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The committee for Margaret Roserika Brooks certifies that this is the approved version of the following thesis:

UNDERSTANDING THE NEUROADAPTATIONS UNDERLYING THE EFFECTS OF (+)-METHYLENEDIOXYMETHAMPHETAMINE [(+)-MDMA; ECSTASY]: IMPLICATIONS FOR THE SEROTONIN 5-HT_{2A} RECEPTOR AND ITS BINDING PARTNERS

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by
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To my family and friends for their love and support, and to my Uncle Ernest for always cheering me on.

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Repeated administration of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") induces "behavioral sensitization," an enhancement of its behavioral effects. The molecular changes underlying behavioral sensitization may be linked to neuropsychological sequelae (e.g., depression, anxiety) seen upon MDMA withdrawal. Microarray analyses were conducted following administration of a behaviorally sensitizing, non-neurotoxic regimen of (+)-MDMA (4 mg/kg/day, 7 days) to identify patterns of neuroadaptations within the brain "reward" circuit. Differential expression of the mRNA for the serotonin 5-HT_{2A} receptor, a known modulator of the behavioral effects of MDMA, and its binding partners, were observed at 24 hrs withdrawal. However, these results were not validated by quantitative RT-PCR nor did Western blot analyses reveal alterations in the abundance of these proteins. Thus, altered expression of the proteins studied herein does not appear to underlie the expression of behavioral sensitization to (+)-MDMA. Future experiments are required to analyze the function of binding partners in the regulation of 5-HT_{2A}R.

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CHAPTER 1: INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a widely abused recreational drug which elicits a number of socially desirable feelings that encourage its use among teens and young adults. Acutely, oral ingestion of (±)-MDMA, the isomeric form identified in street preparations, is associated with mood elevation, perceptual distortions, mental stimulation, closeness to and empathy for others, and decreased anxiety (Vollenweider et al., 1998). However, withdrawal from chronic MDMA use is associated with a number of psychological consequences such as depression, sleep disturbances, cognitive dysfunction, and anxiety (Morgan, 2000). For example, a transient depression of mood has been reported by users to persist for several days after taking ecstasy (Parrott et al., 2002), and cognitive dysfunctions such as poor information processing and memory impairments are present at least 7 days after taking MDMA (Parrott and Lasky, 1998; Morgan, 1998). In addition, increases in aggression and anger have been shown to persist up to 7 days after use of the drug (Curran et al., 2004). Although these consequences appear to resolve within a week of taking MDMA, the long-term consequences of this drug are not fully understood.

The ability of MDMA to alter serotonin (5-HT) neurotransmission may be important in the development of withdrawal sequelae as 5-HT is involved in the regulation of a number of the withdrawal-related processes (Dubovsky and Thomas, 1995). MDMA binds to the reuptake transporters for serotonin, dopamine (DA), and norepinephrine, demonstrating the highest affinity (nM) for the 5-HT transporter (SERT) (Battaglia et al., 1988). MDMA exerts its primary action by disrupting the ability of SERT to reuptake 5-HT and inducing 5-HT release by a transport-mediated exchange (Rudnick and Wall, 1992). By pumping 5-HT into the synaptic cleft, a rapid MDMA-induced increase of extracellular 5-HT occurs, and enhanced 5-HT is thus available for subsequent actions at pre- and post-synaptic 5-HT receptors. In addition, MDMA also increases DA efflux due to similar actions at the DA transporter (DAT) (Johnson et al., 1991) as well as via receptor-mediated facilitatory influences of 5-HT on DA release (Benloucif et al., 1991; Parsons et al., 1993). Like other amphetamine analogs, MDMA inhibits the enzyme monoamine oxidase (MAO) (Leonardi and Azmitia, 1994) and as a

consequence results in decreased metabolism of both 5-HT and DA, contributing to even higher extracellular concentrations of these neurotransmitters. Interestingly, regimens of acute or repeated high dose MDMA have been shown to be neurotoxic to 5-HT systems, reducing expression of the SERT, and depleting levels of 5-HT and its metabolite, 5-hydroxyindole acetic acid (5-HIAA), in nerve terminals in a region-specific manner (Commins et al., 2005; Stone et al., 1987). The mechanisms underlying MDMA-evoked neurotoxicity are not fully understood but clearly include modifications in the 5-HT system.

Serotonin is involved in the regulation of sleep, mood, eating, sexual behavior, memory, and learning. Disorders associated with aberrant serotonergic function include schizophrenia, depression, obsessive-compulsive disorder, and anxiety (Boot et al., 2000). Serotonin is unique among the monoamines in that its effects are carried out by as many as 15 distinct G-protein coupled receptors (GPCRs) and one ligand-gated ion channel (Hoyer et al., 2002). In particular, the 5-HT_2R family (5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}R) has been found to play an important role in the regulation of psychological functions such as mood and cognition, and abnormalities of 5-HT_2R function have been implicated in neuropsychological and neurological conditions such as depression and dementia, respectively (Busatto, 1997). Specifically, the 5-HT_{2A}R subtype, which appears to mediate the subjective effects of most *d*-lysergic acid diethylamide (LSD)-like hallucinogens (Glennon et al., 1984), also has been found to have a prominent role in the behavioral effects associated with acute exposure to MDMA. For example, 5-HT_{2A}R antagonists block and 5-HT_{2C}R antagonists facilitate MDMA-evoked hyperactivity (McCreary et al., 1999; Fletcher et al., 2002; Bankson and Cunningham, 2002; Herin et al., 2005), respectively. In addition, pretreatment with a 5-HT_{2A}R antagonist has been shown to inhibit the neurotoxic effects of MDMA (Nash et al., 1990), and in humans, (±)-MDMA-induced perceptual and emotional changes are diminished after treatment with the 5-$HT_{2A/2C}$R antagonist ketanserin (Liechti, 2000).

The mesocorticoaccumbens DA circuit is an important neuroanatomical component of the brain reward system that regulates responses to natural reinforcers like food, drink, sex, and social interactions in addition to the rewarding effects of drugs of abuse. The reward pathway is composed of DA neurons originating in the ventral

tegmental area (VTA) which project to the nucleus accumbens (NAc) and prefrontal cortex (PFC). Serotonergic cell bodies in the dorsal and median raphe nuclei also innervate these nuclei in which multiple 5-HT receptors (e.g., 5-HT_{1B}R, 5-HT_{2A}R, 5-HT_{2C}R, and 5-HT_3R) are located. Serotonergic innervation of this pathway may contribute to the unique behavioral profile of MDMA (Bankson and Cunningham, 2001) as MDMA has been shown to modify DA release via 5-HT innervation of the mesolimbic DA system (Koch and Galloway, 1997), and a role for DA in its behavioral effects has been reported (Callaway and Geyer, 1992; Schechter, 1996; Bubar et al., 2004). Furthermore, this pathway is thought to underlie the addictive properties of drugs of abuse as it is involved in the reinforcing, stimulus and hyperlocomotive effects of psychostimulants (Callahan et al., 1997; Delfs et al., 1990; Pettit et al., 1984), and appears to be critical for the initiation and expression of behavioral sensitization (Kalivas et al. 1993; Vanderschuren and Kalivas 2000).

Behavioral sensitization is a progressive enhancement of behaviors evoked by several chemical classes of psychostimulants that develops upon repeated, intermittent administration of the drug (Kalivas et al., 1993; Vanderschuren and Kalivas, 2000). Indeed, repeated administration of both (+)- (McCreary et al., 1999) and (±)-isomers (Spanos and Yamamoto, 1989; Kalivas et al., 1998; Itzhak et al., 2004) of MDMA has been shown to result in behavioral sensitization. The molecular changes underlying behavioral sensitization are thought to be linked to the development of addiction and relapse during abstinence as well as the onset of various neuropsychological symptoms (e.g., depression, anxiety) seen upon withdrawal of chronic drug use (Nestler, 2001; Robinson and Berridge, 2001). Importantly, the ability of MDMA to alter 5-HT neurotransmission may be critical in this progression as 5-HT is involved in the regulation of a number of these psychological processes (Dubovsky and Thomas, 1995). A multitude of both transient and enduring changes have been observed within several neurotransmitter systems in response to chronic psychostimulant administration (Robinson and Becker, 1986; Kalivas et al., 1998; Vanderschuren and Kalivas, 2000), including (±)-MDMA (McNamara et al., 1995; Obradovic et al., 1998). For example, short- (1-4 days) and long-term (9-15 days) withdrawal from repeated exposure to a neurotoxic regimen of (±)-MDMA is associated with a reduction in the inhibitory actions

of 5-HT and DA on glutamate-evoked firing in the NAc (Obradovic et al., 1998). Importantly, increased (±)-MDMA-evoked DA release in the NAc arising from adaptations within the reward pathway appears to be an important feature of behavioral sensitization to (±)-MDMA (Kalivas et al., 1998). Identifying the molecular underpinnings of such neuroadaptations are important to the process of discovering genes and their protein products whose aberrant expression after chronic drug use may contribute to a variety of drug-induced states such as withdrawal.

Due to the impact of MDMA on the reward system and the importance of this pathway in the rewarding and reinforcing effects of drugs of abuse, it is key to investigate neural substrates within this pathway. In particular, further studies of 5-HT_{2A}R protein expression and signaling in the reward pathway following administration of MDMA may help elucidate the role of this receptor in the behavioral effects of MDMA. For our studies, the (+)-isomer of MDMA was chosen as this isomer has been shown to be more potent than the (-)-isomer at releasing DA (Hiramatsu et al., 1990) and inducing hyperactivity (Callaway et al., 1990). The following set of experiments was developed in order to more completely understand the complex changes that occur upon withdrawal from repeated use of MDMA. Specifically, using a global method of identifying patterns of gene expression may provide further insight into the neural basis and mechanisms for MDMA to elicit its behavioral effects. These studies seek to identify patterns of neuroadaptation within the reward pathway that occur following repeated (+)-MDMA administration at time points of withdrawal that are characterized by specific behavioral phenotypes such as sensitization.

CHAPTER 2:

REPEATED, INTERMITTENT ADMINISTRATION OF (+)-MDMA ALTERS TRANCRIPTS IN SEVERAL BIOLOGICALLY FUNCTIONAL CATEGORIES

INTRODUCTION

Despite the prevalence of drug addiction in our society, our understanding of the neurobiology underlying drug abuse, craving, withdrawal and other drug-induced states is limited and the molecular bases for these behavioral manifestations remains unclear. As outlined in the previous chapter, the development and expression of behavioral sensitization to (+)- and (±)-MDMA are likely to be mediated by altered neurotransmission and subsequent neuroadaptations within the reward pathway. Studies suggest that such neuroadaptations, including changes in the expression of particular transcripts in the brain, may underlie the transition from casual, controlled drug use to an inability to control consumption of the drug (Robinson and Becker, 1986; Robinson and Berridge, 2001; Nestler, 2001). In addition, these changes may also contribute to the development of withdrawal sequelae that occur during abstinence from drug abuse. By applying genomics to the study of drugs of abuse, we have the potential to identify genes and their protein products that control reward pathways of the brain and adaptations to drugs of abuse.

As discussed in Chapter 1, a number of alterations in neurotransmission occur within the reward circuit in response to chronic psychostimulant administration (Robinson and Becker, 1986; Kalivas et al., 1998; Vanderschuren and Kalivas, 2000), including (±)-MDMA (McNamara et al., 1995; Obradovic et al., 1998). Indeed, studies have shown that many drugs of abuse including (±)-MDMA (Miller, 2005), indirectly stimulate transcription of specific genes by increasing intracellular cAMP, which results in the activation of multi-functional protein kinases and phosphorylation of several cellular proteins (Nestler, 1992). The initiation of gene transcription by drugs of abuse also might affect the synthesis of proteins which are able to activate several networks of

neurochemical pathways in brain neurons. Unfortunately, studying the effects of drugs of abuse on gene expression is hampered by a number of factors including the localization, onset and duration of gene expression. These factors make it difficult to link a drug-induced behavior such as craving or withdrawal to a particular gene. It is possible that single genes can be expressed in specific brain regions, each of which may contribute either directly or indirectly to a number of drug-induced states. However, it is more likely that changes in patterns of gene expression occur during exposure to psychostimulants, and that these patterns of gene expression contribute to the manifestation of behavioral states (e.g., sensitization, withdrawal sequelae) associated with repeated exposure to MDMA.

The recent advances in the cloning of the human, mouse and rat genomes enable investigators to assess global transcript changes in a single experiment through the use of genomic technologies such as serial analysis of gene expression (SAGE), subtraction by hybridization (SBH), and DNA microarrays. Microarrays have become the most commonly used technology to profile the expression of thousands of genes at once. For example, this assay has been used successfully to identify aberrant gene expression in cancer (Gyorffy et al., 2005; Iacobuzio-Donahue et al., 2003) and diseased liver tissues (Schnabl et al., 2005) where gene expression changes are marked by large-fold differences. Small changes in the expression of specific transcripts in the brain often have significant implications, however, the limits of detection for microarrays challenge the efforts of researchers to uncover subtle changes that may potentially occur in the brain during diseased states. Numerous studies have utilized microarrays to evaluate the gene expression profiles of transcripts altered by neuropsychological disorders such as Alzheimer's disease, autism, schizophrenia, and bipolar disorder (Yao et al., 2003; Purcell et al., 2001; Mirnics et al., 2001), as well as the consequences of acute administration of psychoactive drugs including cocaine, methamphetamine, morphine, nicotine and ethanol (Lewohl et al., 2001; Thibault et al., 2000; Freeman et al., 2002; Loguinov et al., 2001; Cadet, 2001; Li et al., 2002).

Microarrays have been utilized recently to identify genes that may be involved in the neurotoxic effects of MDMA in rodents. Cadet and colleagues investigated drug-induced changes in the expression of 28 genes from diverse categories in rat cortex, up to

7 days after termination of a single high dose of (±)-MDMA (20 mg/kg dose) which results in 5-HT neurotoxicity (Thiriet et al., 2002). These categories included receptors, signaling pathways and transcription control, cytoskeletal and matrix proteins, and metabolic pathways to name a few. In another study, the effects of an acute neurotoxic dose of (±)-MDMA (47 mg/kg) on gene expression was studied in mice at varying time points after treatment (Xie et al., 2004). Increased transcription and translation of metallothionein (Mt)-1 and Mt-2, reported to act as reactive oxygen species (ROS) scavengers (Hussain et al., 1996) were found in the ventral midbrain shortly after MDMA treatment (Xie et al., 2004). The authors suggested that this up-regulation may serve as part of a neuroprotective mechanism, as some studies have demonstrated a role for ROS in protection against MDMA-induced neurotoxicity (Cadet, 2001).

Single and repeated exposure to high doses of (±)-MDMA results in a long-term depletion of 5-HT markers [e.g., 5-HT, 5-HIAA, and SERT] (Commins et al., 2005; Stone et al., 1987) and degeneration of 5-HT axons and terminals, known as "neurotoxicity" (O'Hearn et al., 1988). Humans, however, do not take the large doses of MDMA described to produce neurotoxicity in rodent models. Furthermore, the changes in gene transcription that may underlie behavioral sensitization (**see Chapter 1**), which can occur at non-neurotoxic, low-to-moderate doses, may be obscured by MDMA-induced damage to 5-HT systems. This point is important given the evidence to suggest that sensitization develops in the absence of 5-HT neurotoxicity (Kalivas et al., 1998; McCreary et al., 1999; Itzhak et al., 2003; Bubar, Thomas and Cunningham, unpublished observation). Therefore, it is important to examine the processes underlying sensitization in the absence of overlying neurotoxic effects of MDMA.

A regimen of (+)-MDMA (4 mg/kg/day, 7 days) exposure has been established in our lab to result in behavioral sensitization without depletion of 5-HT and its metabolite 5-HIAA (Bubar, Thomas and Cunningham, unpublished observation). An enhancement of MDMA-evoked hyperactivity was observed 24 hrs, but not 2 wks, after the last repeated injection of this regimen of (+)-MDMA (Bubar, Thomas and Cunningham, unpublished observation). This suggests that there may be a differential pattern of gene expression at the two time points that may correlate with the observed pattern of behavioral changes. In the present studies, our goal was to use microarray technology to

discover patterns of gene expression in order to identify potential neuroadaptations within the reward pathway (NAc, VTA) that are linked to behavior at relevant time points.

The most advantageous feature of DNA microarray technology is the ability to simultaneously assess global changes in gene expression. However, due to the nature of this technology, large amounts of data are generated, which require investigators to utilize computer programs and software to facilitate the processing of data into meaningful conclusions. In addition, the validation of microarray data is imperative to confirm that changes can be replicated. Microarrays and quantitative real-time polymerase chain reaction (qRT-PCR) are emerging as complementary technologies to assess gene expression. Even though microarrays offer a quick, cost-effective approach to scan the expression from the entire genome, they are hindered by the requirement of larger amounts of sample than needed for qRT-PCR, and suffer from experimental variability and data standardization issues. Our process for organizing observed transcript changes, including the advantages and limitations of the resources utilized will be reviewed in the Discussion. A discussion of technical and sample variability issues associated with microarrays will be briefly introduced and addressed in further detail in the Discussion of Chapter 3.

METHODS

Animals

Adult male Sprague-Dawley rats (N = 32; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 225-350g at the beginning of the experimental procedures were used. The animals were housed four to a cage in a temperature (21-23ºC) and humidity (40-50%) controlled environment and lighting was maintained under a 12-h light-dark cycle (lights on at 7:00 a.m.-7:00 p.m.). Food and water were available ad libitum. All experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1986) and with the approval of the Institutional Animal Care and Use Committee.

Drugs

(+)-MDMA (National Institute on Drug Abuse; Research Triangle, NC) and chloral hydrate (Sigma-Aldrich, St. Louis, MO) were utilized in the study. Drugs were dissolved in sterile saline (0.9% NaCl) and administered subcutaneously [SC, (+)-MDMA)] or intraperitoneally (IP, chloral hydrate); doses refer to the weight of the salt.

Experimental Protocols

Microarray Analysis of Transcripts Following Repeated Administration (+)-MDMA

Pretreatment Protocol. Naive rats (n=8/group) were removed from their home cage between 8:00-8:30 a.m., weighed, and injected with either (+)-MDMA (4 mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage each day for 7 consecutive days.

RNA Extraction. At 24 hrs or 2 wks following the last repeated injection, rats were anesthetized using chloral hydrate (800 mg/kg, IP) and decapitated. The VTA and NAc issue were microdissected (Heffner et al., 1980; Bubar et al., 2004; Herin et al., 2005) on a cool tray (4°C), and stored in RNA*later*® (Ambion) at -80ºC. Total RNA from tissues was prepared according to manufacturers instruction (RNAqueous,® Ambion). For each rat (N=32), total RNA was extracted from the NAc and VTA. RNA

was pooled according to treatment [(+)-MDMA, saline], brain region (NAc, VTA), and time point (24 hrs, 2 wks) to form 8 separate pools (n=8 rats in each pool).

Pooled samples were sent to the Molecular Genomics Core Facility of the University of Texas Medical Branch for further sample preparation and microarray hybridizations using standard Affymetrix® protocols. A total of 16 microarray hybridizations were performed using the Affymetrix® Rat Expression Array 230A (Santa Clara, CA) representing approximately 25,000 rat genes. Sample preparation begins by generating first strand cDNA from each pooled RNA sample using 5X first strand cDNA buffer (Invitrogen), dithiothreitol (DTT) (Invitrogen), 10 mM di-nucleotide triphosphate (dNTP) mix (Promega), and SuperScript™ reverse transcriptase (Invitrogen). The first strand reaction mix is then mixed with diethylpyrocarbonate (DEPC) treated water, 5X second strand buffer (Invitrogen), 10 mM dNTP mix (Promega), DNA ligase, DNA polymerase I, and RNase H. Following clean-up of the double-stranded cDNA with phenol and chloroform, the antisense cRNA is biotin labeled. The reagents used for this reaction include DEPC treated water, 10X HY reaction buffer, 10X biotin labeled NTPs, 10X DTT, RNase inhibitor, cDNA and 20X T_7 RNA polymerase. Following clean-up of the biotinylated cRNA, cRNA is fragmented using target RNA, 5X fragmentation buffer (Affymetrix), and RNase-free water (Ambion). The prepared fragmented RNA samples are then added to a hybridization cocktail containing control oligonucleotide B2 (Affymetrix), 100X control cRNA, herring sperm DNA (Promega), acetylated bovine serum albumin (BSA) (Invitrogen), MES hybridization buffer and water. The sample mixture is added to the chip and incubated for 16-24 hrs at 45°C. Immediately after hybridization, the arrays undergo an automated washing and staining process on the Affymetrix® Gene Chip Fluidics Station 400. This protocol includes a low stringency wash, a high stringency wash with streptavidin/phycoerythrin (SAPE) stain (Molecular Probes), a second low stringency wash, anti-streptavidin antibody (Vector Labs) stain, a second high stringency wash with SAPE stain, and a final low stringency wash. After staining and washing, each chip is placed in an Affymetrix® Gene Chip Scanner for image capture and conversion to numerical output using the Microarray Suite Analysis Suite version 5.0. Specific experimental information is defined by the software for the probe cell and computes the intensity for each cell. A comparison between chips using

the Affymetrix® protocol was performed using "base-line chip" intensity values, normalized to the average signal intensity.

Data analysis. Data analyses were initiated by the UTMB Program in Bioinformatics. The changes in gene expression relative to the baseline samples (animals receiving saline) were recorded as signal log ratios (SLR) and converted to relative fold changes (FC) by using the calculation 2^n = FC, where *n* equals the SLR, since the log scale used is base 2. Genes were categorized into "up," "down," or "no change" categories with respect to the baseline in each experiment by using the Student's *t* test. A 2-fold increase or decrease in the ratio of intensities as the criteria for a change in gene expression is often used by investigators; therefore, a list containing all genes exhibiting a ≥2-fold change was generated and returned to our lab. Although numerous web-based programs exist, the Affymetrix® NETAFFX Analysis Center (http://www.affymetrix.com), Gene Microarray Pathway Profiler (GenMAPP, version 1.0), and Ingenuity Pathway Analysis™ were used for subsequent data analyses. Transcripts exhibiting a fold-change ≥2 were organized into biologically functional categories. Information found in the National Center for Biotechnology Information (NCBI) facilitated placement of differentially expressed transcripts [(+)-MDMA vs. saline] into a particular category.

RESULTS AND DISCUSSION

Microarray Analyses

cDNA microarrays were used to identify gene expression profiles in the rat NAc and VTA at 24 hrs and 2 wks withdrawal from a repeated regimen of (+)-MDMA (4 mg/kg/day, 7 days). Fluorescently labeled cDNA prepared from the NAc and VTA of two treatment groups [(+)-MDMA, saline] at two withdrawal time points (24 hrs, 2 wks) was hybridized to two Affymetrix® Rat Expression Array 230A, resulting in 16 microarrays (chips) used in this study. This process identified a total of 308 genes exhibiting a ≥2-fold change. Of these 308 genes, only 20 genes exhibited a fold-change ≥5.

Organization of Microarray Data

Microarray technology has important applications in biology and medicine, but presents considerable challenges to data mining, including analyzing data with many characteristics, availability of biological knowledge of the genes, complexity of integrating this information, understanding gene-gene interactions, and understanding the influence of variation and error that contributes to each step of the microarray process (Ojaniemi et al, 2003; Balagurunathan et al., 2004).

In order to make meaningful conclusions from the vast amounts of data generated from microarray studies, the results must be organized in a fashion that reveals patterns of gene expression changes between two sample groups. In our case, microarray changes were initially organized by array, brain region (NAc or VTA), and withdrawal time point (24 hrs or 2 wks). This organization did not reveal a readily apparent pattern of gene expression. Up- and down-regulated genes were relatively equally distributed within each brain area and time point (**Table 1**), and very few transcripts were shown to change in both replicate runs in the same directional manner (**Table 2**). These early analyses led to the use of several computer-based programs to further process our microarray data.

Table 1. Distribution of up-and down-regulated genes

Brain area	Time point	Up-regulated	Down-regulated
NAc	24 hrs	47	39
NAc	2 wks	48	35
VTA	24 hrs	39	27
VTA	2 wks	36	37

Table 2. Genes differentially expressed in duplicate runs

GenBank No.	Gene	Brain area, Time point	Fold-change
NM_053525	ATP-dependent, helicase	NAc, 24 hrs	-3.1, -2.2
M25649	Oxytocin	NAc, 24 hrs	11.0, 3.3
L13041	Calcitonin receptor	NAc, 2 wks	2.0, 2.4
BI285575	Collagen type 1, α 1	NAc, 2 wks	-2.5, -2.1
BI275716	Collagen type III, α 1	NAc, 2 wks	-2.2, -2.0
M75281	Cystatin S	NAc, 2 wks	6.8, 2.4
NM_133294	Erythroid, differentiation gene	NAc, 2 wks	2.0, 2.7
NM_053763	P450, subfamily 27b	NAc, 2 wks	2.6, 2.3
NM_031549	Transgelin	NAc, 2 wks	-2.6, -2.2
NM_053918	Glycoprotein hormones, α	VTA, 24 hrs	2.2, 2.1
BI287300	Hemoglobin β chain complex	VTA, 24 hrs	-2.3, -2.6
AJ302054	Macrophage protein DLM-1	VTA, 24 hrs	2.0, 2.0
NM_031549	Transgelin	VTA, 24 hrs	-2.1, -2.3
NM_013091	TNF superfamily, member 1a	VTA, 24 hrs	-2.2, -2.0
NM_012681	Transthyretin	VTA, 2 wks	-6.1, -9.4
AF071204	Stem-cell factor KL-1	VTA, 2 wks	-2.2, -2.1

Gene Microarray Pathway Profiler (GenMAPP, version 1.0) (Gladstone Institutes, University of California at San Francisco) is a computer program designed and used to view and examine gene expression data within biological pathways. Our data set was imported into the program and GenMAPP was used to convert the expression data into illustrations showing presence and degree of fold-change for the genes represented on the microarray. This program consists of most well-characterized biological pathways (e.g., fatty acid synthesis, signal transduction cascades) called MAPPs, and enables the user to distinguish particular characteristics of genes by color. For example, in our analyses, green represented genes observed to be up-regulated and red represented genes observed to be down-regulated (**Fig. 1**). Unfortunately, these MAPPs only provide static illustrations of pathways where the user is unable to work interactively with the software, and the pathways are not frequently updated. In addition, when our data set was entered, the program did not recognize all of the entries and did not contain a comprehensive list of all known biological pathways.

Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, Mountain View, California) is a marketed web-based application that allows investigators to draw new biological conclusions from their data by creating biological networks based on their experimental data. These networks are generated through the use of the IPA Knowledge Base (Ingenuity® Systems, www.ingenuity.com), which is continually updated to reflect the most current scientific literature. Our data set containing gene names and expression values were uploaded into the application, and subsequently matched to corresponding gene information in the IPA Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity. By selecting a particular network the user is able to visualize and examine specific pathway details (**Fig. 2**). Like GenMAPP, IPA allows the user to distinguish up- (green) and down-regulated (red) genes by color, and additionally identifies the degree of fold-change by the intensity of color (the more intense the color, the larger the fold-change). The proximity of the genes within a network is determined by their relatedness in the knowledge base which is established by scientific publications. Thus the closer the two genes are in the functional

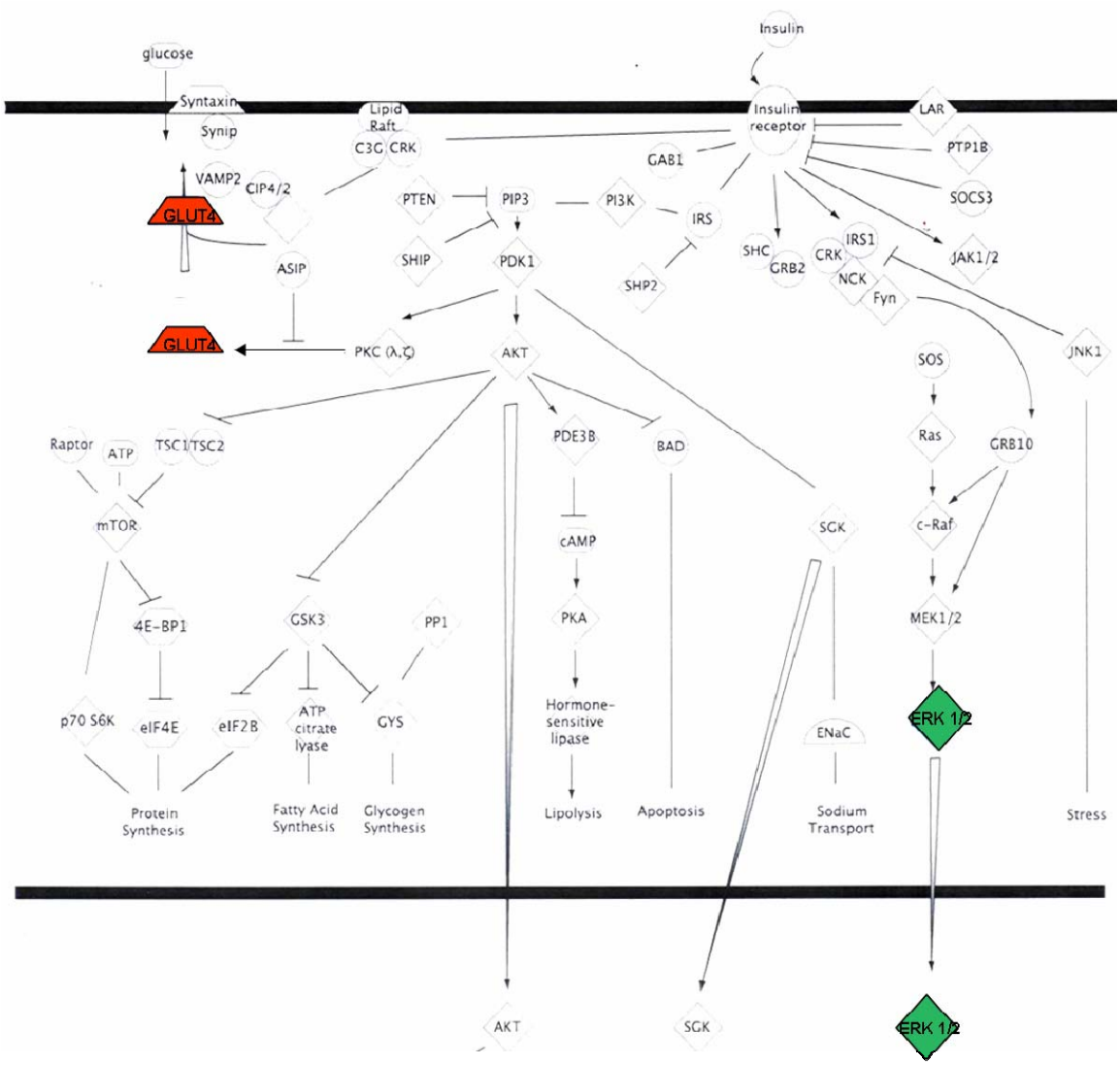

Figure 1. GenMAPP pathway integrating our expression data. The insulin pathway contains genes observed to change in the VTA at 2 weeks from repeated (+)-MDMA (MAPPFinder version 1.0, www.GenMAPP.org). According to our microarray studies, molecules shown in green were up-regulated, and those shown in red were down-regulated.

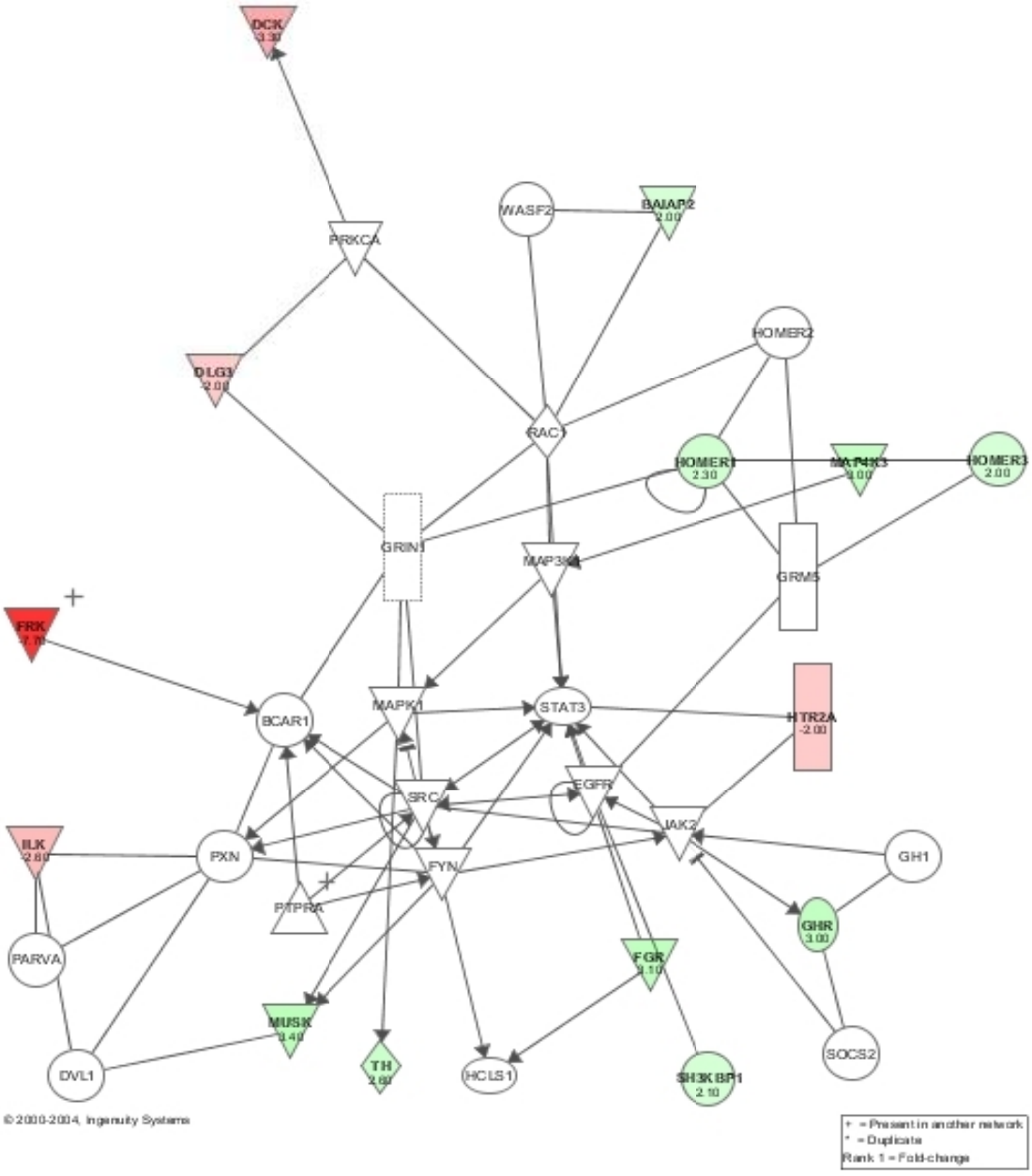

Figure 2. Network generated using Ingenuity Pathway Analysis. Network contains genes observed to change in NAc at 24 hrs from repeated (+)-MDMA (Ingenuity® Systems, **www.ingenuity.com**). According to our microarray studies, molecules shown in green were up-regulated, and those shown in red were down-regulated with greater intensity of color correlating to a larger the fold-change.

network representation, the larger the amount of literature that exists to relate these genes to one another. The IPA software provided a tremendous amount of information on genes and their proposed function, and allowed us to better understand the transcripts reported to have changed; however, attempts to identify a meaningful pattern of gene expression changes were inconclusive.

Data generated from microarray experiments are frequently organized into established biologically functional categories. Previous attempts at organizing our set of gene expression changes by brain region, withdrawal time point, and magnitude and direction of fold-change did not reveal an apparent differential pattern of expression; thus, we chose to collapse our data set to contain genes differentially expressed between (+)-MDMA vs. saline regardless of the brain area and withdrawal time point in which they were observed to change. Research into gene function was conducted using their GenBank accession number [National Center for Biotechnology Information (NCBI)], **www.ncbi.nlm.nih.gov**) and the most recent functional name from the Affymetrix® NetAffx database (**www.affymetrix.com**). The accumulation of gene-function relationships guided the development of 13 functional categories (**Table 3**). Examples of transcripts placed in each category are found in Table 4.

Table 3. Biologically Functional Categories: Number of genes in each category

Growth	6
Immune Response	13
Intracellular Phosphorylation Networks	35
Ionic Homeostasis	9
Metabolism	22
Neuroendocrine Proteins/Glycoproteins	18
Proteolysis and Peptidolysis	19
Receptors/Transporters/Enzymes	31
Replication	12
Scaffolding/Vesicle/Membrane Trafficking	42
Signal Transduction	10
Transcription	33
Miscellaneous	58

A number of data interpretation issues were faced during our data mining. One of the most difficult issues to overcome was the arduous task of deciphering multiple entries of information for a particular gene. For example, the gene encoding synapse associated protein of 102kDa is also found in the literature as the Drosphila disc-large homolog 4 (dlgh-4). This is due in part to the time and nature of discovery, and the fact that partial sequences are often published before the entire gene sequence has

been established. This problem is amplified by the fact that a system for merging this information has not been created; in addition, there is no common system for sharing data between researchers or for comparing data across platforms (Tan et al., 2003).

Discordance between microarray results and post-hoc quantitative validation can be attributed to both technical and biological (sample) variability (Murphy, 2002). Any of these factors alone or in combination can make it difficult to validate results using a different assay. Technical variability in microarray analyses may arise from array-to-array variability, statistical analysis of microarray data, false positives, or the general limits of fluorescent technology; thus it is important to minimize the possibility of their occurance (Murphy, 2002). Replicate microarray experiments must be conducted in order to assess array-to-array variability, and consequently enhance data consistency. In addition, the reliability of each individual probe set must be evaluated to account for probe set-specific variance (Budhraja et al., 2003). The assessment of probe-set variability may decrease the number of false positives observed since multiple members of a gene are often represented on an array. The cross-hybridization of probes to these highly homologous, but biologically distinct genes, often results in false positive results for some genes.

Sample variability in microarrays and quantitative RT-PCR may arise from inconsistent tissue processing (as the size of brain regions we examined are very small), the efficiency of RNA isolation, or successful amplification methods of RT-PCR (Hedge et al., 2000). Due to the number of variables associated with technical variance, sample variation and data processing, it is essential to validate microarray experiments utilizing another assay to study gene expression (Rajeevan et al., 2001). Further discussion of the issues regarding microarray processing and data collection will be addressed in greater detail in the discussion of Chapter 3.

Table 4. Examples of transcripts assigned to biologically functional categories	
Growth	• fibroblast growth factor 23 • growth hormone receptor • glia maturation factor, beta
Immune Response	• lymphotoxin A • insulin substrate 1 and 3
Intracellular Phosphorylation Networks	• brain enriched membrane associated protein • A kinase achor protein 5 • mitogen activated protein kinase
Ionic Transport/ Homeostasis	• Cl^- ion pump • GIRK-K^+ inwardly rectifying channel • K^+ channel, TREK-2
Metabolism	• cytochrome P450, family 27b, polypeptide 1 • glutathione-S-transferase mu 1
Neuroendocrine Proteins/ Glycoproteins	• calcitonin receptor • growth hormone receptor • thyroid stimulating hormone receptor
Proteolysis and Peptidolysis	• alanyl aminopeptidase • dipeptidase 1 • matrix metalloproteinase 3 and 13
Receptors/ Transporters/ Enzymes	• serotonin 2A ($5\text{-}HT_{2A}$) and $5\text{-}HT_4$ receptors • dopamine 3 receptor • tyrosine hydroxylase
Replication	• ATP-dependent helicase • DNA ligase I • DNA polymerase, gamma
Scaffolding/ Vesicle/ Trafficking	• synapse associated protein of 102 kDa • synaptotagmin III and VI • vesicle associated membrane proteins (8 and B/C)
Signal Transduction	• p21-activated kinase 1 • phophodiesterase 1c • phospholipase B
Transcription	• homer 1 and homer 3 • tumor-necrosis factor receptor, member 1a • transcription factor 12
Miscellaneous	• taste bud receptor TB567 and rT2R12 • morphine related protein 1

CHAPTER 3:

ASSESSMENT OF MICROARRAY RESULTS USING QUANTITATIVE RT-PCR

INTRODUCTION

In the previous chapter, genes indicated by microarray analyses to be altered during withdrawal from repeated (+)-MDMA exposure were organized into biologically functional categories (**see Chapter 2**). The category including transcripts related to scaffolding, vesicle function, and membrane trafficking contained the highest number of differentially expressed transcripts [(+)-MDMA vs. saline]. A number of functions rely on membrane composition, including synaptic vesicle trafficking and receptor localization via membrane trafficking (Buckley et al., 2000). Due to the importance of appropriate pre- and post-synaptic membrane composition for proper signal transmission (Davis and Bezprozvanny, 2001), transcripts from this category were chosen for validation and further investigation.

Synaptic vesicle reactions are responsible for the release of neurotransmitter from the pre-synaptic membrane consequent to action potential generation (Matthews, 1996). This process begins with the mobilization of synaptic vesicles at the pre-synaptic membrane and ends with fusion of the vesicle with the pre-synaptic membrane and release of the vesicle contents into the synaptic cleft (Sudhof, 2004). Thus, neurotransmitter release relies on the orchestrated actions of multiple vesicle trafficking reactions and the proteins involved in this process. The life cycle of a transport vesicle can be divided into four phases which include budding, transport, docking and fusion (Bock et al., 2001). Within each phase, various protein families are involved with executing particular functions. Disruption of proteins such as golgi SNAP receptor complex proteins (SNAREs) and vesicle associated membrane proteins (VAMPs) which are involved with docking and fusion, leads to significant changes in vesicle release

(Augustine et al., 1999). Thus, the potential for altered neurotransmission by disruption of vesicle reactions resides with a variety proteins.

As a methoxylated amphetamine derivative, MDMA can affect multiple steps within the cascade of synaptic activity, including the storage and release of synaptic vesicles. Indeed, studies have shown that (±)-MDMA reduces vesicular and synaptosomal uptake of 5-HT (Fleckenstein et al., 2000; Bogen et al., 2003). Fleckenstein and collegues reported a 40% reduction of 5-HT uptake into rat synaptosomes *ex vivo* one hour after treating rats with (±)-MDMA (4 × 15 mg/kg, every 2 hours) (Fleckenstein et al., 2000). Additional studies of (±)-MDMA (2 × 10 mg/kg/day, 4 days) *ex vivo* have shown reduced synaptosomal and vesicular 5-HT uptake 66 hrs after treatment (Bogen et al., 2003). The basis of altered vesicle release and storage that occur following MDMA administration may provide insight into the persistent state of altered neurotransmission that occurs during withdrawal from (+)-MDMA (McNamara et al., 1995; Obradovic et al., 1998; Kalivas et al., 1998).

One way to regulate receptor activity is through membrane trafficking, which refers to the movement of receptors from one membrane region to another via vesicular carriers and protein complexes (Kim and von Zastrow, 2003). The targeting of receptors to specific subcellular compartments is critical for their appropriate incorporation and localization, as well as the processing of neural signals (Gage et al., 2001). Moreover, it is likely that various points of cell-cell contact are associated with unique sets of proteins with which they interact. Specifically, membrane associated guanylate kinases (MAGUKs) are a family of proteins that contain multiple protein-binding domains that allow for the organization of specific multi-protein complexes at sites of cell-cell contact, and are thus recognized for their structural and signaling roles (Kim et al., 1997). In addition, post-synaptic density protein of 97kDa (PSD-97) has been shown to interact with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits to localize these receptors to the post-synaptic density for signaling interactions (Leonard et al., 1998). A third MAGUK, synapse associated protein of 102kDA (SAP102), is involved with the clustering of various proteins at the synapse, and has been found to associate with a number of glutamate receptors at the post-synaptic density (Montgomery

et al., 2004; Kornau and Seeburg, 1997). The **P**SD-95/**D**isc-large/**Z**onula occludens-1 (PDZ) domain-containing proteins PSD-95 and SAP102 are of particular interest to the present proposal. PSD-95 has been reported to have a specific interaction with the 5-HT_{2A}R when they are associated *in vitro* (Xia et al., 2003). When PSD-95 and 5-HT_{2A}R were expressed in HEK293 cells, increased 5-HT_{2A}R-mediated signaling and decreased agonist-induced internalization of the receptor were observed, when compared to PSD-95 co-expression with a mutant 5-HT_{2A}R (lacking PDZ domain) (Xia et al., 2003). These data suggest that the protein-protein interaction between PSD-95 and 5-HT_{2A}R via PDZ domain interactions may be critical in 5-HT_{2A}R regulation. An interaction between SAP102 with the 5-HT_{2A}R has not been reported; however, the role of SAP102 in molecular scaffolding and its involvement with neurotransmitter receptors at the post synaptic density make it a likely candidate for 5-HT_{2A}R modulation (Sans et al., 2001).

Numerous proteins found to complex with GPCRs contain PDZ domains. Indeed, the 5-HT_{2A}R expresses a PDZ domain at its C-terminus, and has been shown to interact with a number of PDZ binding proteins, including the multiple PDZ domain-containing protein (MUPP-1), PSD-95, channel-interacting protein (CIPP), and MAGUK 3 (MPP3) (Ullmer et al., 1998; Bécamel et al., 2004). Interestingly, our microarray data indicated that the gene encoding the 5-HT_{2A}R was observed to decrease 2-fold in the NAc at 24 hrs withdrawal. This observation may be particularly important considering the role of 5-HT_{2A}R in the behavioral effects of MDMA (McCreary et al., 1999; Fletcher et al., 2002; Bankson and Cunningham, 2002; Herin et al., 2005) and the ability of 5-HT_{2A}R to bind PDZ domain-containing proteins that have been shown to alter signaling and localization of GPCRs through domain binding interactions.

The 5-HT_{2A}R is a seven-transmembrane GPCR positively coupled to phospholipase C-β (PLC- β) through the $G\alpha_{q/11}$ G-protein. Activation of this receptor leads to the accumulation of inositol phosphates, increases in intracellular Ca^{+2}, and neuronal depolarization (Raymond et al., 2001; Hoyer et al., 2002). The 5-HT_{2A}R has also been shown to activate other phopholipases, such as phospholipase A_2 (PLA_2), leading to arachidonic acid release (Berg et al., 1998). The 5-HT_{2A}R is extensively distributed throughout the central nervous system with dense localization in the cerebral

cortex and, importantly, in areas of the reward pathway, which is involved in the behavioral effects of psychostimulants, such as the NAc and VTA (Cornea-Hebert et al., 1999; Xu and Pandey, 2000). The mRNA and protein for the 5-HT_{2A}R have been reported to be distributed to the same brain regions, suggesting that the 5-HT_{2A}R is being expressed in the same areas in which it is produced (Pompeiano et al., 1994). The 5-HT_{2A}R in brain has been shown to undergo rapid desensitization and down-regulation in response to chronic agonist (Buckholtz et al., 1988; Anji et al., 2000) and antagonist exposure (Blackshear and Sanders-Bush, 1982; Hensler and Truett, 1998). As a result, it is possible that continued availability of 5-HT in response to MDMA exposure could similarly regulate the 5-HT_{2A}R. Indeed, an acute dose of (±)-MDMA (20 mg/kg) has been shown to transiently down-regulate the 5-HT_{2A}R in rat cortex for up to seven days (Scheffel et al., 1992), and in more recent studies, the expression of the 5-HT_{2A}R was decreased in several cortical regions following treatment with (±)-MDMA (2 X 10 mg/kg/day, 4 days) at 6 hrs withdrawal, and in the occipital cortex for up to three days (Reneman et al., 2002). Based on these numerous observations and the impact of MDMA on neurotransmitter release and storage, proteins involved with scaffolding, and vesicle or membrane trafficking may be important in mediating the actions of (+)-MDMA at 5-HT_{2A}R by altering receptor signaling for the 5-HT_{2A}R.

Prior to initiating further studies regarding the functional role of these molecules in the effects of MDMA, we first needed to validate the changes in transcription for scaffolding and vesicle/membrane trafficking as seen in microarray experiments (**see Chapter 2**). One reason for validating the observed changes was to ensure that the findings were reproducible in a different set of samples. In addition, artifacts producing false positives can be introduced in the numerous preparative steps of microarray hybridization (Balagurunathan et al., 2004). Finally, it is necessary to verify that the observed outcomes are not the result of problems associated with the technology itself. One way to verify that the genes of interest are truly differentially expressed is through qRT-PCR. This method is one of the most sensitive for detecting low-abundance mRNA (Bustin, 2000), and is based on the quantitative relationship that exists between the amount of starting material (cDNA) and the amount of product at any given cycle number during the logarithmic phase of replication (Provenzano et al., 2001).

In order to validate the transcript changes indicated by microarray data, a second set of animals was treated with the same sensitizing, sub-neurotoxic regimen of (+)-MDMA (4 mg/kg/day, 7 days). A subset of transcripts within the scaffolding, vesicle and membrane trafficking associated category were chosen for further studies and included cask-interacting protein (caskin-1), dynamin 1-like protein (dnm1-l), MAGUK-interacting protein 2 (MAGUIN-2), SAP102, SNARE GOSR 1, VAMP B/C, and VAMP 8. These transcripts were all shown to be differentially expressed [(+)-MDMA vs. saline] in either the NAc or VTA at 24 hrs withdrawal and included both up- and down-regulated genes (**Table 5**). The genes encoding the 5-HT_{2A}R, and the closely related 5-HT_{2C}R (Hartig, 1989), were also evaluated for relative transcript expression. Quantitative RT-PCR (qRT-PCR), using Taqman® gene expression assays, was used to assess the relative expression of chosen transcripts observed to change in microarray studies.

Table 5. Genes to be investigated

GenBank Acc. No.	Gene	Description	Relative FC, 24 hrs
NM_017254	**5-HT$_{2A}$R**	5-HT receptor involved in behavioral effects of MDMA	↓ 2.0, NAc
NM_012765	**5-HT$_{2C}$R**	5-HT receptor with high homology to 5-HT$_{2A}$R	No change
NM_080690	**caskin-1**	Brain specific adaptor protein	↓ 2.1, NAc
AF132727	**dnm1-l**	GTPase implicated in tabulation and fission events at cellular membranes	↓ 2.3, NAc
AF102854	**MAGUIN-2**	Neuronal protein found to assemble to components at synaptic junctions	↓ 2.4, VTA
U53367	**SAP102**	PDZ domain-containing, scaffolding protein	↓ 2.0, NAc
NM_053584	**SNARE GOSR 1**	Priming/fusion of transmitter vesicles to membrane for release	↓ 2.3, NAc
NM_021847	**VAMP B/C**	Membrane traffic regulatory protein	↑ 2.3, NAc
NM_031827	**VAMP 8**	Endosomal fusion protein	↓ 2.0, VTA

FC=Fold-Change

METHODS

Animals

Adult male Sprague-Dawley rats (N = 32; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 175-250g at the beginning of the experimental procedures were used. The animals were housed four to a cage in a temperature (21-23ºC) and humidity (40-50%) controlled environment and lighting was maintained under a 12-h light-dark cycle (lights on at 7:00 a.m.-7:00 p.m.). Food and water were available ad libitum. All experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1986) and with the approval by the Institutional Animal Care and Use Committee.

Drugs

(+)-MDMA (National Institute on Drug Abuse; Research Triangle, NC) and chloral hydrate (Sigma-Aldrich, St. Louis, MO) were utilized in the study. Drugs were dissolved in sterile saline (0.9% NaCl) and administered subcutaneously [SC, (+)-MDMA)] or intraperitoneally (IP, chloral hydrate); doses refer to the weight of the salt.

Experimental Protocols

Pretreatment Protocol. Naive rats (n=8/group) were removed from their home cage between 8:00-8:30 a.m., weighed, and injected with the identical regimen of either (+)-MDMA (4 mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage each day for 7 consecutive days.

RNA Extraction. At 24 hrs or 2 wks following the last repeated injection, rats were anesthetized using chloral hydrate (800 mg/kg, IP) and decapitated. The VTA and NAc tissue were microdissected (Heffner et al., 1980; Bubar et al., 2004; Herin et al., 2005) on a cool tray (4°C), and stored in RNA*later*® (Ambion) at -80ºC. Total RNA from tissues was prepared using standard methods (RNAqueous®, Ambion). The quality of total RNA was verified by electrophoresis and spectrophotometry.

Quantitative RT-PCR. Reverse transcription was performed using the TaqMan® Reverse Transcription Kit and the Perkin Elmer GeneAmp® PCR System 9600 (PE Biosystems, Foster City, CA). The reverse transcription program consisted of 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Random hexamers were used to prime all reverse transcription reactions. cDNA was quantified by TaqMan® gene expression assays (Applied Biosystems, Foster City, CA) using an ABI PRISM® 7700 Sequence Detection System (PE Biosystems, Foster City, CA). The amplification program consisted of a 6 min activation step at 95°C followed by 42 cycles of denaturation (95°C for 15 sec), annealing (60°C for 30 sec), and elongation (72°C for 30 sec). The Taqman® gene expression assays utilized for qRT-PCR experiments include: **5-HT$_{2A}$R** (Rn_00568473_m1), **5-HT$_{2C}$R** (Rn_00562748_m1), **caskin-1** (Rn_00589889_m1), **dnm1-l** (Rn_00586466_m1), **MAGUIN-2** (Rn_00572430_m1), **SAP102** (Rn_00581244_m1), **SNARE GOSR1** (Rn_00585841_m1), **VAMP 8**

(Rn_00582868_m1) and **VAMP B/C** (Rn_00573032). A negative control with no template (NTC) was used to assess overall specificity. Data from each sample were normalized to the respective β-actin.

Data Collection & Analysis. All qRT-PCR experiments were conducted on the ABI PRISM® 7700 and data were collected using Sequence Detection System Version 1.7. For our qRT-PCR experiments we chose to use Taqman® gene expression assays developed by ABI. The assays have been optimized (for primer/probe concentrations and annealing temperature) and include a primer pair and a labeled probe which are provided in a single tube. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher attached. After denaturation, both primers and probe annealed to the cDNA strands. This dependence on polymerization ensured that cleavage of the probe occurred only if the target sequence was being amplified. While the probe was intact, the proximity of the quencher greatly reduced the fluorescence emitted by the reporter dye. Cleavage of the probe by the 5' nuclease activity of the Taq polymerase separated the reporter dye from quencher dye, increasing the reporter fluorescence. Inclusion of the elongation step allowed primer extension to continue to the end of the template strand. The amount of fluorescence increased logarithmically and recorded for a brief interval at the end of the extension phase of each cycle.

At the end of the run (40-42 cycles), the crossing threshold (Ct, the cycle at which the fluorescence level crosses a baseline set above noise level and during logarithmic amplification) was determined for each sample. For normalization, ΔCt was calculated for each gene being studied: ΔCt = Ct (gene) - Ct (housekeeping gene). The mean ΔCt +/- SEM was determined for each group. ANOVA or t-test (depending on the number of groups under study) was used to determine whether difference between the appropriate groups was significant. The magnitude of the difference was calculated by: ΔΔCt = ΔCt (control) – ΔCt (test); Fold increase (or decrease) = $2^{-\Delta\Delta C}$

SYBR® Green I based qRT-PCR (Applied Biosystems, Foster City, CA) was used to detect the relative expression of the oxytocin transcript. Reverse transcription, detection and data collection procedures are described above. The amplification program consisted of a 2 min activation step at 95°C followed by 42 cycles of denaturation (95°C for 15 sec), annealing (60°C for 30 sec), and elongation (72°C for 30 sec). The DNA

intercalator dye SYBR® Green bound double-stranded DNA and detection was monitored by measuring the increase in fluorescence throughout the cycle. At the end of the run (42 cycles), the crossing threshold (Ct) was determined for each sample as described above. For normalization, ΔCt was calculated for each gene being studied: ΔCt = Ct (gene) - Ct (housekeeping gene). The mean ΔCt +/- SEM was determined for each group. ANOVA or t-test (depending on the number of groups under study) was used to determine whether difference between the appropriate groups was significant.

RESULTS

In order to validate microarray findings, qRT-PCR was carried out for eight transcripts within the scaffolding, vesicle and membrane trafficking associated category observed to change at 24 hrs withdrawal from (+)-MDMA in the NAc: caskin-1, dnm1-l, PSD-95, SAP102, SNARE GOSR 1, VAMP B/C, or VTA: MAGUIN-2, VAMP 8 (**Table 5**). In addition, expression of the gene encoding the 5-HT_{2A}R, observed to decrease 2-fold in the Nac at 24 hrs withdrawal, and the closely related 5-HT_{2C}R were examined (**Table 5**).

RNA from each rat (n=6/group) was reverse transcribed into cDNA and two independent qRT-PCR trials were conducted for each template source. In each trial, duplicate samples of the template source were analyzed. The housekeeping gene β-actin was not differentially expressed between (+)-MDMA and saline treated samples by either microarray or qRT-PCR. No amplicons were generated in template-free negative controls.

Interestingly, RNA derived from VTA tissue was found to be less concentrated than that derived from the NAc, and may reflect poorer RNA isolation from the smaller tissue sample. As seen in Figure 3, the relative expression of 5-HT_{2A}R and β-actin transcripts in saline samples from the NAc exhibited low variability between crossing thresholds (**Fig. 3A, 3B**). However, the variability of crossing thresholds for MAGUIN-2 and β-actin transcripts observed in saline samples from the VTA was high (**Fig. 3C, 3D**). It is possible that this variability was inherent to the VTA tissue sample since this was observed for both the transcript of interest and housekeeping gene.

Results of qRT-PCR analysis are summarized in **Figure 4**. No differences were found in the expression of transcripts indicated by microarray data to be differentially [(+)-MDMA vs. saline] expressed. However, expression of 5-HT_{2C}R mRNA was found to be significantly decreased in (+)-MDMA samples (p=.04) using qRT-PCR which was not observed in microarray studies.

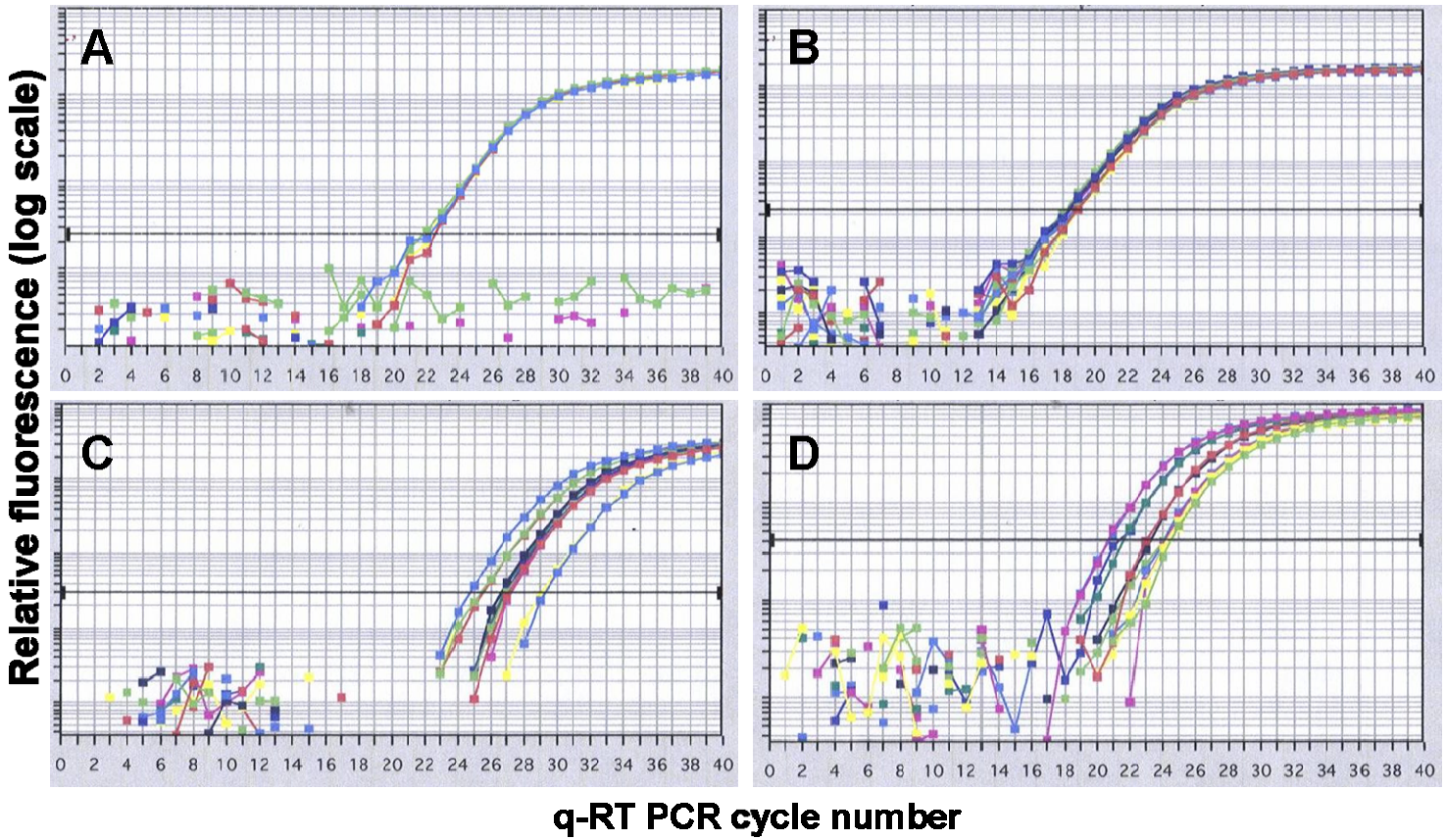

Figure 3. qRT-PCR amplification plots. Crossing thresholds for 5-HT_{2A}R [A] and β-actin [B] mRNA in the NAc, and MAGUIN-2 [C] and β-actin [D] mRNA in the VTA at 24 hrs withdrawal from (+)-MDMA (4 mg/kg/day, 7 days)

The results from the previous experiment led us to question the sensitivity of the microarray experiments since the transcripts investigated by qRT-PCR were all observed to change 2-2.4-fold in microarray experiments. Thus, we sought to validate changes in the oxytocin transcript, which was observed on microarrays to decrease 3.3- and 11-fold in the NAc at 24 hrs withdrawal. The results of qRT-PCR analyses using SYBR Green (DNA intercalator) or a Taqman® gene expression assay for the oxytocin transcript are summarized in **Figure 5**.

The present results indicate that even large fold-changes observed in our microarray experiments were not validated when a more sensitive assay was performed. The inability to validate our microarray studies suggests that microarray protocols may not have been optimized at the time of our studies.

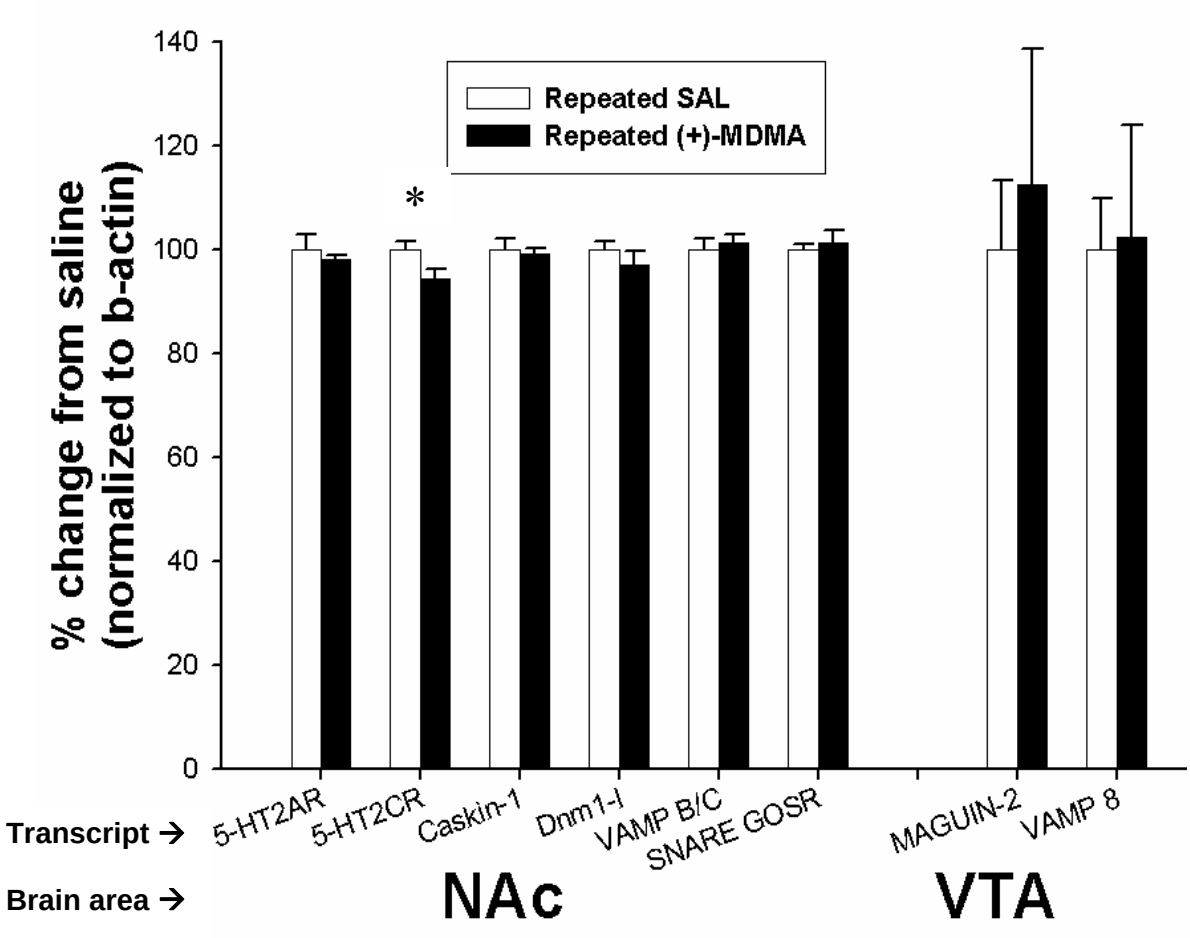

Figure 4. Real-time PCR quantitation of mRNA levels in NAc and VTA at 24 hrs withdrawal from repeated (+)-MDMA. Relative levels were determined from real-time crossing thresholds as described in Methods. Values are expressed as a percentage of the mean control value ± SEM (n = 6). * $p < 0.05$ vs. saline.

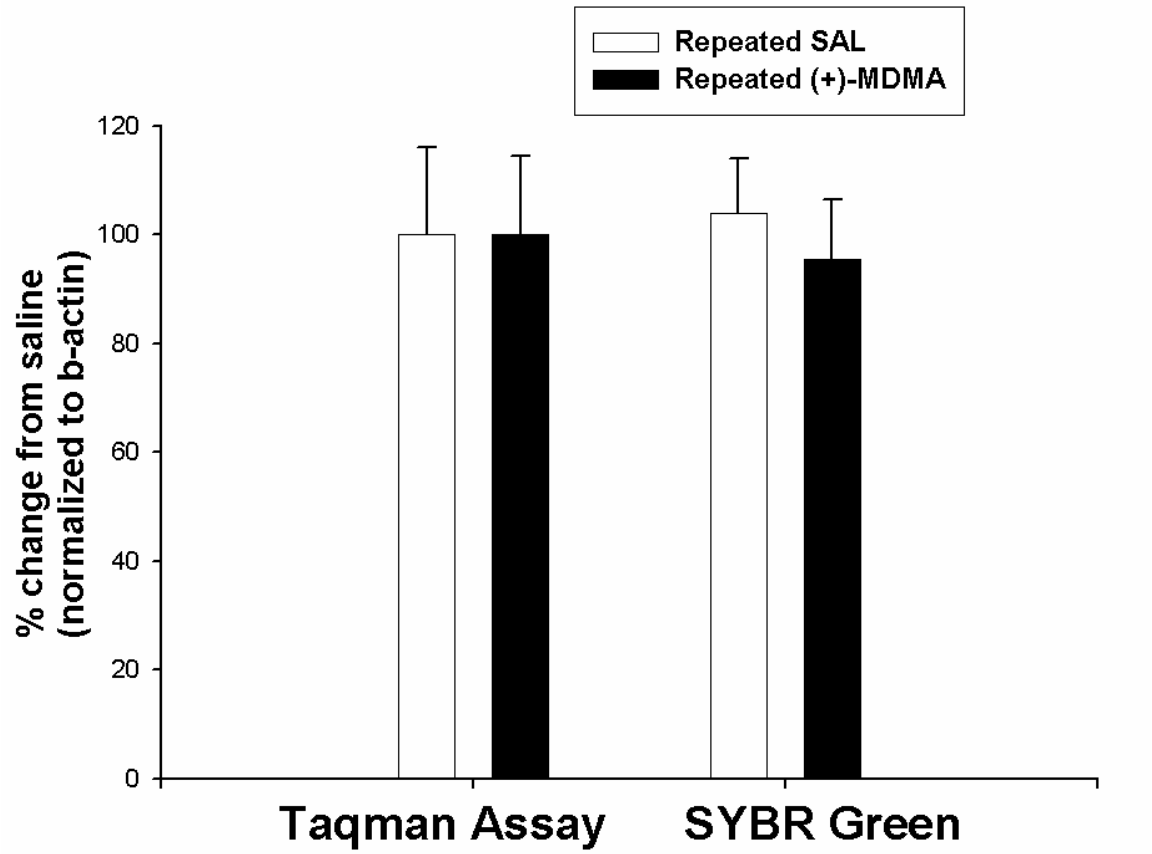

Figure 5. Real-time PCR quantitation of mRNA levels of oxytocin in NAc at 24 hrs withdrawal from repeated (+)-MDMA. Relative levels were determined from real-time crossing thresholds as described in Methods. Values are expressed as a percentage of the mean control value ± SEM (n = 6).

DISCUSSION

Assessment of Microarray Data by Quantitative RT-PCR

Quantitative RT-PCR assays are widely used for validation of transcript changes seen on DNA microarrays and are characterized by a wide dynamic range of quantification (7–8 logarithmic decades), high technical sensitivity (< 5 copies) and high precision (< 2% standard deviation) (Bustin, 2000). This method has been used to validate gene expression changes in the majority of published microarray studies due to its speed, sensitivity, high throughput capacity and minimal amounts of test material required for qRT-PCR. We employed qRT-PCR techniques in a similar fashion to assess the relative expression of 5-HT_{2A}R, caskin-1, dnm1-l, MAGUIN-2, SAP102, SNARE GORS1, VAMP 8 and VAMP B/C in NAc and VTA tissues taken 24 hrs and 2 wks after termination of repeated (+)-MDMA treatment (**Table 5**). These transcripts belong to a category of transcripts involved with scaffolding, vesicle and membrane trafficking which all were shown to be changed by microarray analysis at 24 hrs withdrawal. However, these microarray data were not validated when the sensitive qRT-PCR assay was performed. Surprisingly, 5-HT_{2C}R mRNA was found to be significantly decreased in (+)-MDMA samples (p=0.04), even though this change was not observed in microarray studies.

The SAP102 and MAGUIN-2 transcripts examined by qRT-PCR have protein products that belong to the MAGUK family. As previously described, this family of proteins contains multiple binding domains (e.g., PDZ binding domains) which are used to form multi-protein complexes and are recognized for their structural and signaling roles (Montgomery et al., 2004). In particular, SAP102 and MAGUIN-2 may participate in the regulation of 5-HT_{2A}R function through PDZ domain interactions with this receptor. Our present results suggest that differential expression [(+)-MDMA vs. saline] of SAP102, MAGUIN-2 and 5-HT_{2A}R mRNA was not found by qRT-PCR.

We have suggested that altered vesicle release and storage may provide insight into the persistent state of altered neurotransmission that occurs during withdrawal from repeated exposure to (+)-MDMA (McNamara et al., 1995; Obradovic et al., 1998;

Kalivas et al., 1998). For example, the altered expression of the mRNA for some SNARE and VAMPs has been reported to lead to significant changes in vesicle release (Augustine et al., 1999) and may contribute to the effects of (+)-MDMA on neurotransmitter release. However, it is important to consider that different transcripts have different rates of onset and duration (half-life), which may contribute to our inability to detect changes. The inability to detect changes in this group of transcripts does not exclude these molecules from contributing to vesicle and membrane trafficking reactions but indicates that changes in the expression of their mRNA may not be a requirement. For example, post-translational modifications including glycosylation, phosphorylation, ubiquitination, and methylation do not require mRNA changes, and may facilitate the processes of fine-tuning or rapidly switching protein functions quickly, without the need to synthesize new protein (Mata et al., 2005). We propose that the molecules involved in scaffolding and vesicle or membrane trafficking that we chose to investigate may still be important in 1) mediating the actions of (+)-MDMA at 5-HT_{2A}R by altering receptor signaling for the 5-HT_{2A}R, or 2) participating in neurotransmitter release and storage. This issue will be addressed in further detail in Chapter 4.

DNA Microarray Analyses

Discordance between microarray results and post-hoc quantitative validation can be attributed to both technical (array-to-array variability, analysis of microarray data, false positives, or the general limits of fluorescent technology) and biological (sample) variability (Murphy, 2002). The combination of these factors alone or in combination can make it difficult to validate results using a different assay.

The complexity of analyzing microarray data in order to reveal true differences in gene expression and the importance of conducting replicate arrays is becoming well-recognized (Mirnics et al., 2001; Yuferov et al., 2003). The use of triplicate microarray analyses, where the same sample is assayed on three chips, has been reported to greatly diminish the number of genes observed to change in response to acute “binge” cocaine treatment (Yuferov et al., 2003). By using triplicate microarray analyses, the authors were able to reduce their list of differentially expressed genes by 3-fold when compared to

single chip analysis. The consideration of genes to those which changed in all three arrays greatly diminished the possibility of false positives. In our studies, duplicate microarray experiments were performed for each group, and it is possible that our microarray data may have contained a higher number of false positives due to the lower number of replicate arrays performed. Indeed, very few transcripts were observed to change in the same direction in the duplicate runs.

Sample variability in microarrays and qRT-PCR may arise from inconsistent tissue processing, the efficiency of RNA isolation, or successful amplification of cDNA (Murphy, 2002). With regard to qRT-PCR sample variability, in **Figure 4** there is a notable difference in the magnitude of the standard error of the mean between samples from the NAc and those examined from the VTA. This variability may stem from inherent inconsistency of the reverse transcription reaction as it is highly dependent on the concentrations of the sample RNA. In addition, uniformity between the first and second set of arrays may not have been ideal since each sample group was pooled just prior to hybridization. Regardless of the cause, high variability often prevents the detection of true differential expression, since it decreases the power of statistical tests (Steibel et al., 2005).

Importantly, the processing of small tissue samples in which we are unable to distinguish distinct cell types may hinder our ability to reveal small changes in transcript expression as differences may be washed out when all cell types are examined together.

The low sensitivity of microarrays to detect small changes also adds to this problem. In an attempt to address this issue, we chose to validate the transcript from our microarray data exhibiting the largest fold-change. The expression of oxytocin, observed to decrease 3.3- and 11-fold in the NAc at 24 hrs withdrawal, was evaluated by qRT-PCR using two detection methods. Taqman and SYBR® Green methods were both utilized to ensure that the findings by qRT-PCR were consistent. However, the relative expression of oxytocin was not found to be differentially expressed in (+)-MDMA vs. saline control samples (**Fig. 5**). Because we were unable to validate a transcript exhibiting a large fold-change, microarray sensitivity is not likely to be the sole reason that we were unable to validate small fold-changes in the transcripts studied by qRT-PCR.

In summary, the variability of both sample and microarray processing further emphasize the need for uniform protocols across laboratories for sample preparation, microarray hybridization and data analyses.

CHAPTER 4:

ROLE OF 5-HT_{2A}R AND BINDING PROTEINS IN BEHAVIORAL SENSITIZATION TO (+)-MDMA

INTRODUCTION

The 5-HT_{2A}R has been implicated in a number of physiological and psychological conditions including depression, schizophrenia, obsessive compulsive disorder, and anxiety (Dubovsky and Thomas, 1995). In addition, this GPCR has been shown to play a role in the effects of MDMA including hyperactivity, and hyperthermia (McCreary et al., 1999; Bankson and Cunningham, 2002; Fletcher et al., 2002; Herin et al., 2005). The 5-HT_{2A}R is extensively distributed throughout the central nervous system with dense localization in the cerebral cortex and, in areas of the DA mesoaccumbens reward pathway, which is naturally involved in the behavioral effects of psychostimulants, including the NAc and VTA (Cornea-Hebert et al., 1999; Xu and Pandey, 2000). This pathway appears to be critical for the initiation and expression of behavioral sensitization seen upon withdrawal from repeated administration of psychostimulants including MDMA (**see Chapter 1**) (Kalivas et al. 1993; Vanderschuren and Kalivas, 2000). It is possible that the 5-HT_{2A}R, found in regions of the reward pathway, could play an essential role in the development of behavioral sensitization to repeated (+)- and (±)-MDMA observed upon drug cessation, and may mediate the manifestation of behavioral withdrawal sequelae (e.g., depression and anxiety) (Morgan, 2000).

In the previous chapter, we attempted to validate microarray data suggesting the differential expression [(+)-MDMA vs. saline] of the transcript for 5-HT_{2A}R and a number of scaffolding, vesicle and membrane trafficking molecules. These transcripts were suggested in microarray analyses to be altered (up- or down-regulated) at 24 hrs withdrawal from the repeated regimen of (+)-MDMA (4 mg/kg/day, 7 days). A transient enhancement of MDMA-evoked hyperactivity (behavioral sensitization) was also observed at this time point. Although our microarray data were not validated (**see**

Chapter 3), a number of studies have shown down-regulation of 5-HT_{2A}R (Reneman et al., 2002) and 5-HT_2R (Scheffel et al., 1992; McGregor et al., 2003) binding sites following repeated regimens of (±)-MDMA. The absence of 5-HT_{2A}R mRNA changes in our qRT-PCR experiments does not exclude the possibility of changes in 5-HT_{2A}R protein expression.

A number of factors make the gene-protein relationship difficult to predict. For instance, the time elapsed between transcript generation and protein synthesis, and the variable durations of their respective half-lives of expression, make it difficult to draw conclusions about temporal relationships. One study has reported that increased 5-HT_{1B}R density occurs in the absence of changes in 5-HT_{1B}R mRNA, leading to the conclusion that the increase may have been associated with post-transcriptional changes in receptor regulation and not with changes in the transcription of the mRNA (Sexton et al., 1998). Thus, it is possible that 5-HT_{2A}R protein expression may be altered by the repeated regimen of (+)-MDMA employed, despite the lack of mRNA change observed with qRT-PCR. The circumstances surrounding the down-regulation of 5-HT_{2A}R in response to (+)-MDMA and (±)-MDMA have been generally studied but not fully characterized. As previously discussed, the 5-HT_{2A}R has been shown to undergo rapid desensitization and down-regulation in response to both chronic agonist (Buckholtz et al., 1988; Anji et al., 2000) and antagonist exposure (Blackshear and Sanders-Bush, 1982; Hensler and Truett, 1998). It is feasible that continued availability of 5-HT due to MDMA-induced reversal of SERT and activation of 5-HT_{2A}R by MDMA itself (Battaglia et al., 1988) could induce regulation of 5-HT_{2A}R through similar mechanisms.

The GPCRs are desensitized in a matter of minutes upon agonist stimulation (Hausdorff et al., 1990). Desensitization can occur as a result of internalization (sequestration of the receptor away from the membrane) or receptor uncoupling from G-protein or down-regulation (loss of total receptor number) (Hausdorff, 1990; Bohm et al., 1997). By using the β-adrenergic receptor as a prototype, the mechanisms underlying desensitization by ligand-induced internalization have been studied for GPCRs (Bunemann and Hosey, 1999). Within this integrated "classical" pathway, the agonist-occupied receptor is phosphorylated by a second messenger-dependent kinase (e.g., protein kinase A) and/or a GPCR kinase (GRK) (Stoffel et al., 1997). Binding of the

scaffold protein arrestin leads to uncoupling of the receptor from the G-protein, steric inhibition of G-protein re-coupling and targeting of the receptor to clathrin-coated pits for internalization (Ferguson, 2001). This process of internalization is dependent on the small GTPase dynamin which is ultimately responsible for pinching off the endocytotic vesicle (Hausdorff et al., 1991). The receptor will be targeted either to the endosome for de-phosphorylation and recycling to the membrane or to lysosomes for degradation (Hausdorff et al., 1991).

The regulation of the 5-HT_{2A}R does not appear to follow this classical method of internalization. Evidence suggests that internalization of the 5-HT_{2A}R does not require the expression of GRKs *in vitro* (Gray and Roth, 2001). In addition, although the scaffolding protein arrestin has been found to bind 5-HT_{2A}R, arrestin and 5-HT_{2A}R are differentially sorted upon agonist exposure to separate membrane compartments (Gelber et al., 1999). Studies also have shown that 5-HT_{2A}R internalization *in vitro* is arrestin-independent and dynamin-dependent, providing further evidence that arrestin is not a necessary regulatory protein for the internalization of 5-HT_{2A}R (Bhatnager et al., 2001). Although these studies demonstrate that the 5-HT_{2A}R is not regulated by a number of classical regulating proteins, the molecular mechanisms underlying 5-HT_{2A}R regulation, to include internalization and down-regulation, remain unidentified.

The down-regulation, internalization, and intracellular signaling for GPCRs are modulated by proteins that bind to their intracellular binding domains (Kreienkamp, 2002). These binding partners are multi-domain containing proteins capable of modulating receptor function (e.g., signaling) and providing scaffolds for protein-protein interactions. Many of the proteins identified as binding partners of GPCRs contain PDZ domains which bind to short C-terminal sequences on numerous membrane proteins (Hung and Sheng, 2002). One example of receptor function modulation by PDZ domain-containing proteins involves the β_2-adrenergic receptor. Activation of this receptor leads to increased cAMP levels, which subsequently lead to the inhibition of the Na+/H+ exchanger (NHE) (Hall et al., 1998). However, binding of the NHE regulatory factor (NHE-RF) via one of its PDZ binding domains to the agonist-occupied NHE results in the opposite action and increased exchanger activity (Hall et al., 1998). This suggests that

the binding of PDZ domain-containing proteins to GPCRs has the potential to alter predetermined internal signaling pathways.

The PDZ domain-containing proteins also serve as scaffolds for protein-protein interactions. For example, the N-methyl-D-aspartate (NMDA) receptor is anchored by PSD-95 beneath the post-synaptic membrane, and PSD-95 is anchored by guanylate-kinase-associated proteins (GKAPs) (Kim et al., 1997). It is clear that the ability of PDZ proteins to provide scaffolds and participate in the modulation of neurotransmitter receptor complexes will remain the focus of many future studies; the interactions of PDZ domain-containing proteins with the 5-HT_{2A}R and, the closely related 5-HT_{2C}R, have already been initiated (Bécamel et al., 2002; Bécamel et al., 2004).

The last four amino acids of the 5-HT_{2A}R constitute a Type I PDZ binding domain that has been shown to interact with PDZ domain-containing proteins. The prototypic PDZ domain protein PSD-95 has been reported to have a specific interaction with the 5-HT_{2A}R that results in increased 5-HT_{2A}R-mediated signaling, clustering of 5-HT_{2A}R at the cell membrane and decreased agonist-induced internalization of the receptor *in vitro* (Xia et al., 2003). The 5-HT_{2A}R has also been found to interact with other PDZ domain-containing proteins (Bécamel et al., 2004), such as activin-receptor interacting protein (ARIP-1), SAP97, channel interacting PDZ domain protein (CIPP), and membrane palmitoylated protein member 3 (MPP-3). The ARIP-1 scaffolding protein unites activin receptors with intracellular signaling molecules to regulate activin-mediated signaling in neuronal cells (Tsuchida et al., 2004) while SAP97 is reported to be involved in the formation and stabilization of synaptic junctions in nerve terminals and is thought to play a role in the trafficking of AMPARs (Sans et al., 2001). Containing four PDZ domains, channel interacting PDZ protein (CIPP) is reported to interact with NMDA NR2 subunits and inward rectifying potassium channels (Kurschner et al., 1998). The actions of MPP-3, a member of the MAGUK subfamily, have not been well characterized, but are likely to resemble the actions of other MAGUKs. These proteins serve as molecular scaffolds to coordinate the membrane-associated cytoskeleton linking diverse functional molecules of the postsynaptic density to assist in post-synaptic signaling (Jing-Ping et al., 2005). These data suggest that PDZ domain-containing proteins serve as molecular scaffolds to bring structurally diverse but functionally-

connected proteins together at the synapse to facilitate signal transduction. It is possible that PDZ domain-containing proteins may have similar interactions with 5-HT_{2A}R; however, this issue has not been extensively studied.

As described in this chapter, a number of PDZ domain-containing proteins have been shown to associate with 5-HT_{2A}R; however, the full implications of these interactions remain unknown, and the role that these molecules may play in the regulation of 5-HT_{2A}R is only beginning to be explored. In addition, the role of the 5-HT_{2A}R in behavioral sensitization has yet to be fully characterized. In Chapter 1, we reported that a repeated regimen of (+)-MDMA (4 mg/kg/day, 7 days) was shown to produce transient behavioral sensitization (demonstrated by increased (+)-MDMA evoked hyperactivity) at 24 hrs withdrawal, but not 2 wks. Thus, in the present studies western blot analysis was utilized to determine whether the transient sensitization observed at 24 hrs withdrawal was associated with alterations in 5-HT_{2A}R, PSD-95 or SAP102 protein expression in the NAc and VTA. Although an interaction for SAP102 with the 5-HT_{2A}R has not been reported, the role of SAP102 in molecular scaffolding and its involvement with neurotransmitter receptors at the post synaptic density, make it a likely candidate for 5-HT_{2A}R modulation. In addition, due to the close homology between the 5-HT_{2A}R and 5-HT_{2C}R (Hartig, 1989), we also investigated whether withdrawal from the repeated (+)-MDMA regimen would be associated with altered expression of 5-HT_{2C}R protein in these brain areas. In order to investigate alterations in the expression of the 5-HT_{2A}R, 5-HT_{2C}R, PSD-95 and SAP102 proteins following withdrawal from the repeated regimen of (+)-MDMA, a third set of animals was treated using the same sensitizing, non-neurotoxic regimen of (+)-MDMA (4 mg/kg/day, 7 days).

METHODS

Animals

Adult male Sprague-Dawley rats (N = 32; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 225-350g at the beginning of the experimental procedures were used. The animals were housed four to a cage in a temperature (21-23ºC) and humidity (40-50%) controlled environment and lighting was maintained under a 12-h light-dark cycle (lights on at 7:00 a.m.-7:00 p.m.). Food and water were available ad libitum. All experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1986) and with the approval by the Institutional Animal Care and Use Committee.

Drugs

(+)-MDMA (National Institutes on Drug Abuse; Research Triangle, NC) and chloral hydrate (Sigma-Aldrich, St. Louis, MO) were utilized in the study. Drugs were dissolved in sterile saline (0.9% NaCl) and administered subcutaneously (SC) or intraperitoneally (IP); doses refer to the weight of the salt.

Experimental Protocols

5-HT_{2A}R, 5-HT_{2C}R, PSD-95 and SAP102 Protein Expression during Withdrawal

Pretreatment Protocol. Naive rats (n=8/group) were removed from their home cage between 8:00-8:30 a.m., weighed, and injected with the identical regimen of either (+)-MDMA (4 mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage each day for 7 consecutive days.

Western Blot. At 24 hrs or 2 wks following the last repeated injection, rats were anesthetized using chloral hydrate (800 mg/kg, IP) and decapitated. The VTA and NAc issue were microdissected (Heffner et al., 1980; Bubar et al., 2004; Herin et al., 2005) on a cool tray (4°C) and homogenized in HEPES containing, EDTA, EGTA, dithiothreitol (DTT), and protease inhibitor cocktail. Insoluble matter (nuclear enriched pellet) was

removed by microcentrifugation at 1000 x g at 4°C for 10 min. The resulting supernatant was centrifuged at 20000 x g for 30 min to obtain a membrane-enriched pellet. The membrane enriched pellet was re-suspended in 1% SDS and frozen at -80°C until protein analysis was conducted. Total membrane-bound protein concentration was determined using a BCA protein determination kit (Pierce, Rockford, IL). Total protein (20-30 μg) was reduced with an appropriate volume of Laemmli sample buffer with DTT for 1 min/ 100°C and separated on a 10% Tris-glycine gradient gel (Novex, San Diego, CA) using SDS-PAGE. Following gel electrophoresis, proteins were transferred to a PVDF membrane (BIORAD, Hercules, CA) via semi-dry electroblotting (Alltech, Deerfield, IL). The membrane was blocked with 5% non-fat dry milk for 1h, followed by incubation in 1% non-fat dry milk with either the monoclonal mouse anti-5-HT_{2A}R antibody (1:5000; RT; BD PharMingen, San Diego, CA), the polyclonal goat anti-5-HT_{2C}R antibody (1:500; overnight 4°C; Santa Cruz), the monoclonal mouse anti-PSD-95 antibody (1:2000; RT; Chemicon International, Temecula CA), the polyclonal rabbit anti-SAP102 antibody (1:400; RT; Oncogene Research Products, Cambridge, MA) or the monoclonal mouse anti-β-actin antibody (1:5000; RT 1hr; Chemicon International, Temecula, CA). After 3 x 20 min washes with TBS-T, the blot was incubated in horseradish peroxidase (HRP) conjugated sheep anti-mouse (1:5000-1:10000; Chemicon International, Temecula, CA), mouse anti-goat (1:2000; Santa Cruz), or goat anti-rabbit (1:2000; Chemicon International, Temecula, CA) in 1% non-fat dry milk for 1hr at RT. Following 3 x 10 min washes in TBS-T and an overnight wash in TBS (4°C), the membrane was incubated in ECL Plus Detection® Reagents (Amersham Biosciences) for 5 min and then exposed to film (Kodak MXR® Hyperfilm) for 1 sec to 10 min, depending upon the antibody being detected, and developed. Each membrane was probed with all four antibodies; antibodies were stripped from the membrane via incubation in Re-blot Plus Mild® (Chemicon) for 7-15 min prior to re-probing with each additional antibody according to the procedures described above.

Films were scanned and subjected to densitometric analysis using Scion Image Analysis Software (Scion Corporation, Fredrick, MD). The density of each band was calculated as the actual band density minus the background density of an adjacent area of

the same size. Following background subtraction, the ratio of 5-HT_{2A}R, PSD-95 or SAP102 band density to actin band density was determined for each sample to normalize for the amount of protein loaded. A Student's *t*-test was used to compare the resultant 5-HT_{2A}R, 5-HT_{2C}R, PSD-95 or SAP102 band densities from saline-treated vs. (+)-MDMA-treated rats for each brain area (NAc or VTA) at each withdrawal time point (24 hrs or 2 wks) with the error rate (α) set at $p < 0.05$. Data are presented as the mean (± SEM) 5-HT_{2A}R, 5-HT_{2C}R, PSD-95 or SAP102 protein expression expressed as percent change from repeated saline-treated controls.

RESULTS

5-HT_{2A}R, 5-HT_{2C}R, SAP102 and PSD-95 Protein Expression

Analysis of membrane-bound protein levels revealed no significant differences in **5-HT_{2A}R** protein expression between saline- and (+)-MDMA-treated rats in NAc at 24 hrs ($p = 0.77$) or 2 wks ($p = 0.36$) withdrawal, or in the VTA at 24 hrs ($p = 0.79$) or 2 wks ($p = 0.78$) withdrawal (**Fig. 6A**). Differential protein expression of the **5-HT_{2C}R** was not found between saline- and (+)-MDMA-treated rats in the NAc at 24 hrs ($p = 0.73$) or 2 wks ($p = 0.29$) withdrawal, or in the VTA at 24 hrs ($p = 0.53$) or 2 wks ($p = 0.80$) withdrawal (**Fig. 6B**). No significant differences in **PSD-95** protein expression were observed between saline- and (+)-MDMA-treated rats in the NAc at 24 hrs ($p = 0.55$) or 2 wks ($p = 0.91$) withdrawal, or in the VTA at 24 hrs ($p = 0.49$) or 2 wks ($p = 0.19$) withdrawal (**Fig. 6C**). A similar lack of significant difference in **SAP102** protein expression between saline- and (+)-MDMA-treated rats was observed in the NAc at 24 hrs ($p = 0.19$) and 2 wks ($p = 0.36$) withdrawal, and in the VTA at 24 hrs ($p = 0.93$) and 2 wks ($p = 0.64$) withdrawal (**Fig. 6D**).

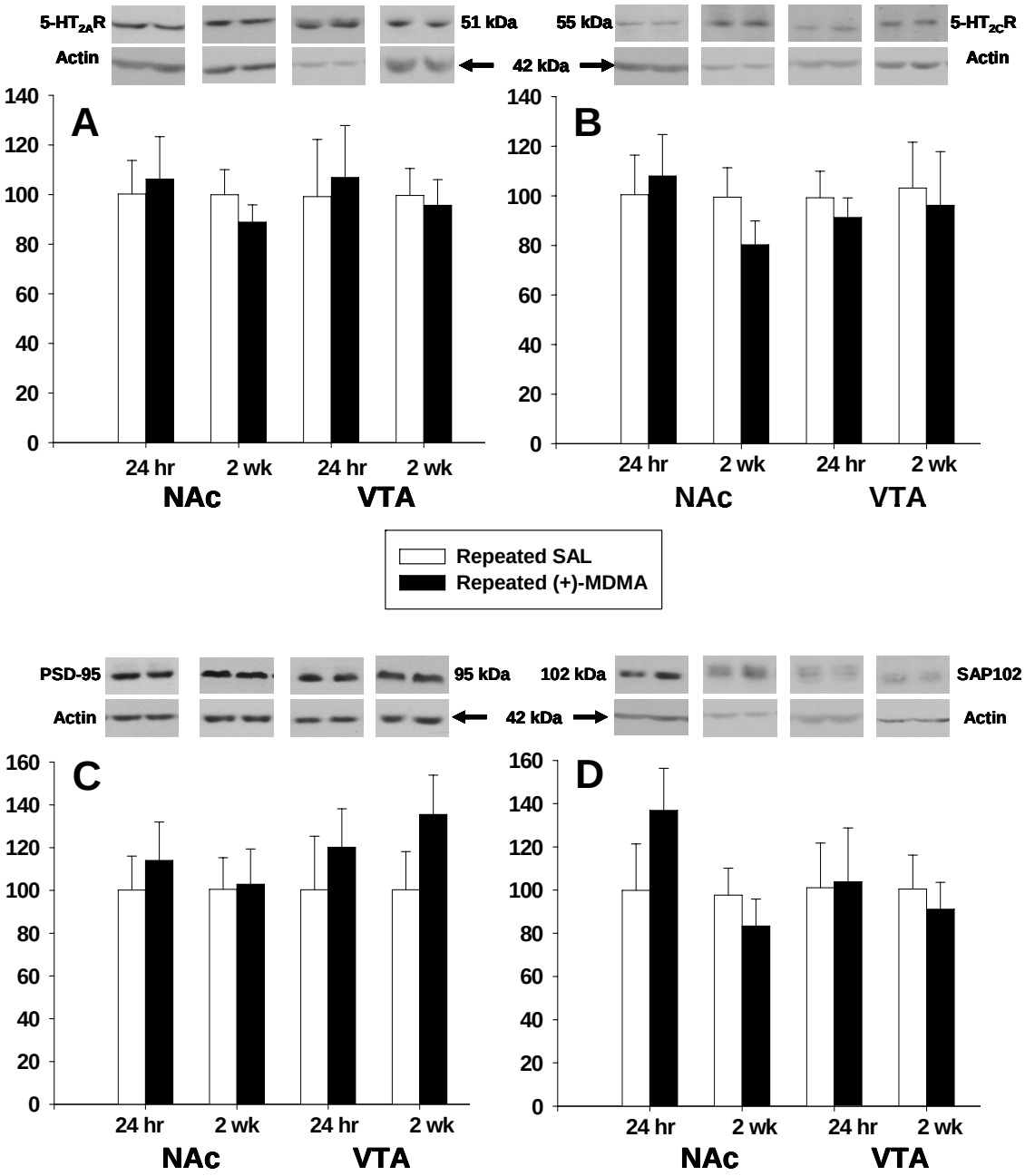

Figure 6. 5-HT_{2A}R, 5-HT_{2C}R, PSD-95 and SAP102 protein expression during withdrawal from repeated (+)-MDMA administration. Representative Western blots of membrane-associated 5-HT_{2A}R protein [A], 5-HT_{2C}R protein [B], PSD-95 [C], and SAP102 [D] in the NAc and VTA at 24 hrs or 2 wks of withdrawal from repeated saline or (+)MDMA (4 mg/kg/day, SC, 7 days) treatment. Results of the densitometric analysis, expressed as percent change from repeated saline controls, represent the mean (± SEM; n = 6-8) protein expression normalized to actin.

DISCUSSION

Behavioral sensitization in response to repeated intermittent administration of (±)- (Spanos and Yamamoto, 1989; Kalivas et al., 1998; Itzhak et al., 2004) or (+)-MDMA (McCreary et al., 1999) is a phenomenon characterized by a progressive enhancement of behavioral effects induced by the stimulant. It has been theorized that behavioral sensitization is linked to the development to drug craving, drug dependence and a number of withdrawal sequelae (Nestler, 2001; Robinson and Berridge, 2001). Thus, determining the neural adaptations underlying behavioral sensitization specifically to (+)-MDMA may facilitate our understanding of behavioral changes (e.g., depression, anxiety) that occur during withdrawal from its repeated use in humans. Our lab has demonstrated that intermittent repeated (+)-MDMA (4 mg/kg/day, 7 days) is not neurotoxic to 5-HT neurons but does evoke a transient behavioral sensitization (Bubar, Thomas and Cunningham, unpublished observation). The important role of the 5-HT_{2A}R in mediating the behavioral effects of (+)-MDMA after acute administration suggests that disruptions in 5-HT_{2A}R signaling may mediate in part the behavioral patterns seen during withdrawal from intermittent treatment with (+)-MDMA.

The 5-HT_{2A}R does not appear to undergo classical methods of desensitization and internalization (Gelber et al., 1999; Gray and Roth, 2001; Bhatnager et al., 2001), and interactions with binding proteins that contain PDZ binding domains to form protein-protein complexes are important in the regulatory mechanisms of this receptor in response to agonist exposure (Xia et al., 2003). Studies conducted *in vitro* suggest that PSD-95 maintains 5-HT_{2A}R insertion at the cell membrane and therefore plays a key role in 5-HT_{2A}R-mediated signaling (Xia et al., 2003). Co-immunoprecipitation studies revealed that the native 5-HT_{2A}R, but not a mutant (lacking the PDZ-binding domain), directly interacts with PSD-95 and that this interaction enhances 5-HT_{2A}R-mediated signaling (Xia et al., 2003). These studies indicate a novel role for PSD-95 in modulating the activity of 5-HT_{2A}R. In addition, the PDZ scaffolding protein SAP102 has been shown to interact with neurotransmitter receptors at the post synaptic density (Sans et al., 2001), and an interaction with the 5-HT_{2A}R is likely. One way to explore the 5-HT_{2A}R-binding partner complex is to evaluate the expression of the component proteins. In the

present studies, 5-HT_{2A}R, PSD-95 and SAP102 protein expression was evaluated in the NAc and VTA in order to determine whether the sensitization observed at 24 hrs is associated with differential protein expression between (+)-MDMA-treated vs. control rats. The results indicate that there were no significant differences in the protein markers at this time point. These observations do not support our hypothesis that perturbations in the 5-HT_{2A}R protein and its binding partners (PSD-95, SAP102) contribute to behavioral sensitization.

As previously described, the 5-HT_{2A}R undergoes rapid desensitization in response to agonist application (Buckholtz et al., 1988; Anji et al., 2000). The regulatory mechanisms behind this desensitization include internalization, G-protein uncoupling, and down-regulation; however, we did not observe changes in 5-HT_{2A}R protein expression. Although studies have reported decreased 5-HT_{2A}R density following (±)-MDMA treatment (Scheffel et al., 1992; Reneman et al., 2002), the (±)-MDMA regimens employed were also noted to evoke neurotoxicity of 5-HT terminals, and were reported in the cortex which has a high density of 5-HT_{2A}R. The brain areas under investigation in our study (NAc, VTA) have not been previously examined for changes in 5-HT_{2A}R density following (±)- or (+)-MDMA treatment. It is possible that differences in the density of 5-HT_{2A}R in these brain regions may explain the differential (±)-MDMA-induced decrease in cortical 5-HT_{2A}Rs, whereas 5-HT_{2A}R density was not changed in the NAc and VTA under non-neurotoxic conditions.

The absence of detectable changes in 5-HT_{2A}R protein expression does not exclude a role for desensitization of this receptor via other mechanisms (e.g., trafficking) as a consequence of repeated administration of (+)-MDMA. It is reasonable to envision that normal functional interactions among 5-HT_{2A}R, PSD-95 and SAP102 may be restructured by (+)-MDMA exposure in a way that alters signal transduction and desensitization of 5-HT_{2A}R, in the absence of overt protein changes. Several protein-protein interactions with GPCRs have been described (Kim et al., 1997; Hall et al., 1998; Kreienkamp, 2002; Bécamel et al., 2002), and the nature of these interactions likely depends on their purpose. These protein complex functions range from transient associations for rapid signaling purposes to more prolonged interactions that could serve to determine the structure and position of a GPCR at the membrane (Milligan and White,

2001). For example, the NMDA receptor is anchored at the membrane by PSD-95 which is in turn anchored by guanylate-kinase-associated proteins (GKAPs), and this protein-protein interaction is important for NMDA signaling (Kim et al., 1997). In this regard, GPCR-binding partner complexes should be considered as dynamic assemblies that involve receptor-receptor interactions as well as receptor-G-protein-binding partner interactions which play a role in receptor regulation and function (e.g., desensitization, membrane localization, signaling) (Milligan and White, 2001). With regard to the regulation of the 5-HT_{2A}R, it is possible that repeated (+)-MDMA treatment may rearrange the 5-HT_{2A}R protein complex in a way that disrupts 5-HT_{2A}R binding (e.g., 5-HT, MDMA) or the ability to activate G-protein cascades.

In the present studies, the expression of the 5-HT_{2C}R protein was examined due to its close homology to 5-HT_{2A}R (Hartig, 1989), and its role in the behavioral effects of MDMA (Bankson and Cunningham, 2001; Fletcher et al., 2002; Herin et al., 2005). The 5-HT_{2C}R appears to have an inhibitory role by limiting the degree of hyperactivity (Bankson and Cunningham, 2002; Fletcher et al., 2002) and DA efflux in the NAc upon MDMA exposure (Bankson and Yamamoto, 2004). Studies from our lab have demonstrated that repeated (+)-MDMA (4 mg/kg/day, 7 days) results in a short-term sensitization that is paralleled by a transient decrease in the functional sensitivity of the 5-HT_{2C}R (Bubar, Thomas, Cunningham, unpublished observation). The present results indicate no significant differences in 5-HT_{2C}R protein between repeated (+)-MDMA and saline treated rats in the NAc or VTA at 24 hrs or 2 wks withdrawal. In light of the discussion above regarding 5-HT_{2A}R regulation, it follows that the 5-HT_{2C}R may also form protein complexes that regulate its localization or function at the membrane. Indeed a number of PDZ domain-containing proteins have been shown to associate with this receptor (Ullmer et al., 1998; Bécamel et al., 2002).

One way to examine the interactions of 5-HT_{2A}R and protein partners is to develop an appropriate *in vitro* model in which to study the localization of these molecules following (+)-MDMA treatment using immunocytochemistry. The RN46A cell line appears to be promising as these serotonergic cells are derived from embryonic day 13 rat medullary raphe neurons, and have been reported to produce 5-HT, and exhibit both release and uptake of 5-HT (Eaton and Whittemore, 1995; Koldzic-Zivanovic et al.,

2004). Importantly, these cells have been shown to contain the mRNA for 5-HT_{2A}R, PSD-95 and SAP102 (Brooks and Cunningham, unpublished observation), and may prove to be a valuable tool in assessing the subcellular distribution and interactions of these molecules with each other.

We have speculated that withdrawal from a repeated regimen of (+)-MDMA will alter the association of binding partners such as PSD-95 or SAP102 with 5-HT_{2A}R, which may lead to impaired receptor function. These alterations could contribute to certain behavioral phenomena seen upon withdrawal, such as sensitization, which may be mediated in part by disrupted 5-HT_{2A}R function. Since activation of the 5-HT_{2A}R leads to increases in $Ca_{i,}^{2+}$ we propose to investigate Ca^{2+} mobilization in RN46A cells after treatment with (+)-MDMA (or vehicle) using the Ca^{+2}-sensitive fluorescent dye Fluo-3. These studies would be useful in determining whether 5-HT_{2A}R function is in fact impaired by (+)-MDMA treatment, and may lead to a better understanding of 5-HT_{2A}R receptor dysfunction by MDMA.

In summary, results from the present study suggest that the involvement of 5-HT_{2A}R in the short-term sensitization that develops at 24 hrs withdrawal from repeated (+)-MDMA employed in our studies does not reflect changes in 5-HT_{2A}R, 5-HT_{2C}R, PSD-95 or SAP102 protein expression. It is reasonable to speculate that altered 5-HT_{2A}R sensitivity and/or function may be related to more complex mechanisms such as receptor trafficking or modifications in receptor-protein complexes.

CHAPTER 5:

CONCLUSIONS

The studies described here were carried out in order to gain further insight into the complex molecular changes that occur upon withdrawal from repeated (+)-MDMA administration and whether these adaptations may contribute to behavioral sensitization. The 5-HT_{2A}R, a known modulator of the behavioral effects of (+)- and (±)-MDMA, and binding partners (PSD-95, SAP102) were examined to determine their role in behavioral sensitization. A link between altered 5-HT_{2A}R, PSD-95 and SAP102 protein expression, and behavioral manifestations associated with withdrawal from (+)-MDMA was not confirmed. However, it is possible that the molecular changes underlying behavioral sensitization do not require alterations in the expression of the proteins studied here, but modifications in the function or structure of the protein complex with which they associate.

The down-regulation and internalization of GPCRs is altered by proteins that bind to their intracellular binding domains (Kreienkamp, 2002), and as we have discussed, proteins that contain PDZ binding domains play a general role in the localization and scaffolding of channels and receptors to cytosketal or signaling proteins (Craven and Bredt, 1998). Recent studies have demonstrated a role for PDZ domain-containing proteins in the regulation of withdrawal sequelae to alcohol (Shirley et al., 2004). The multiple PDZ domain protein 1 (MPDZ/MUPP1) contains 13 PDZ domains and is an adaptor protein that clusters and links 5-HT_2Rs to other proteins (Sitek et al., 2003). This protein is also involved with trafficking of γ-aminobutyric acid (GABA) type B receptors from the cytoplasm to the membrane (Milligan and White, 2001). The gene for MPDZ/MUPP1 has been reported to correlate with the severity of withdrawal in mice from alcohol, such that animals with lower expression of MPDZ/MUPP1 mRNA are associated with greater seizure susceptibility (Shirley et al., 2004). The authors suggest that the organization of MPDZ/MUPP1 with its binding partners could affect withdrawal by altering the rate and efficiency of signal transduction, since this protein has been found to regulate glutamate and GABA release (Shen and Johnson, 1997). These data suggest that protein complexes may serve an important role in the manifestation of

particular behaviors both during and after drug abuse. Of importance to this study, the 5-HT_{2A}R-binding protein complex may be rearranged in way that alters signal transduction and desensitization of 5-HT_{2A}R upon withdrawal from repeated (+)-MDMA. This is particularly important since the 5-HT_{2A}R has been found to have a prominent role in the behavioral effects associated with acute exposure to MDMA (McCreary et al., 1999; Fletcher et al., 2002; Bankson and Cunningham; 2002, Herin et al., 2005). In humans, (±)-MDMA-induced perceptual and emotional changes are diminished after treatment with the 5-$HT_{2A/2C}$R antagonist ketanserin (Liechti, 2000). Imaging studies in humans have also demonstrated that recent users (those who had their last experience with MDMA two weeks prior to the study) had significantly lower cortical 5-HT_{2A}R density when compared to naïve control subjects, while ex-MDMA users (those who had their last experience with MDMA 18 weeks before the study), exhibited higher 5-HT_{2A}R density (Reneman et al., 2002). The authors suggest that down-regulation of 5-HT_{2A}R in current MDMA users could reflect MDMA-induced release of 5-HT, and that compensatory up-regulation of post-synaptic receptors in ex-MDMA users may reflect a state of MDMA-induced depletion. However it is difficult to imagine that changes solely in the expression of 5-HT_{2A}R could be responsible for the various drug-induced states observed during withdrawal (**see Chapter 1**). The dynamic range of protein assemblies with which 5-HT_{2A}R complexes, require further study in order to determine their role in 5-HT_{2A}R regulation.

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Abstract Publications

M.R. Brooks, M.J. Bubar, K.M. Pack, M.L. Thomas and K.A. Cunningham (2004) Gene Expression Analysis in Rats Treated with Repeated (+)-3,4-Methylenedioxymethamphetamine [(+)-MDMA; Ecstasy]. *Soc. Neurosci. Abst.* 29: 578.7, October, 2004.

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M.R. Brooks, M.J. Bubar, K.M. Pack, M.L. Thomas and K.A. Cunningham (2004) Analysis of Differential Gene Expression in Rats Treated with Repeated (+)-3,4-Methylenedioxymethamphetamine [(+)-MDMA; Ecstasy]. National Science Research Forum, Galveston, TX, April 2005.