the dramatic effects this compound may have on the genetic expression of proteins important for serotonergic functioning. In contrast to reductions in SERT protein levels, MDMA caused little change in the relative levels of VMAT-2 in the previous experiment irrespective of MDMA-induced reductions in VMAT-2 gene expression at this time point (for similar example, see Takechi et al., 1998). This dichotomy may again have resulted from aforementioned factors such as post-administration increases in VMAT-2 translation despite a decrease in transcription, alterations in post-translational modification of the VMAT-2 product (Surratt et al., 1993), or as a consequence of slow turnover rates of the VMAT-2 protein, though these possibilities have yet to be investigated for this marker following exposure to MDMA.

Importantly, our finding of reduced VMAT-2 mRNA following MDMA exposure argues against the notion that VMAT-2 may have been compensatorily upregulated in spared terminals following MDMA-induced neurodegeneration, which in turn could have contributed to deceptive normalization in regional VMAT-2 levels as documented in experiment II. On the contrary, given the modest changes in VMAT-2 protein levels amidst massive reductions in protein and transcript levels of SERT, this finding suggests that the depleting effects of this compound on various serotonergic markers (i.e. SERT and 5-HT) may occur as a consequence of biochemical downregulation and not necessarily axotomy. Again, it is possible that enduring reductions in the genetic expression of VMAT-2 following MDMA may have ultimately resulted in lowered protein levels of this marker if analyzed at a later time point, though in this case we would not be able to discriminate whether such reductions occurred as a result of damage, compromised genetic expression, or both. Finally, as the results from experiment IV suggest, MDMA may cause acute, reactive synaptogenesis as evidenced by a significant increase in 5-HT-specific synaptosomes. If this were the case, perhaps levels of VMAT-2 would have been expected to proportionally increase (~38%), assuming that these new synapses were functional. As such, our finding of marginally lowered VMAT-2 content following MDMA may have actually underestimated the extent of VMAT-2 depletions in the previous experiment, reductions that may have indeed resulted from decreases in VMAT-2 genetic expression.

Overall, the results from this experiment indicate that MDMA can have substantial effects on the genetic expression of serotonergic markers, bringing into question the validity of assuming neurodegeneration as the only possible explanation for MDMA-induced reductions in levels of these proteins.

# CHAPTER 5

# EXPERIMENT IV: EFFECTS OF MDMA ON TPH EXPRESSION AND QUANTITY OF SEROTONERGIC TERMINALS

## Effects of MDMA on Protein and Genetic Expression of TPH

### Background and Rationale

As discussed in the previous chapters, our findings from experiments I-III suggest that MDMA may downregulate the expression of certain serotonergic markers (i.e. SERT) without necessarily inducing neurodegeneration.  Given this possibility, we investigated whether other serotonergic markers known to be depleted following MDMA exposure are subject to similar neuroregulatory changes as were documented for SERT and VMAT-2 in our studies.  To this end, we determined the effects of MDMA on the protein and genetic expression of tryptophan hydroxylase, the rate-limiting enzyme involved in the biosynthesis of 5-HT.  Importantly, deficits in expression of this marker following MDMA have been inferred mainly from studies showing drug-induced decreases in TPH activity (Schmidt and Taylor, 1987, 1988; Stone et al., 1988), though a few studies have also found post-MDMA reductions in serotonergic fiber density when stained for this protein (Adori et al., 2006; Xie et al., 2006; Bonkale and Austin, 2008). To date, no study has ever used immunoblotting to measure TPH levels after exposure to this compound, and as such, we measured changes in TPH protein levels with this technique using synaptosomal tissue (Sal/Sal and Sal/MDMA groups) from experiment II.

Additionally only a few studies have investigated the effects of MDMA on TPH gene expression, yielding contradictory findings. For example, two weeks following repeated administration of MDMA to Sprague-Dawley rats (20mg/kg twice daily for 4 days), Bonkale and Austin (2008) noted increased levels of TPH mRNA in dorsal raphe by in-situ hybridization. In contrast, Garcia-Osta et al. (2004) found TPH mRNA levels to be decreased in the hippocampus 2h and 48h following a single high dose of MDMA (15mg/kg) to Dark Agouti rats, though the functional significance of distal transcripts in axons and terminals is unclear. Given these ambiguities, we additionally measured the transcript levels of TPH by qRT-PCR using the same dorsal/median raphe RNA extracts as used in the previous experiment.

## Experimental Design

For validation of TPH-immunoblotting, protein band patterns in the hippocampus were compared between animals previously treated with 5,7-DHT or saline (n=4 per group) (see experiment I(a)). Levels of the TPH protein were measured by immunoblotting while transcript levels (TPH-2, predominant isoform in the rat brain (Sakowski et al., 2006)) were measured by qRT-PCR using synaptosomal tissue and RNA samples from experiments II and III, respectively. For TPH-immunoblotting, the Sal/Sal group was compared to the Sal/MDMA group.

## Statistical Analysis

All analyses between treatment groups were performed by means of Student's t-tests. A $p<0.05$ was used to determine statistical significance.

Analysis of protein band patterns in the hippocampus of 5,7-DHT (or saline) treated animals revealed a TPH-positive band of ~47 kDa (Figure 22(a); Figure 23; (t (6) = 2.327, p=0.0589)). This band was used to quantify TPH levels in all subsequent analyses. MDMA had no effect on protein expression of TPH in he hippocampus when analyzed two weeks following MDMA treatment (Figure 22(b,c); Figure 24). Transcript levels of TPH were significantly reduced (~15-fold) in dorsal/median raphe of MDMA-treated animals compared to controls (t (15) = 2.217, p=0.0425) (Figure 25).

Figure 22. Immunoblots comparing TPH levels in the hippocampus between animals treated with (a) 5,7-DHT or saline, and (b,c) MDMA or saline.

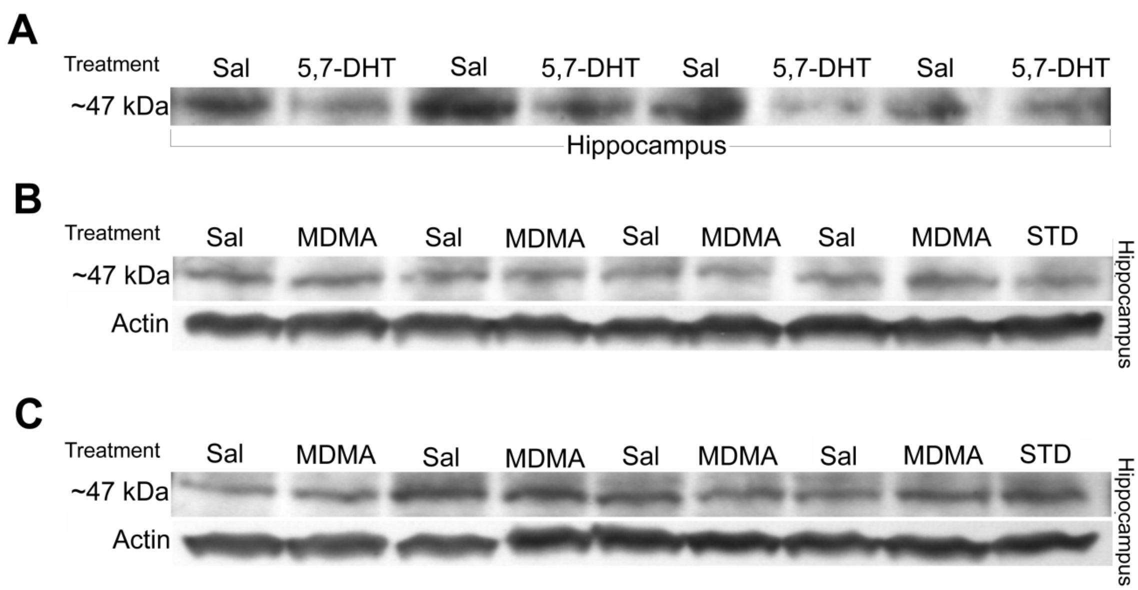


Figure 23. TPH levels in the hippocampus four weeks following 5,7-DHT (or saline) treatment.

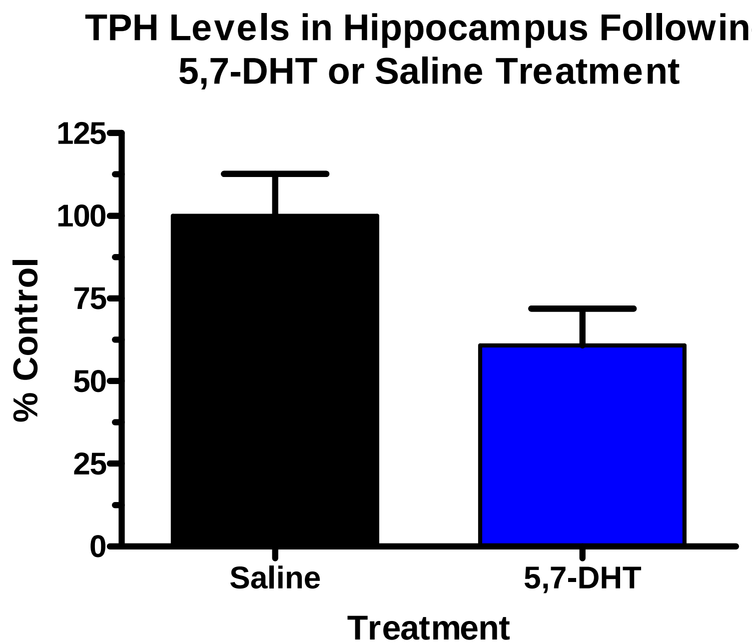



Figure 24. TPH levels in the hippocampus two weeks following MDMA (or saline) treatment.

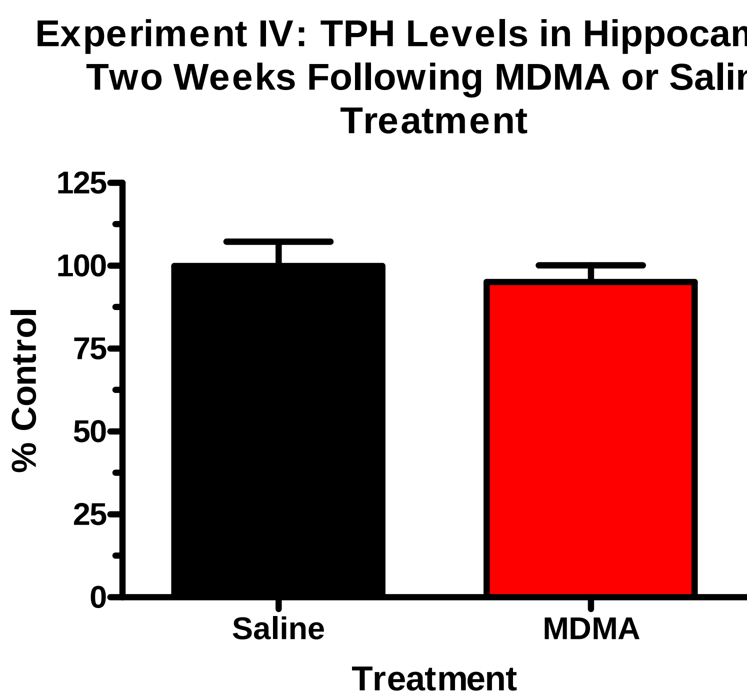
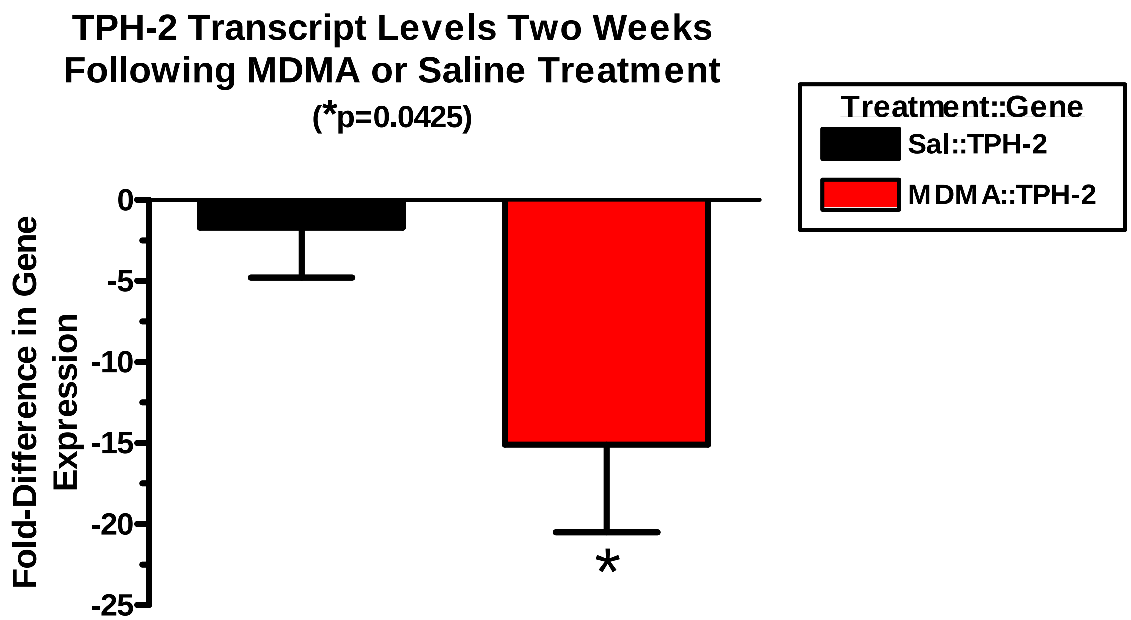


Figure 25. TPH-2 transcript levels two weeks following MDMA (or saline) treatment.




Discussion

Two weeks following repeated MDMA treatment, we were surprised to find that TPH protein levels by immunoblotting were completely unchanged in the hippocampus of Sal/MDMA treated animals when compared to Sal/Sal controls. Additionally, transcript levels of this marker were significantly reduced in the dorsal/median raphe as assessed by qRT-PCR. These results remarkably parallel our finding of marginally altered VMAT-2 content in the hippocampus of MDMA-treated animals at the same time point, with similar reductions (10-fold) in VMAT-2 mRNA noted in the raphe. Taken together, these results further indicate that the depleting effects of MDMA on serotonergic markers may not necessarily occur in the presence of distal axotomy.

Interestingly, the majority of evidence demonstrating that TPH levels are compromised following treatment with MDMA comes from studies showing acute and long-term decreases in drug-induced activity of this enzyme. For example, Schmidt and Taylor (1987) first showed that MDMA exposure (20mg/kg, single injection) in rats significantly reduced TPH activity 3h, 12h, and one week following dosing, with partial recovery noted at the one week time point. Although these deficits may have resulted from MDMA-induced axotomy and/or compromised expression of this protein, it also possible that TPH may have simply been inactivated without concurrent loss of fibers (or loss of marker), potentially explaining the null effect of MDMA on expression of this protein in our study. In support of the latter possibility, two studies have shown that MDMA has no effect on TPH activity *in-vitro* or when administered directly into the brain (Schmidt and Taylor, 1987, 1988), suggesting that a metabolite of MDMA may indeed inactivate the protein. Importantly, certain MDMA metabolites can form quinone structures capable of forming disulfide bonds with cysteine residues in proteins (Monks et al., 2004), presumably enabling them to covalently bond with TPH. This could in turn inactivate the enzyme acutely, and possibly damage the protein in the long-term, a contention supported by another study showing that MDMA-induced decreases in TPH could be reversed *in-vitro* by incubating TPH-containing samples in dithiothreitol/$Fe^{+2}$ or 2-mercapto-ethanol under anaerobic conditions, but only up to 3 days following dosing (Stone et al., 1988). Again, taken together with our results demonstrating a lack of effect of MDMA on levels of TPH (and VMAT-2) protein, reductions in TPH activity following MDMA exposure as documented in these studies may not necessarily reflect loss of marker or compromised fiber integrity.

As mentioned in the introduction, there exist only a handful of studies measuring MDMA effects on serotonergic fiber density when stained for TPH, and none have ever measured TPH protein levels by immunoblotting. In 2006, Adori et al. administered one dose of either 7.5, 15, or 30mg/kg MDMA to Dark Agouti rats and found regional decreases in TPH immunoreactive fiber density at 3 days (caudate only for lowest dose) and 7 days following doing. Affected regions included the striatum, hippocampus, and several cortical areas. Similar decrements have been documented in the dorsal raphe (Bonkale and Austin, 2008), cerebral cortex, and the hippocampus (Xie et al., 2006) two weeks following repeated administration of the compound to Sprague-Dawley rats. It is thus curious that in light of these studies we found no change in protein levels of this marker between treatment groups when measured by immunoblotting. This disparity may be related to how proteins are biochemically treated in each respective method, and the effect this may have on antigenicity of the TPH protein. For example, before removing cerebral tissue for subsequent sectioning for IHC, animals are transcardially perfused with a fixative, usually paraformaldehyde. By forming covalent bridges between lysine residues, this process acts to stabilize cytosolic and membrane bound proteins within tissues (including the brain) by mutual cross linking, as well as by anchoring them to cytoskeletal elements (Schmiedeberg et al., 2009). Since it is possible that the bonding of certain MDMA metabolites to the TPH protein (see above) may occlude or disrupt the epitope of anti-TPH antibodies, the use of a fixative in this method may stabilize this interaction and thus prevent antibody binding to this marker in brains of MDMA-treated animals. If this were the case, TPH-stained fibers would fail to appear following MDMA exposure (if the signal consequently fell below thresholds for detection), but would become visible following synthesis of new protein over time. Indeed, when Kovács et al. (2007) measured long-term effects of MDMA on regional TPH-immunoreactive fiber density, normalization was noted at 180 days (compared to 7 days) following a single injection of 15mg/kg to Dark Agouti rats.

In contrast to studies using the IHC method, immunoblotting has the advantage of introducing reducing agents (such as sodium dodecyl sulfate (SDS)) which act to denature proteins during mechanical homogenization and subsequent tissue processing. During electrophoresis, this denatured state is maintained as polyacrylamide gels also contain SDS, after which these proteins are electrotransferred onto membranes for antibody probing. As such, if indeed an MDMA metabolite was occluding antibody epitopes on the TPH protein, it is possible that this may have been reversed following protein denaturation and subsequent epitope re-exposure in our immunoblots. Thus, immunoblotting may have reinstated TPH antigenicity in our study, potentially explaining the differences in measured levels of TPH protein following MDMA as assessed by either method.

Finally, it is interesting to note that MDMA exposure in our animals lowered TPH transcript levels nearly 15-fold as assessed by qRT-PCR, without a concomitant change in protein levels of this marker. As discussed before, this disconnect may reflect post-transcriptional or post-translational modification of TPH mRNA or protein product, respectively, potentially normalizing the expression of this marker in neurons at this time point. Our finding of a far greater deficit in TPH gene expression than what was documented by Garcia-Osta et al. (2004) may be reflective of the much greater sensitivity of qRT-PCR analysis compared to more descriptive gel analysis of transcripts amplified by reverse-transcriptase PCR. Additionally, although Bonkale and Austin (2008) found increases in TPH gene expression in the dorsal raphe, they did not investigate expression within the median raphe, which may have yielded opposite results. As such, since we pooled both populations of raphe nuclei in our studies, it is possible that severe decrements in TPH gene expression within the median raphe may have resulted in an overall decrease in measured levels of the TPH transcript irrespective of potentially opposite changes within the dorsal raphe. Given this limitation, future studies should utilize qRT-PCR to measure MDMA-induced changes in transcript levels of various serotonergic markers in discrete cell populations within the raphe system.

## Effects of MDMA on Quantity of Serotonergic Terminals

### Background and Rationale

As previously discussed, little direct evidence exists unequivocally demonstrating that the adverse neurochemical effects of MDMA on 5-HT nerve terminals are neurodegenerative in nature. The few direct methods for detecting degenerating neurons following MDMA exposure (i.e. silver staining and Fluoro Jade B staining) have indeed suggested this eventuality, though, unfortunately, dual-labeling studies have never been performed to confirm the neurochemical identity of the affected neurons. In order to directly determine the potential for this compound to alter the quantity of serotonergic terminals following exposure, we employed flow cytometry to count and compare the number of 5-HT synaptosomes derived from the hippocampus of MDMA- and saline-treated animals two weeks following treatment. Although flow cytometry has historically been used almost exclusively in the fields of immunology and derivatives thereof, research by Wolf and Kapatos (1989a, 1989b) and Gylys et al. (2000) has established the validity of using purified as well as crude synaptosomal preparations, respectively, for computational parametric analyses as well as fluorescence-activated cell-sorting (FACS analysis). Importantly, modern flow cytometers can utilize two or more lasers to distinguish proteins of interest labeled with various fluorescent markers, allowing not only the analysis of changes in each marker's fluorescence intensity following treatments or manipulations, but the independent parametric analysis of discrete populations of differentially labeled synaptosomes originating from neurons of specific neurochemical subtypes (Wolf and Kapatos, 1989b). Flow cytometry thus allowed the direct counting of 5-HT synaptosomes, and the comparison of how MDMA may affect their quantity two weeks following exposure to the compound.

An important consideration in this study is that it relied on using serotonergic markers in order to identify and subsequently count this population of terminals. As discussed before, expression of these proteins may be amenable to regulation by MDMA itself, or as an indirect consequence of MDMA exposure. It was thus conceivable that the degree to which this method could count these structures "directly" in part relied on the effects of MDMA on protein expression of the chosen marker. For example, if MDMA treatment in our animals significantly lowered levels of a chosen 5-HT marker in terminals, it is possible that insufficient staining during subsequent flow analysis of individual synaptosomes could have in turn "missed" the affected 5-HT synaptosomes even if intact and unaltered in quantity, again equivocating on whether the effects of the compound are neurodegenerative or neuroregulatory in nature. Conversely, if MDMA had no effect on protein expression of such marker, this would enable the direct counting of serotonergic terminals between treatment groups and the assessment of whether their quantity is altered as a function of MDMA exposure. As such, given that levels of the TPH protein were found to be unaffected following MDMA in the previous experiment, we used this marker to identify 5-HT synaptosomes in this study. Additionally, the use of TPH was advantageous as it is a more direct marker for serotonergic terminals than VMAT-2, and since the use of SERT (known to be depleted by MDMA) would preclude direct measurement of individual synaptosomes as discussed above.

In this study, synaptosomes were dual-labeled for two terminal-specific proteins, synaptosome-associated protein of 25 kDa (SNAP-25) and TPH. As each sample was subsequently analyzed by the flow cytometer, the use of these particular markers allowed identification of specific populations of interest as follows (see Table 3 below): (1) SNAP-25 positive synaptosomes were considered to originate from presynaptic terminals, (2) dual-labeled (SNAP-25/TPH positive) synaptosomes were considered presynaptic serotonergic terminals, and (3) SNAP-25 positive but TPH negative synaptosomes were deemed as originating from non-5-HT terminals. Another advantage to double staining for both markers was the inherent ability to exclude gliosomes, debris, and postsynaptic synaptosomes. Additionally, since both SNAP-25 and TPH are predominantly present in terminal endings, staining for these markers presumably allowed high specificity and thus the sensitivity of our analyses, which is important given that crude synaptosomal preparations may yield axonal fragments.

Table 3. Synaptosomal populations as identified by coincidence of terminal markers.

| SNAP-25 | TPH | Synaptosomal Population |
|---|---|---|
| + | - | Non-serotonergic |
| + | + | Serotonergic |
| - | +/- | All other combinations will denote synaptosomal fragments/debris |

## Experimental Design

Briefly, adult rats (n=8) were treated with binge-MDMA as before, while control rats (n=8) received the saline vehicle only. Temperature measurements during dosing were taken as described in Appendix B. Two weeks following drug administration, all animals were lightly anesthetized with $CO_2$ and decapitated. Brains were quickly removed, and dissected hippocampi immediately processed for flow cytometry as discussed in Appendix B.

## Statistical Analysis

All analyses between treatment groups were performed by means of Student's t-tests. A $p<0.05$ was used to determine statistical significance.

## Results

As documented in the previous three experiments, animals given MDMA during dosing experienced significant elevations in body temperature compared to animals receiving the saline vehicle ($t\ (13) = 5.911$, $p<0.0001$) (Figures 26 and 27). Animals treated with MDMA showed a significant increase (~38%) in the number of SNAP-25/TPH positive synaptosomes compared to controls (t (12) = 2.605, p=0.023) (Figure 29), without a significant change in the total number of SNAP-25 positive synaptosomes (Figure 28).

Figure 26. Body temperature during, and following MDMA (or saline) administration in experiment IV(b).

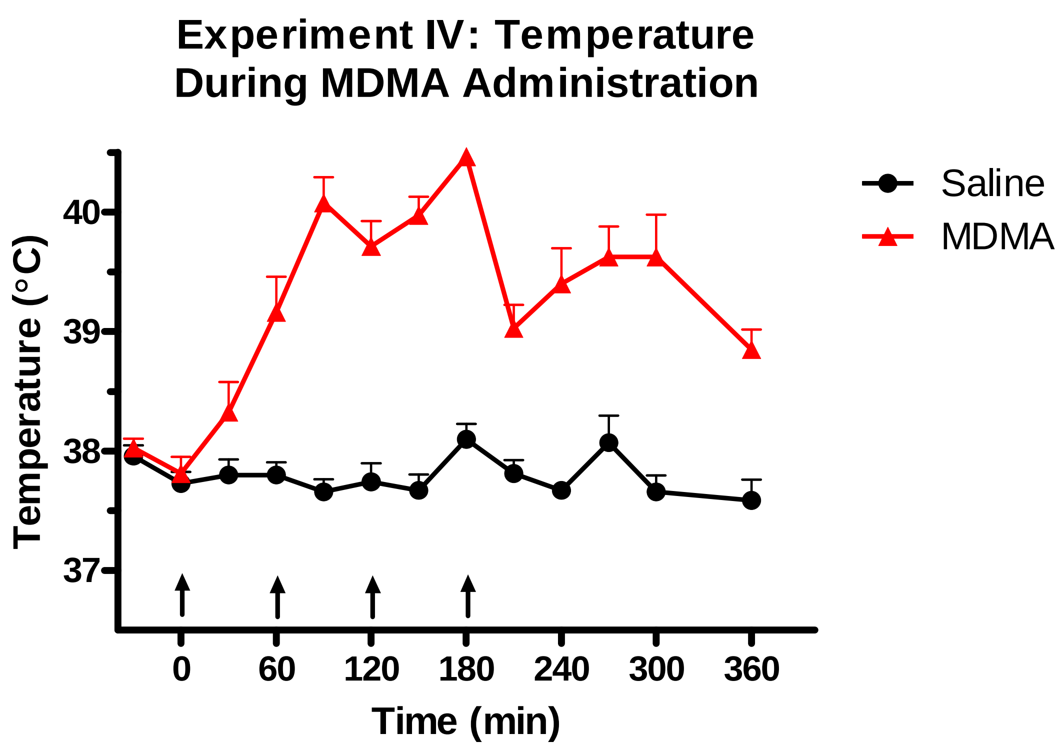


Figure 27. Area under the curve analysis of body temperature during MDMA (or saline) administration in experiment IV(b).

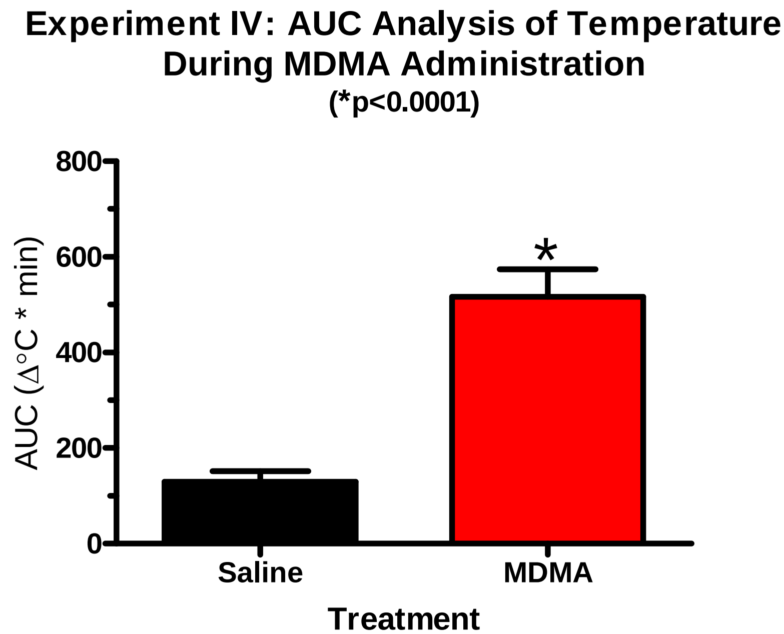



Figure 28. Quantity of presynaptic (SNAP-25 postive) synaptosomes two weeks following MDMA (or saline) treatment.

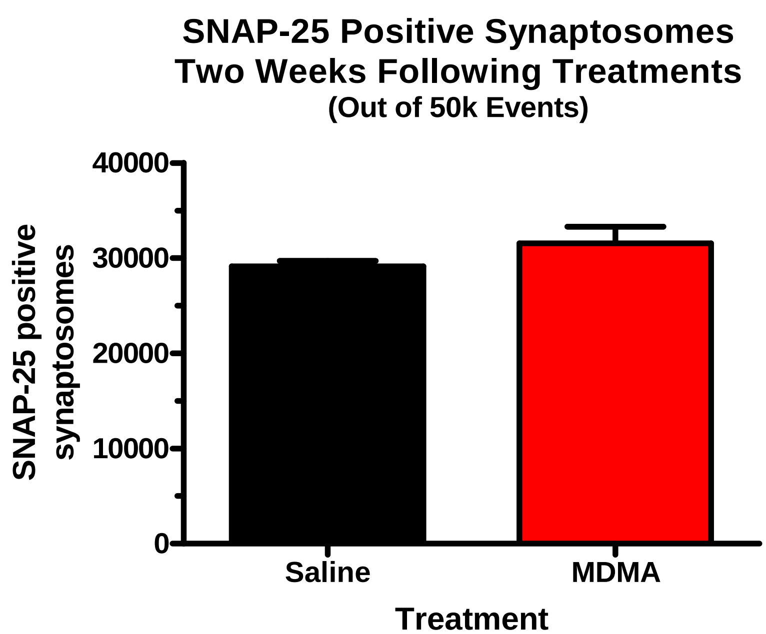
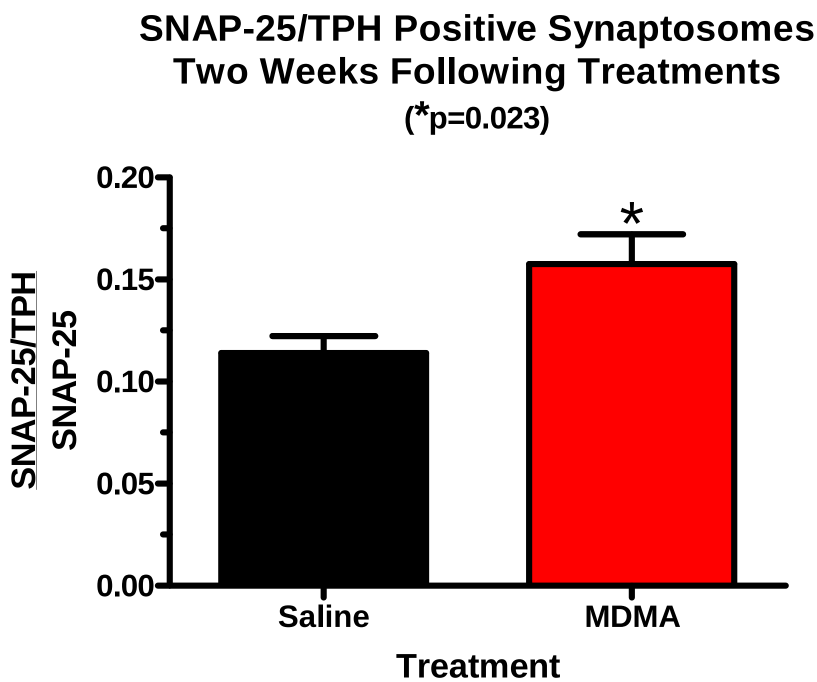


Figure 29. Fraction of serotonergic (SNAP-25/TPH positive) synaptosomes two weeks following MDMA (or saline) treatment.




## Discussion

Two weeks following repeated treatment with MDMA (or saline), synaptosomes generated from the hippocampus of MDMA- or saline-treated animals were initially analyzed by the flow cytometer for the presence of SNAP-25. Structures positive for this marker were considered presynaptic terminals and thus allowed exclusion of gliosomes, postsynaptic terminals, and general debris from further analyses. It is noteworthy that nearly 60% of analyzed tissue was positively identified as presynaptic synaptosomes, which very closely agrees with previous estimates of synaptosomal quantity (~66%) following crude preparations (Gylys et al., 2000). When these synaptosomes were further analyzed for presence of TPH, we were surprised to find that the number of

SNAP-25/TPH (i.e. serotonergic) terminals was significantly increased (38%) in the MDMA-treated group, suggesting substantial reactive sprouting of serotonergic terminals following MDMA exposure. Indeed, terminal sprouting has been regarded as the underlying factor in long-term regional reinnervation of 5-HT fibers following MDMA-induced “lesion” (Battaglia et al., 1988; Molliver et al., 1990), though as findings from our previous experiments suggest, this may alternatively be explained by gradual repletion of 5-HT markers in intact axons.  More pertinently, if proven accurate, our finding may potentially help explain the mechanism of serotonergic hyperinnervation documented to occur months or even years following acute MDMA exposure (Fischer et al., 1995, Meyer et al., 2004; Ricaurte et al., 1992).  For example, Meyer et al. administered MDMA (10mg/kg) twice daily to neonatal pups from PD1-4, and noted a region-specific alteration in SERT-fiber density compared to controls when examined 9 months later.  More specifically, while fiber decrement or normalization was noted in the a few cortical regions as well as the hippocampus, increased fiber density was found in caudate-putamen and nucleus accumbens.  Finding a different pattern of hyperinnervation, Fischer et al. documented increases in the density of 5-HT fibers in the hypothalamus (small subset of rats and most squirrel monkeys) and globus pallidus (most squirrel monkeys) 52 and 72 weeks (rats and monkeys, respectively) following acute, repeated administration of MDMA to these animals.   Though these studies differ in what regions may be hyperinnervated in response to MDMA, they highlight the possibility that reactive synaptogenesis may account for this phenomenon following exposure to this drug.

Importantly, although the above studies interpreted serotonergic hyperinnervation as a consequence of initial pruning followed by long-term sprouting, our data from experiments II-IV suggest that reactive synaptogenesis may occur very quickly following MDMA treatment in animals, but that it cannot be detected by visualization of 5-HT fibers (by IHC) until drug-induced marker depletion has been reversed many months later. Although this is an intriguing possibility, it should be noted that we only measured the quantity of 5-HT synaptosomes only at two weeks following dosing, and thus it remains to be seen whether similar increases are evident at a much later time point. Additionally, evidence in support of reactive, drug-induced synaptogenesis is very limited, though a few studies have noted applicable phenomena with MDMA and other structurally related drugs of abuse. For example, Dawirs et al. (1993) documented an 80% increase in density of dendritic spines in layers III and IV of prefrontal cortex 7 days following METH (25mg/kg x 1) administration to gerbils, though the relevance of post-synaptic sprouting in that region to our observed MDMA-induced increases in hippocampal 5-HT terminals is unclear. In another study, within 24h following a single dose of METH (4mg/kg) to rats, Ujike et al. (2002) noted increases in synaptophysin and stathmin mRNA, considered as markers for synaptogenesis and neuritic sprouting, in the accumbens, striatum, hippocampus, and several cortices, including the medial frontal cortex. Measuring a neurotrophin implicated in synaptogenesis and axonal sprouting, Piper et al. (2009) additionally documented increases in levels of brain-derived neurotrophic factor in the occipital cortex two months following neonatal MDMA treatment (10mg/kg x 2, PD1-4). Finally, Stroemer et al. (1998) found significantly elevated growth-associated protein 43 (GAP-43) and synaptophysin immunoreactive fiber density near the site of damage 7-14 days following AMPH (2mg/kg i.p.) administration to Wistar rats with previously induced ischemia. Taken together, these studies give at least some credence to our findings in that some potential may exist for reactive synaptogenesis following treatment with the amphetamine class of drugs, further supported by a recent review by Yuferov et al. (2005) indicating that psychostimulants may cause increases in the expression of genes implicated in synaptogenesis and neuritic sprouting.

Nevertheless, caution should be taken in extracting significance from our findings in that we documented an unprecedented phenomenon using an approach seldom used in neuropharmacology research. As such, other studies should attempt to replicate and perhaps corroborate our data by other means. For example, following a similar dosing regimen and synaptosomal preparation, it may be possible to isolate TPH-tagged synaptosomes though the use of immunomagnophoretic beads conjugated to protein A, after which relative quantity between treatment groups could be analyzed by SNAP-25 immunoblotting. Additionally, studies should also investigate the potential for MDMA to cause reactive synaptogenesis by measuring protein/transcript levels or immunoreactivity of pertinent markers such as synaptophysin and GAP-43. Until these studies are performed, the results from this study should at most raise the possibility that MDMA may be causing acute, reactive alterations to the serotonergic system.

# CHAPTER 6

## GENERAL DISCUSSION

In experiments I-IV(a), we examined the influence of MDMA on three different markers of 5-HT fibers and terminals to test whether drug-induced serotonergic deficits are reflective of neurodegeneration. To this end, we compared relative changes in protein levels of SERT, VMAT-2, and TPH at 2 weeks following a binge regimen of MDMA. Given the sparse DA innervation of the hippocampus (Ciliax et al., 1995; Kitahama et al., 2007), we aimed to limit VMAT-2 expression in this area mainly to serotonergic sources by initially lesioning NE input with the known noradrenergic neurotoxin DSP-4 (Jaim-Etcheverry and Zieher, 1980). In the hippocampus of DSP-4/MDMA treated animals, immunoblot analysis revealed only a small, nonsignificant effect of MDMA on VMAT-2 protein expression compared to the expression seen in DSP-4/saline controls, while regional levels of SERT were substantially reduced in all MDMA-treated animals. In addition, we also found no change in hippocampal levels of TPH by immunoblotting between animals treated with saline or MDMA (Table 4). Taken together, our results indicate that MDMA may deplete SERT without necessarily inducing terminal loss. In experiments III and IV(a), we explored the contribution of altered gene expression to MDMA-induced changes in the levels of SERT, VMAT-2, and TPH protein by measuring transcript levels of these markers in dorsal/median raphe punches 2 weeks following MDMA or saline administration. Amidst smaller reductions in levels of VMAT-2 and TPH-2 transcripts, MDMA treatment caused a tremendous decrease in SERT gene expression (Table 4) relative to controls, again suggesting that this compound may compromise the levels of certain serotonergic markers without necessarily inducing distal axotomy.

Table 4. Effects of MDMA on protein and transcript levels of SERT, VMAT-2, and TPH two weeks following treatment.

| 10mg/kg MDMA x 4, Measures Examined 2 Weeks Later | **SERT** | **VMAT-2** | **TPH** |
|---|---|---|---|
| Protein Expression | ↓↓↓↓ | ↓ | – |
| Genetic Expression | ↓↓↓↓↓ | ↓↓ | ↓↓ |

To our knowledge, the present studies are the first to use VMAT-2 as an index of serotonergic terminal integrity following MDMA administration. Importantly, the use of this protein was particularly advantageous since it has been well established as a marker of terminal loss in other models of neurodegeneration. For example, postmortem studies have revealed significant reductions in both VMAT-2 protein expression and VMAT-2-immunoreactive fiber density in the caudate and putamen of Parkinson's disease patients compared to age-matched controls (Miller et al., 1999). Furthermore, the extent of these depletions has been shown to parallel reductions in the levels of the dopamine transporter in the same regions, another measure of DA-terminal integrity (Miller et al., 1997). Finally, non-human primates treated with the selective nigrostriatal DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine have shown similar decreases in both VMAT-2 and DAT (Miller, 1999). Thus, quantification of VMAT-2 allowed us to determine whether MDMA-induced changes in SERT levels were accompanied by changes in an established marker of terminal integrity.

Our finding of reduced SERT protein expression following MDMA exposure is consistent with the previous report of Xie et al. (2006), though the concomitant lack of an effect of MDMA on VMAT-2 and TPH expression suggests that this reduction may be related to a down-regulation phenomenon and not necessarily dependent on neurodegeneration. Such an interpretation is supported by other research indicating a failure of MDMA to invoke glial activity thought to occur in response to neural damage. Importantly, O'Callaghan and Miller (1993) and Pubill et al. (2003) both found no MDMA-induced increases in GFAP, an astroglial marker that is elevated following treatment with METH, 5,7-DHT, or other recognized neurotoxins (O'Callaghan et al., 1995). Another measure of astrogliosis, the expression of heat shock protein 27, followed the same trend. Finally, MDMA similarly failed to produce evidence for microglial activation as measured by OX-6 immunoreactivity or [$^3$H]PK-11195 binding to the peripheral benzodiazepine receptor (Pubill et al., 2003). It should be noted, however, that at least two studies have documented increases in GFAP expression in the hippocampus (but not other brain regions) following MDMA treatment, suggesting that this region may be particularly vulnerable to MDMA-induced neural damage (Aguirre et al., 1999; Adori et al., 2006). Another study showed post-MDMA increases in GFAP immunoreactivity in the hippocampus, cortex, and cerebellum, though the relevance of these findings is questionable given the extremely high dose (40mg/kg i.p.) chosen for administration (Sharma and Ali, 2008). Nevertheless, it is important to mention that these studies did not assess the neurochemical identify of the affected neurons, equivocating on whether glial activity following MDMA occurred in response to serotonergic or non-serotonergic damage, a limitation that should be addressed in future studies.

Importantly, our interpretation of the results from experiment II can only hold true assuming the success of DSP-4 pretreatment in lesioning hippocampal NE fibers of MDMA-treated animals. As documented by Jaim-Etcheverry and Zieher (1980) and Fritschy and Grzanna (1992), DSP-4 is an alkylating agent which targets the NET as well as structures within NE terminals, causing pruning of locus coeruleus afferents throughout the brain, an effect which peaks at 2-4 weeks following administration. Interestingly, the notion that DSP-4 induces neurodegeneration of NE fibers is based on similar, indirect measures used to address the neurotoxic capacity of MDMA, which include severe depletions in NE, *in-vivo* inhibition of [$^{3}$H]-NE uptake, and most convincingly, lasting reductions in dopamine-β-hydroxylase immunoreactivity (Landa et al., 1984; Fritschy et al., 1990). Using NET as an alternative marker for NE neurons, we achieved a significant reduction in hippocampal levels of this protein following DSP-4 pretreatment, implying success in lesioning of NE input to this region. However, as is the case with DβH, NET is yet another marker liable to regulation, bringing into question the validity of considering reductions in these proteins as evidence for neurodegeneration (see Booze et al., 1988). Notably, we found no change in regional VMAT-2 expression in the DSP-4/saline group, which may have been expected to decrease given that NE fibers express significant quantities of this protein. Given the coexistence of 5-HT and DA nerve endings in the striatum and parietal cortex (Reader et al., 1989; Kabani et al., 1990), the relative contribution of VMAT-2 from NE sources in these regions may have been insufficient to allow detection of loss following DSP-4 treatment. This however, may not hold true for the hippocampus, since the number of noradrenergic and serotonergic varicosities bearing VMAT-2 may be similar (Oleskevich et al., 1989; Oleskevich and Descarries, 1990), though this has never been directly examined. It is thus unexpected that levels of VMAT-2 following DSP-4 exposure would be unaffected in this region, and as such, it cannot be ruled out that failure of DSP-4 to lesion NE fibers in the DSP-4/MDMA group contributed to the null effect of MDMA on the expression of VMAT-2, since it is known that this drug does not adversely affect this population of neurons (Green et al., 2003). However, given the extensive depletion of hippocampal NET in this group, assuming this reflects DSP-4-induced neurodegeneration, it also cannot be ruled out that normalization of the VMAT-2 signal could have occurred from compensatory upregulation of the protein in spared NE terminals. In either case, it is noteworthy that in the event of MDMA-induced terminal loss, if serotonergic levels of VMAT-2 were subject to the same magnitude of reduction as was found for SERT (~60%), significant reductions (~30%) in hippocampal VMAT-2 content would be expected in the Saline/MDMA groups even if NE-sources of this protein were intact. This possibility was not supported by our finding of only modest reductions (~7%) in VMAT-2 levels in this treatment group. Additionally, our corroborative finding of unaltered synaptosomal levels of TPH in the hippocampus of Sal/MDMA animals relative to controls reinforces the notion that serotonergic terminals may indeed be spared following treatment with this compound, though additional experiments are necessary to confirm our findings by use of more direct measures not dependent on marker staining. Such experiments should investigate ways to directly highlight and subsequently measure changes in 5-HT fiber density in response to MDMA, such as through the use of anterograde or retrograde tracers, or by using reporter genes (e.g. GFP) driven by promoters active in only 5-HT neurons.

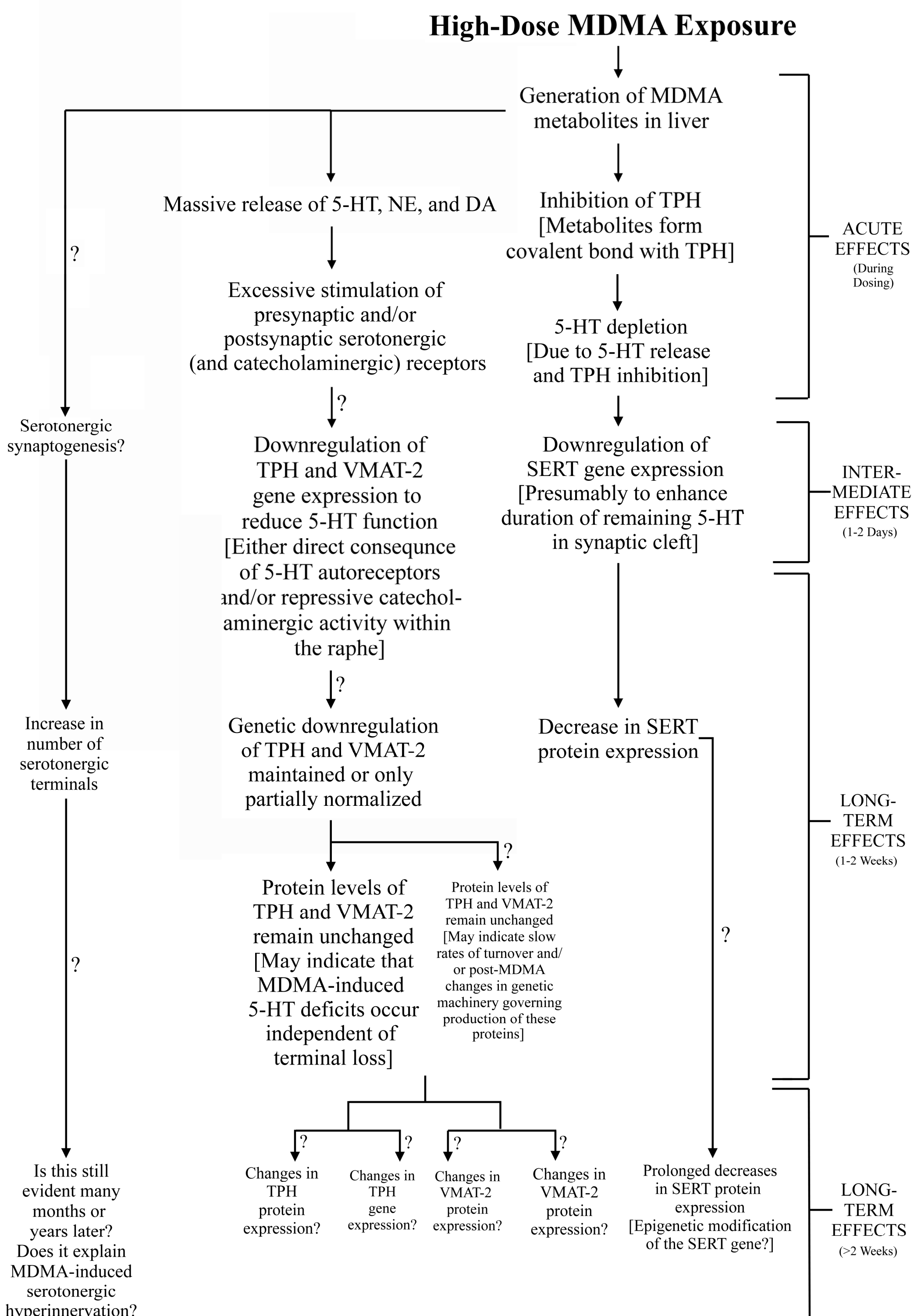
Despite much focus on the neurochemical effects of MDMA on serotonergic fibers, relatively little research exists regarding changes in the gene expression of neuronal markers following exposure to this compound. There has been only one comprehensive study examining MDMA effects on the expression of various 5-HT receptors (Kindlundh-Högberg et al., 2006), while only two such efforts have described such changes in SERT (Li et al., 2006; Kirilly et al., 2007) and TPH (Garcia-Osta et al., 2004; Bonkale and Austin, 2008). Given the existing evidence that MDMA can produce changes in the expression of 5-HT-related genes, we measured transcript levels of SERT, VMAT-2, and TPH by qRT-PCR to determine the potential contribution of altered gene expression to the effects of MDMA on protein levels of these markers seen following immunoblotting. For example, to explore whether genetic downregulation of SERT could (at least, in part) account for MDMA-induced depletions in SERT protein, we investigated the potential of this compound to alter SERT mRNA expression two weeks following dosing. Indeed, MDMA exposure led to a striking reduction in SERT transcript levels relative to controls, suggesting that decreases in SERT protein expression (as documented in experiments I(b) and II) following MDMA exposure may occur as a consequence of biochemical downregulation and not necessarily as a result of damage. Additionally, we measured transcript levels of both TPH and VMAT-2 to test whether an alternative explanation could account for the relatively unchanged levels of these markers in the hippocampus of MDMA-treated animals. More specifically, given that these markers are not a direct measure of serotonergic terminal integrity, it was possible that normalized expression of both proteins in our immunoblots may have resulted from compensatory increases in protein production following MDMA-induced axotomy, presumably to enhance 5-HT synthesis and sequestration in remaining terminals.  This scenario may have been evidenced by increased levels of TPH and VMAT-2 transcripts in the MDMA group relative to controls, which instead were reduced 10-15 fold in these animals.  As such, our results indicate that the effects of MDMA on SERT levels may indeed occur irrespective of axotomy.  It should be mentioned, however, that we measured transcript levels of VMAT-2 and TPH at only one time point following MDMA treatment (2 weeks), and thus the contribution of altered gene expression to protein levels of these markers prior to, and following this time point are unknown, and should be investigated in future studies.

Another intriguing finding in our studies is that despite MDMA-induced reductions in transcript levels of all three markers, protein expression of VMAT-2 and TPH in the hippocampus of MDMA-treated animals remained relatively unchanged two weeks following exposure to this compound.  This apparent disconnect has previously been noted for SERT (Rattray et al., 1996), and may reflect either low inherent turnover rates of these markers, or enhanced marker stability following specific post-translational modifications of the mRNA product resulting from MDMA exposure.  Likewise, increased efficiency of VMAT-2 and TPH transcript translation following MDMA treatment may have contributed to normalized levels of protein translation despite a decrease in transcription.  These possibilities may also in part explain the disproportionate reduction in SERT gene expression (~50-fold) relative to protein levels of this marker (~50%) in MDMA-treated animals.  Future studies should consider this and other effects of MDMA on the molecular machinery governing production of proteins used to identify monoaminergic and other neural subtypes.

In the last experiment (IV(b)), we investigated whether MDMA alters the quantity of 5-HT terminals two weeks following drug exposure. This was achieved by comparing relative changes in the number of hippocampus-derived synaptosomes positively stained for SNAP-25 and TPH (i.e. serotonergic in origin) between MDMA and saline treated animals. In order to count these terminals directly, we made novel use of flow cytometry, a technique which can count cells (and synaptosomes) according to size, granularity, and coincident detection of fluorescently labeled antigens. In the hippocampus of MDMA-treated animals, we were surprised to find that the quantity of 5-HT positive synaptosomes significantly increased relative to controls, suggesting that MDMA may cause acute, reactive synaptogenesis. This phenomenon may in turn partly explain the mechanism underlying long-term hyperinnervation of certain brain regions in previously drug-treated animals (Fischer et al., 1995, Meyer et al., 2004; Ricaurte et al., 1992), presumably undetected by IHC studies in the short term due to MDMA-induced marker depletion. Given the novelty of our findings in the absence of corroborative studies, however, it is important to caution that our data should at most be considered preliminary evidence of possible acute alterations to 5-HT innervation following exposure to this compound. Other studies should investigate the potential for MDMA to induce synaptogenesis by other means, such as quantitation of appropriate markers (e.g. synaptophysin, GAP-43, etc.), or by measuring potential changes in the number of 5-HT terminals using alternative approaches (e.g. immunomagnetophoresis).

Despite many years of intensive research, the exact nature of MDMA neurotoxicity remains controversial. Substantial evidence has been offered both for (Molliver et al., 1990; Ricaurte et al., 2000; Green et al., 2003) and against (O'Callaghan et al., 1995; Pubill et al., 2003; Baumann et al., 2007; Wang et al., 2007) the neurodegeneration hypothesis. Wang and coworkers (2007) have proposed three possible models of MDMA neurotoxicity: (1) neurodegeneration, (2) neuroadaptation, and (3) a mixed model involving both kinds of changes (i.e., significant loss of serotonergic nerve terminals along with adaptive changes in the functioning of the remaining terminals). The present results demonstrating a profound down-regulation of SERT gene expression accompanied by a significant reduction in expression of both the VMAT-2 and the TPH genes certainly confirms that the serotonergic neurons of the dorsal and median raphe nuclei undergo major adaptive changes following a high-dose MDMA treatment regimen. Moreover, even with the caveats expressed earlier, the lack of a significant decrease in synaptosomal VMAT-2 and TPH protein expression suggests at the very least that MDMA does not produce a massive loss of serotonergic nerve terminals. Therefore our findings are consistent either with the neuroadaptation (see Figure 30 for example) or mixed model of Wang et al. (2007), since we cannot exclude the possibility of a partial degenerative response in the MDMA-treated animals.

Figure 30. Tentative model for explaining MDMA-induced serotonergic deficits in the absence of neurodegeneration. Question marks denote phenomena that require further testing.



The overarching implication of our findings is that drug-induced depletion in protein markers liable to regulation may not necessarily reflect neurodegeneration. Other factors, such as changes in genetic expression of these proteins, must be addressed in order to determine the nature of adverse effects of any specific compound. Given that all our methods for detecting MDMA neurotoxicity in the present experiments were indirect (i.e. relied on quantifying protein/gene expression of markers liable to regulation) and measured at only one time point, the current studies are not definitive proof that MDMA affects 5-HT terminals without inducing neurodegeneration, but they do exemplify the dramatic effects this compound can have on regulation of 5-HT markers, questioning the need to invoke distal axotomy as the only explanation for MDMA-induced 5-HT dysfunction. As such, future studies should use more direct approaches to unequivocally assess the effects of MDMA on serotonergic terminal integrity.

# APPENDIX A

## ANIMALS AND MDMA ADMINISTRATION

### Vertebrate Animals

Adult male Sprague-Dawley rats (experiments I-IV) or pregnant dams (experiment I(a)) were obtained from Charles River Laboratories (Kingston, NY). Male wild-type and SERT-KO BJ.6 mice (experiment I(a)) were obtained from Taconic Farms (Hudson, NY). All animals were housed in standard plastic tubs with a bedding of wood shavings, with food and water available *ad lib*. Pregnant dams were singly housed under a standard 12:12h light-dark cycle, while all other animals were pair-housed under a reverse 12:12h light-dark cycle. Animal rooms were maintained at 23°C ± 1 throughout all experiments. All male rats and mice were habituated to the experimenters by gentle handling for approximately 1 min each day for at least 3 days prior to the beginning of drug administration or euthanasia. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996), and the experimental protocols were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee.

### MDMA Dosing Paradigm

All animals in Experiments I(b), II, III, and IV(b) were administered the same binge regimen of (±)-MDMA HCl (RTI International, Research Triangle Park, NC), and sacrificed two weeks following administration for analysis. This paradigm is typically used in many MDMA studies and reflects a post-drug period during which major decrements in markers of 5-HT axons and terminals have been documented (Green et al., 2003). The regimen consisted of four subcutaneous (s.c.) injections of 10mg/kg MDMA with an interdose interval of 1h, delivered in a 0.9% physiological saline vehicle. All control animals received the same regimen of saline vehicle only.

MDMA Dosing Rationale

Given our interest in examining the neurotoxic potential of MDMA on the serotonergic system, all experiments involving MDMA administration used rats rather than mice since it is well known that MDMA adversely affects different monoaminergic systems in these species. More specifically, MDMA exposure in rat causes long-term decrements in markers of 5-HT axons and terminals, whereas in mice, the dopaminergic system is primarily affected (Green et al., 2003). Using the conversion principles of interspecies scaling ($Human_{Dose} = [Animal_{Dose} * (Human_{Weight} / Animal_{Weight})^{Scaling\ Coefficient}]/Human_{Weight}$) with a scaling coefficient of 0.66, the doses we administered to 400g adult rats (4 x 10mg/kg, interdose interval of 1h) roughly approximated the binge-ingestion of two 150mg tablets of pure MDMA by a 70 kg human ($Human_{Dose}$ = 4.32mg/kg), every few hours for a total of four uses (Piper and Meyer, 2004). Thus, our dosing schedule took into account that it is not unusual for a human to ingest multiple MDMA tablets at once (“stacking”), and then repeat this process several hours later to prolong the effects of the drug (“boosting”) (Piper and Meyer, 2004). It is important to note that the interspecies scaling formula considers differences in only the weight and metabolic rate of species of comparison, and allows at most a very rough approximation of how a certain drug dose in one species may compare in relative level to another. It is thus not without flaw, and relative comparisons should only be considered preliminary.

More evidential, empirically, is how the pharmacokinetics of a 10mg/kg administration of MDMA to rat compares with that of a human taking a single, or multiple tablets of MDMA, in both a laboratory and real-world setting. Recent findings from our laboratory (Meyer et al., 2008) suggest that the pharmacokinetics of both MDMA and its metabolite MDA, following this dose in adult rat, differ from that of humans administered one tablet (100mg; <2 mg/kg) of pure MDMA in a laboratory setting (de la Torre et al., 2004). This is evidenced by a much faster half-life (1h vs. 9h), $t_{max}$ (0.71h vs. 2.3h), and higher $C_{max}$ (1753ng/ml vs. 222ng/ml) in rats, resulting from both a much higher single dose and presumably a faster overall ADME profile in rat than in human (Meyer et al., 2008). Effectively, following our dose, rats experience a much higher circulating level of MDMA than humans taking only a single tablet, but over a much shorter time span. As such, if one was to consider employing area under the curve analysis of a hypothetical plasma-concentration time curve, comparing a rat treated with 10mg/kg to a human treated with less than 2mg/kg may reveal that both could be experiencing similar levels of overall drug exposure. Importantly, it should be recognized that the de la Torre et al. (2004) study (one 100mg dose to a ~70kg human) does not adequately represent human self-administration in a real-world environment, which may include instances of stacking and boosting as discussed above (Meyer et al., 2008). In consideration of such factors, Irvine and colleagues (2006) collected blood samples from volunteers the morning after a rave in southern Australia, where between 1-7 Ecstasy tablets were knowingly consumed by each participant. Although it is unlikely that MDMA and its metabolites were assessed at their peak concentration, it was nonetheless striking that plasma levels of MDMA averaged around 310 ng/ml, with at least a few participants reaching upwards of 750 ng/ml or more.  It is therefore not unreasonable to assume that the peak concentration of MDMA in rats administered 10mg/kg of the compound in our studies may have approximated the $C_{max}$ of, at least, some recreational users of Ecstasy, again supporting the strength of our treatment regimen in simulating an MDMA binge in such individuals.

# APPENDIX B

# EXPERIMENTAL PROCEDURES

## Core Temperature Measurements

Changes in animal body temperature during drug administration were monitored using a rectal probe (RET-2, Physitemp Instruments, Clifton, NJ) connected to a digital thermometer (Thermalert TH-5, Physitemp Instruments, Clifton, NJ). Temperature measurements were taken 30 min prior to, and then every 30 min during and following drug administration, terminating at 180 min after the final injection. All rats exceeding 40.5°C during dosing were briefly cooled by application of ice packs to either side of the animal.

## Animal Euthanasia and Tissue Dissection: Experiments I, II, IV(a)

On the day of sacrifice, animals were lightly anesthetized with $CO_2$ and decapitated. Brains were rapidly removed, chilled by immersion in ice-cold 0.9% NaCl for 1 min, and then placed into an acrylic brain block. A 2-mm thick slice beginning 4 mm from the anterior pole was removed, and the parietal cortex was separated from the underlying striatum. Where applicable, the hippocampus, occipital cortex, and cerebellum were dissected free-hand from remaining tissue. All brain regions were frozen on dry ice and stored at -70°C until time of analysis.

## Animal Euthanasia and Tissue Dissection: Experiment III, IV(a)

On the day of sacrifice, all animals were anesthetized and decapitated as before. Whole brains were removed and immediately frozen in 2-methyl-butane previously cooled in powdered dry ice. Frozen brains were then stored at -70°C until day of processing.

Animal Euthanasia and Tissue Dissection: Experiment IV(b)

Animals were euthanized and their hippocampus dissected as described above for experiments I and II, with the exception that tissue was immediately homogenized, and thus did not undergo any freeze-thaw cycles.

Immunoblotting

Preparation of Whole-Cell Lysates/Protein Extraction (Experiment I). Tissues were weighed and homogenized using a Teflon-glass motorized pestle in 30 vols of ice-cold radioimmunoprecipitation (RIPA) buffer (pH, 7.4) containing 0.05 M Tris, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1% of a commercially available protease inhibitor cocktail (P8340, Sigma). Following homogenization, samples were agitated on a rocker for 30 min at 4°C, and then centrifuged at the same temperature for 15 min at 22,000 x *g*. The resulting supernatant was collected and frozen at -70°C for subsequent polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Protein concentration of samples was determined using the Bio-Rad© DC Protein Assay (Hercules, CA) with bovine gamma globulin (BGG) as the standard.

Preparation of Synaptosomes/Protein Extraction (Experiment II, IV(a)). Tissues were weighed and homogenized in 30 vols of ice-cold 0.32 M sucrose using a Teflon-glass motorized pestle as before. The homogenate was then centrifuged at 1000 x *g* to remove nuclei and cellular debris, and the resulting supernatant was then centrifuged at

16,000 x *g* to yield a crude synaptosomal fraction. Synaptosomes were resuspended and homogenized in RIPA buffer (30 vols for striatal samples, 5 vols for hippocampal samples) to solubilize membrane-bound proteins. After a final centrifugation at 22,000 x *g*, the resulting supernatant was collected and frozen at -70°C for later SDS-PAGE. Protein concentrations were determined as above.

SDS-PAGE. On day of analysis, loading buffer was added to a volume of sample calculated to contain 30-60 μg of protein (Table 5), and the resulting mixture was boiled for 5 min using a dry bath. Molecular weight ladders (MagicMark™) were prepared according to manufacturer's recommendations (Invitrogen, Carlsbad, CA). All samples were run on 10% precast polyacrylamide gels (Pierce, Rockford, IL) at 100V for approximately 60 min. Proteins were electrotransferred at 100V onto Immobilon™ polyvinylidine difluoride (PVDF) membranes (Millipore, Billerica, MA) for 80 min at 4°C. Following transfer, PVDF membranes were washed in Tris-buffered saline (TBS) for 10 min and blocked in 5% nonfat milk buffer in TBS for 1 h at room temperature. Membranes were then probed with primary antibodies against SERT (Calbiochem, San Diego CA; cat# PC177L), VMAT-2 (Calbiochem; cat# AB1767), or TPH (Millipore; cat# AB1541) (see Table 5 below) overnight at 4°C, with the exception of membranes containing striatal protein, which were incubated with VMAT-2 antibody for 1 h at room temperature (Table 5). Membranes were rinsed twice with TBS containing 0.2% Tween-20 (TBS-T), and then washed in TBS-T for 10 min followed by three 5-min washes. Membranes were then incubated with secondary antibodies labeled with horseradish peroxidase (HRP) at room temperature for 2 h, washed for 10 min followed by three 5-min washes in TBS-T, and finally immersed in enhanced chemiluminescent reagent

(ECL-Plus, Amersham Biosciences, Piscataway, NJ) for 1 min. The resulting chemiluminescent reaction was exposed on film (Hyperfilm ECL, Amersham Biosciences), after which membranes were stripped and reprobed with antibodies against β-actin (Sigma) in order to verify equal protein loading. As an additional control, an identical "standard sample" was run on all gels to normalize variability in band density across blots in subsequent analyses. Protein bands were quantified using Scion Image™ densitometry software (Scion Corporation, Frederick, MD), and the optical density of bands was expressed as percent of control.

Table 5. Antibody vendors and epitope variants used in listed immunoblotting experiments.

| **Experiment** | **I** | **II** | **IV** |
|---|---|---|---|
| **SERT 1°** | 1) Immunostar: (579-599)<br>2) Calbiochem: (579-599; PC177L)<br>3) Santa Cruz: H-115<br>4) Santa Cruz: C-20<br>5) Santa Cruz: N-14<br>6) Santa Cruz: 24A5 (mono) | 1) Calbiochem: (579-599) | - |
| **SERT 2°** | Sigma | Sigma | - |
| **VMAT-2 1°** | - | Calbiochem (AB1767) | - |
| **VMAT-2 2°** | - | Sigma | - |
| **TPH 1°** | - | - | Millipore (AB1541) |
| **TPH 2°** | - | - | Calbiochem (402100) |
| **Beta-Actin 1°** | Sigma | Sigma | Sigma |
| **Beta-Actin 2°** | Sigma | Sigma | Sigma |

Table 6. Antibody concentrations used in listed immunoblotting experiments.

| **Experiment** | **I** (Whole-Cell Lysate) | **II** (Synaptosomes) | | | **IV(a)** (Synap) |
|---|---|---|---|---|---|
| **Brain Region** | All | Striatum | Hippo | Parietal Cortex | Hippo |
| **Protein Loaded** | 30ug | 10ug | 60ug | 60ug | 60ug |
| **SERT 1°** | 1:666 | 1:800 | 1:570 | 1:570 | - |
| **SERT 2°** | 1:2500 | 1:2500 | 1:2500 | 1:2500 | - |
| **VMAT-2 1°** | - | 1:5000* | 1:1000 | 1:1000 | - |
| **VMAT-2 2°** | - | 1:5000 | 1:5000 | 1:5000 | - |
| **TPH 1°** | - | - | - | - | 1:2000 |
| **TPH 2°** | - | - | - | - | 1:5000 |
| **Beta-Actin 1°** | 1:20k | 1:10k* | 1:20k | 1:20k | 1:20k |
| **Beta-Actin 2°** | 1:2500 | 1:2500 | 1:2500 | 1:2500 | 1:2500 |

*****All *primary* antibody incubations were performed overnight, with the exception of those indicated by an asterisk, which were performed for 1h.

Analysis of Protein Bands. In Experiment I(b), immunoblotting analysis was performed by subtracting the background optical density (OD) from the OD of each SERT band, and then dividing this resulting OD by that of beta-actin. Per blot, care was taken to run alternating tissue from control and MDMA-treated subjects across lanes, limited to one brain region. All data were expressed as percent control.

In Experiment II and IV(a), an additional control was run to help eliminate signal variability which may occur between different blots. Each blot thus contained, among tissue from control and MDMA treated animals, the same "standard" loaded into the last well, which was initially collected from several control samples. Following immunoblotting, analysis was performed as above, except that the OD of each SERT, VMAT-2, or beta-actin band was first divided by the OD of the standard. Again, assuming that the standard varied in density as a function of exposure intensity of each blot, this analysis allowed signal normalization and thus legitimate comparison of band densities across blots. All data were expressed as percent control.

Serotonin Transporter Binding Analysis

Serotonin transporter binding analysis was performed as described in Piper et al., 2005. Briefly, tissue was homogenized with a Polytron (Brinkmann, Westbury, NY) in 40 vols of ice-cold buffer (pH, 7.4) containing 10 mM sodium phosphate, 5 mM potassium chloride, and 120 mM sodium chloride, and then centrifuged at 20,000 x *g* for 20 min at 4°C. The homogenization and centrifugation steps were repeated twice. Following the second centrifugation, samples were placed in a dry bath at 30°C for 20 min to facilitate dissociation of endogenous serotonin from SERT to limit interference with radioligand binding. Washed membrane preparations were assayed in triplicate using a 1.0 nM concentration of [$^3$H]citalopram (84.2 Ci/mmol, PerkinElmer, Waltham, MA). Nonspecific binding was determined in the presence of 10 mM unlabeled fluoxetine. The incubation step was carried out at room temperature for 1 hr, after which the reaction was terminated by filtration through Whatman GF/B filters presoaked in 0.05% polyethyleneimine. Filters were washed twice with 5ml ice-cold buffer, placed in 4.3 ml of Scintisafe™ (30%), and counted at least 24h later in a Packard 1900CA liquid scintillation analyzer. Protein levels in each sample were determined by means of the

Bio-Rad© DC protein assay (Hercules, CA) using bovine gamma globulin as the standard. All data were expressed as fmol SERT/mg protein.

Norepinephrine Transporter Binding Analysis

Norepinephrine transporter binding was conducted as described by Tejani-Butt (1992), with minor modifications. Samples were weighed and homogenized with a Polytron in 30 vols of ice-cold assay buffer containing 50 mM Tris, 120 mM NaCl, and 5 mM KCl, pH 7.4. Homogenates were then centrifuged at 20,000 x *g* for 20 min at 4°C and resuspended, followed by three additional homogenizations and washes. Membrane preparations were finally homogenized in ice-cold buffer containing 50 mM Tris, 300 mM NaCl, and 5 mM KCl, pH 7.4, since the increased concentration of NaCl enhances binding of nisoxetine to NET (Tejani-Butt, 1992). The binding reaction was carried out by incubating the washed membranes in triplicate with 2.0 nM [$^3$H]nisoxetine (87.2 Ci/mmol, PerkinElmer, Waltham MA) for 4 h at 4°C. Nonspecific binding was defined using 10 μM desipramine. The reaction was terminated by addition of 5 ml ice-cold buffer and filtration through GF/B filters presoaked in 0.05% polyethyleneimine. Filters were washed twice more with buffer and then counted in a Packard 1900CA liquid scintillation analyzer. The protein concentration of each sample was determined as described above. All data were expressed as fmol NET/mg protein.

Dorsal and Median Raphe Tissue Punches for Real-Time PCR

Whole brains were cut coronally using a cryostat set at -10°C. Approaching the midbrain, the anterior boundaries of the dorsal and median raphe (same plane) were identified using the following two landmarks: 1) complete lateralization of the hippocampus concomitant with 2) rapid enlargement of the cerebral aqueduct (Paxinos and Watson, 1998). A 1-mm section encompassing the entire rostral-caudal extent of both nuclei was then taken and placed on an ionized glass slide (Thermo Electron Corporation, Waltham, MA). The slide was chilled on dry ice for 1 min, after which punches of both nuclei were taken using a sterile needle/expeller with an internal diameter of 1 mm. Tissue punches from each animal were pooled and stored at -70°C until RNA extraction.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA Extraction**.** Total RNA was extracted from combined dorsal/median raphe punches using an RNeasy Micro Kit (Qiagen, Germantown, MD). Briefly, punches were disrupted and homogenized in Qiazol reagent, after which chloroform was added, the samples were centrifuged at 12,000 x *g*, and the clear, upper phase containing total RNA was carefully collected. Samples were then mixed with 70% EtOH and loaded onto microcentrifuge columns. Before final RNA extraction using RNase-free water, columns were subject to multiple washes using proprietary buffers, DNAse I treatment, and at least two further washes in 80% EtOH. The RNA eluate was analyzed for purity ($260_{OD}/280_{OD}$ ratio of 1.9-2.1 in conjunction with curve analysis of $220_{OD} \rightarrow 320_{OD}$ scan) and concentration using a ND-1000 NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Extracted RNA was diluted in RNase-free water as needed and stored at -70°C until real-time PCR analysis.

qRT-PCR Analysis. Quantitative, real-time PCR was performed using a one-step reverse-transcriptase (RT)-PCR Quantitect™ kit from Qiagen, with SYBR green as the amplicon fluorescence marker. Briefly, template RNA (~10ng/reaction) was added to a master mix containing random primers (RT step), Sensiscript$^{TM}$ and Omniscript$^{TM}$ reverse transcriptases (RT step), and for the amplification step, gene-specific forward and reverse primers (Quantitect Primer Assays, Qiagen), Hot-Start$^{TM}$ Taq polymerase, deoxynucleotidetriphosphates (dNTPs), and magnesium buffer. All thermocycling steps, as well as detection of SYBR green fluorescence intensity, were performed in triplicate on an MX3000p PCR instrument (Stratagene, La Jolla, CA). Relative mRNA abundance between treatment groups was calculated by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize RNA loading in each reaction. All data are expressed as fold-difference in gene expression.

## Flow Cytometry

Preparation, Fixation, and Permeabilization of Synaptosomes. Synaptosomes for flow cytometry were prepared using a combined protocol from Wolf and Kapatos (1989a; 1989b) and Gylys et al. (2000), with modifications. Briefly, animals were deeply anesthetized with $CO_2$ and decapitated. Following removal of the brain, the hippocampus was dissected free-hand, weighed, and immediately submerged in 30 vol of ice-cold 0.32M sucrose containing 10 mM Tris (pH, 7.4) and (50 ul/g) tissue protease inhibitor cocktail (Sigma). Homogenization was performed using a Teflon-glass motorized pestle set at speed 6, using 10 up and down strokes, after which the homogenate was centrifuged at 1000 x *g* to remove nuclei and large debris. The supernatant was further centrifuged at 16,000 x *g* to yield the crude-synaptosomal fraction. For fixation/permeabilization, the resulting pellet was resuspended in equal volumes of

Kreb's-Ringer phosphate (KRP) buffer (121.9 mM NaCl, 0.87 mM $CaCl_2$, 4.89 mM KCl, 1.23 mM $MgSO_4$, 1.23 mM $KH_2PO_4$, 10.3 mM sodium phosphate buffer (pH, 7.4), and 11.87 mM glucose) and Zamboni fluid (4% wt/vol parformaldehyde and 15% vol/vol saturated picric acid in 0.1 M phosphate buffer, pH 7.4) for 30 min at room temperature, yielding a final concentration of 2% paraformaldehyde and 7.5% picric acid. Permeabilized synaptosomes were washed 4 times in 15 vol of modified Dulbecco's PBS containing 0.53 mM $MgCl_2$ and *no* $CaCl_{2,}$ and finally resuspended in this medium at an approximate concentration of 5mg/ml. Synaptosomes were stored in this medium at 4°C until processing for flow cytometry.

Dual-Labeling and LSR-II Analysis. For all incubations, synaptosomes were used at a concentration of 1mg/ml. Briefly, on day of assay, synaptosomes were blocked in KRP containing 2% sheep and mouse serum (Invitrogen) for 1h at 4°C, and then incubated with primary antibodies directed against SNAP-25 (mouse-generated, Sigma) and TPH (sheep-generated, Millipore), at a concentration of 1:2500 and 1:100, respectively. Synaptosomes were then washed 4x in KRP containing 2% goat and rabbit serum (Invitrogen) and incubated for 1h (4°C) in anti-mouse and anti-sheep secondary antibodies conjugated to the fluorescent markers phycoerythrin (PE), and fluorescein-5-isothiocyanate (FITC), respectively. Following another 4 washes in KRP only, dual-labeled synaptosomes were suspended (1:20) in this medium for flow analysis.

Flow analysis was performed on a LSR II flow cytometer (BD Biosciences) using FACSDiva$^{TM}$ software. Initially, emission spectral overlap between fluorophores used in this study was compensated for using Calibrite$^{TM}$ beads (BD Biosciences) individually labeled with each marker. Synaptosomal samples were then collected at ~1,000 events per second and analyzed by forward-scatter (measure of size) and side-scatter (measure of granularity) plots to confirm linearity of spread, indicative of synaptosomal integrity (Gylys et al., 2004). Per subject, unlabeled samples as well as samples labeled with secondary antibody only were run to determine background fluorescence for the purpose of setting a gating threshold for detection, defined as the fluorescence intensity above which only 2% of the total events in the sample were identified as PE (SNAP-25) or FITC (TPH) positive. Dual-labeled samples were subsequently run for each subject to identify a SNAP-25 positive population by applying the proper gating threshold from the previous analysis. Synaptosomes deemed SNAP-25 positive were then analyzed for presence (or absence) of TPH, again by applying the relevant threshold gate from the initial background analysis. Ultimately, as discussed in chapter 4, events identified as SNAP-25/TPH positive were considered to originate from presynaptic serotonergic terminals, and the number of SNAP-25/TPH positive synaptosomes was compared between treatment groups.

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