

NICOTINAMIDE CO-ADMINISTRATION WITH
METHAMPHETAMINE: EFFECTS ON THE
P450 AND DOPAMINERGIC SYSTEMS

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ABBREVIATIONS

ACC	Nucleus accumbens
AHMC	Fluorescent metabolite, 3-[2-(N,N-diethyl-N-methylamino) ethyl]-7-hydroxy-4-methylcoumarin
AMA	American Medical Association
AMMC	Nonfluorescent probe, 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin
AMP	Amphetamine
ANOVA	Analysis of variance
AUC	Area under the curve
B3	Vitamin B-3
BFC	Nonfluorescent probe, 7-benzyloxy-4-trifluoromethylcoumarin
Bmax	Transporter density
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
COMBO	50:50 mixture of methamphetamine:nicotinamide
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAT	Dopamine transporter protein

<i>l</i> -DOPA	<i>l</i> -dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
EC ₅₀	50% Effective concentration
GC	Gas chromatograph
HFC	Fluorescent metabolite, 7-benzyloxy-4-trifluoromethylcoumarin
HI	Hydriodic acid
HP	Hewlett Packard
HTS	High throughput inhibitor screening
HVA	Homovanillic acid
IC ₅₀	50% Inhibitory concentration
i.p.	Intraperitoneal
IV	Intravenous
JAMA	Journal of American Medical Association
K _d	Dissociation constant
K _i	Inhibitory constant
K _m	Concentration of substrate that gives "half-maximal activity"
MA	Methamphetamine
<i>d</i> -MA	<i>d</i> -methamphetamine
<i>dl</i> -MA	<i>dl</i> -methamphetamine
<i>l</i> -MA	<i>l</i> -methamphetamine
MAO	Monoamine oxidase
MS	Mass spectrometer

NAD	Nicotinamide adenine dinucleotide
NIC	Nicotinamide
PBS	Phosphate buffered saline
PFPA	Pentafluoropropionic anhydride
PM	Poor metabolizer
RFU	Relative fluorescence units
RLM	Rat liver microsomal
SEM	Standard error mean
STR	Striatum
V _{max}	Transporter velocity
VTA	Ventral tegmental area

CHAPTER I

INTRODUCTION AND LITERATURE SEARCH

1.1 Introduction

The adulteration of illicit methamphetamine (MA) with nicotinamide (NIC) has become so prevalent in the United States that researchers must now consider the possibility that NIC may possess some ability to enhance the psychostimulant effects of MA. A one month study of purported MA samples submitted to the Tulsa Police Department Forensic Laboratory demonstrated that over 95% of the samples with MA contained some portion of NIC (unpublished data). The purpose of this series of studies is to examine NIC's metabolic and neurochemical ability to enhance the psychostimulant effects of MA.

1.2 History of methamphetamine

L. Edeleano (Edeleano, 1887) reportedly first synthesized the compound known as amphetamine (AMP) in 1887. Some speculate that it may have been the German chemist, R. Leuckart, that may have previously synthesized AMP accidentally, while experimenting with his now famous benzene chemistry (Leuckart, 1885), but that remains to be proven. It is difficult to pinpoint its origin because of the variety of names associated with this compound early in its history. The molecule

known today as AMP was named by Gordon Alles, who is credited for the discovery of its molecular structure circa 1933. The word “amphetamine”, which is an abbreviation of its lengthy chemical name, **Alpha-Methyl-PHENyl-ETHyl-AMINE** (Alles, 1933a) has also been referred to as phenylisopropylamine, benzpropaminum phosphoricum, desoxynorephedrine (Moffat, 1986), and many other synonymous names making historical literature searches very difficult. Regardless of the discoverer, after the synthesis of AMP in the late 1800’s, research remained dormant until around the early 1930’s.

Ephedrine was discovered by Carl Schmidt and K. K. Chen, who were looking for a substitute for adrenaline to be used as an anti-asthmatic (Schmidt and Chen, 1924). Chen was curious about Chinese herbal medicine, especially ma huang. He and Lilly chemists quickly isolated ephedrine and verified that it would widen bronchial passages. Since adrenaline could not be taken orally, as well as having severe side effects, ephedrine seemed vastly preferable. The rarity of ma huang sent chemists searching for synthetic ephedrine and sometime in the 1930’s one of them stumbled onto AMP (Snyder, 1996). This was believed to be Alles, Priness, and Miller, as clinical observations were published as early as 1930 (Priness et. al., 1930). The ability of AMP to raise blood pressure, constrict blood vessels, and dilate the small bronchial sacs, encouraged Smith, Kline, and French (now known as Smith, Kline, and Beecham) to market the Benzedrine® inhaler in 1932 (Fancett and Bush, 1998). The sympathomimetic effects and respiratory stimulant effects of AMP were described in 1933 (Alles, 1933a,b) and later used to treat narcolepsy in 1935 (Prinzmetal and Bloomberg, 1935).

Furthermore, an editorial published in the Journal of American Medical Association (JAMA) in 1936 described a study that had just been completed by researchers in the Department of Psychology at Minnesota University. The study provided data that showed students using Benzedrine® while studying for final exams scored higher and reported that it provided them with the pep they needed. Increased energy along with Charles Bradley's discovery of paradoxical effect of AMP on hyperactive children (Gainetdinov and Caron, 2001) led to a tablet form of Benzedrine® in 1937. Soon after the Benzedrine® tablets reached society, there were publications on the potential for abuse (Galdston, 1940). By 1946, a report generated by the pharmaceutical industry noted thirty-nine different disorders for which Benzedrine® was listed as a recommended treatment (Tyler, 1995). The abuse of the inhalers reached such alarming levels that in 1949 the AMP in the inhalers was replaced with propylhexedrine, a drug with weaker stimulant properties (Winger et al., 1992).

The wide use of inhalers and newfound tablet formulation led to an increase in recreational AMP usage during the 30's and 40's. It appears that recreational usage made it easier for some people to manage their lives during times of war, and the Great Depression. In addition to the thirty plus disorders for which it was listed as a treatment, people recognized it acted as an appetite suppressant and it was subsequently used successfully to treat obesity in 1940 (Ersner, 1940). The increased energy along with its appetite suppressant properties made it very appealing to members of society.

The original identification and isolation of MA is fairly vague and controversial within the literature. It, like AMP, has been referenced by numerous names, including: phenylisopropylamine, benzpropaminum phosphoricum, and desoxynorephedrine.

Tatetsu states that Nagai extracted MA, the N-methyl derivative of amphetamine, from a crude drug in 1888 (Tatetsu, 1959). Emde, Connell, and many other researchers (Emde, 1929; Connell, 1962; Brill and Hirose, 1969; Kramer, 1967) have given credit to the Japanese chemist, Ogata (Ogata, 1919), for first synthesizing MA in 1919. Some believe both are incorrect, citing that Schmidt first synthesized MA by the reduction of ephedrine via chloro- and bromo-ephedrines (Schmidt, 1914; Skinner, 1990). Whether MA was extracted by Nagai or synthesized by Ogata or Schmidt, MA as we know it today remained dormant in medicine and society until about 1940.

Many of the original literature articles beginning in the 1940's refer to MA and AMP together as amphetamines. It is difficult to distinguish where the literature begins to merge them and where they again separate. The two drug's nomenclature, molecular, and pharmacological similarity has made it difficult to separate the two from a historical perspective. Not only are the two agents named similarly, they offer many of the same effects. It is worthy noting that most historical reviews of either MA or AMP cite many of the same references, thus, making it harder and harder to trace back to a single origin.

Today, the scientific literature is much better at separating the two compounds. Though there is not an official chemical class of compounds recognized to be amphetamines, members of society and the scientific community commonly refer to AMP and MA as amphetamines, most likely because of their similar physiological and psychostimulant effects. Depending upon the source from which information is cited, the term 'amphetamines' could mean amphetamine, MA, or both.

AMP was first placed on sale in Sweden in 1938, three years after its introduction into the practice of medicine in the U. S. Though sales were small, the potential for

abuse was recognized early. In 1939, Sweden placed amphetamines on a list of drugs available only by prescription, a step the U. S. did not take until 1954 (Gunnar, 1969). By 1942, Swedish physicians were prescribing it to 3% of the population (Gunnar, 1969). A study in 1942 produced results that statistically showed people were using the drug responsibly (Goldberg, 1968). In 1944 the prescribing of amphetamines was placed under even more severe restrictions.

It seems AMP and MA integrated themselves into society circa World War I. During World War II, MA was widely distributed to promote fighting spirits among the Japanese soldiers. Numerous stories have been told about Kamakazi pilots ingesting toxic levels of MA prior to their military missions. Some speculate that their effectiveness and accuracy would have been much better if it had not been for the extremely high levels of MA in their systems. In addition, Hitler was said to have been unable to function without daily injections of MA (Tyler, 1995).

Before 1945, substance abuse in Japan was not a substantial problem, with the exception of alcoholism and a few opium abusers. Yet, from 1945 to 1956, Japan suffered from the new problem of stimulant abuse (Kato, 1990). During the war and many years after, the stimulant of choice became MA. After the war, the military cleared its large surplus of MA by flooding the civilian marketplace with ampules of MA, marketed as a mood-elevating drug. In Japan, MA was known as Wake-amine (Tatetsu, 1959), awaking drug, philopon, pervitin, and phenylmethylaminopropane (Brill and Hirose, 1969). Its abuse rapidly spread throughout Japan. In a 1948 study, it was estimated that 5% of the males between the age of 16 and 25 were addicted to AMP

(Konuma, 1994). The high rate of addiction was mainly attributed to the lack of laws prohibiting the use and sale of this substance.

It is interesting to see that in spite of wartime issues, MA found its way to England around 1940, the same year it gained popularity in Japan. The German government recognized its strong potential for abuse and had it listed as a potential drug of abuse by 1941 (Kalant, 1966). Japan, however, did not pass legislation until 1949, when a law to control the awakening drugs was enacted. An additional law, the Stimulants Control Law, was passed in 1951 and nicknamed the “Awakening Drug Control Law”. Further amendments in 1954 and 1955, together with a vigorous policy of enforcement, began to slow the abuse of MA. Some sources say AMP and MA abuse peaked for the first time in 1950 and 1951, and it was not until 1958 that Japan saw a true decrease in the number of users (Kato, 1990). It then peaked again in 1963 and once again in 1970. The 1970 epidemic lasted 16 years (Kato, 1990). Some will say AMP and methylphenidate (also included in these Stimulant Control Law) were rarely abused in Japan (Suwaki, 1991; Brill and Hirose, 1969). There have been many publications dealing with the various amphetamine/MA problems in Japan during and after the war (Kato, 1990; Morimoto, 1957; Nagahama, 1968; Tatetsu, 1959).

In 1914, the U.S. federal government (Harrison Act) classified cocaine as a narcotic, even though it is not, and outlawed it. The American Heritage Dictionary (1997) defines a narcotic as inducing sleep or stupor; causing narcosis, none of which are characteristics similar to the effects of cocaine. Still, it was not until the early 1920’s that a decline was seen. Some will speculate that this decline was more likely due to the acceptance and substitution of AMP rather than the legislation. Cocaine use continued

to decline slowly throughout the 1960's. Originally, AMP was appealing to the cocaine user because of its similar high, and because it was legal. More significantly, AMP and MA were inexpensive. Cocaine use stayed extremely low in the U.S. until all of the amphetamines became illegal without a prescription.

Starting in 1947, the abuse of Benzedrine® inhalers was well documented. By 1949, efforts to replace AMP with propylhexedrine, a less addictive chemical substitute, were well under way (Winger et al., 1992). During the rise to popularity, oral amphetamines inundated our military and became extremely popular among civilians, including, students, truck drivers, homemakers attempting to lose weight, and recreational drug users. In the United States, AMP tablets were available without a prescription until 1954 (Smith, 1969) and in 1959 the FDA banned the use of all Benzedrine® inhalers, except under use of a physician (Anglin et al., 2000). The tolerant and sometimes humorous view of central nervous system (CNS) stimulants, specifically amphetamine, became clear with the release of Harry "The Hipster" Gibson's hit song in 1944, "Who put the Benzedrine in Mrs. Murphy's Ovaltine?"

As the American public continued to abuse the oral pep teasers, Benzedrine® (amphetamine) and Methedrine® (methamphetamine), the American soldiers took the first step in the historical trend of pharmacological synergism. This was the planned combination of AMP and heroin, originally used to treat depression, to produce an effect different and greater than the two independent drug effects. The combination of a CNS stimulant like AMP, MA, or cocaine and heroin, later would be referred to as "speedballing", marking the first documented intravenous (IV) use of amphetamine/MA

by Americans. Even President John F. Kennedy was known for having a physician inject him several times a day with AMP to combat fatigue (Witkin, 1995).

In the late 1950's, some physicians in San Francisco prescribed AMP and/or MA injections for treatment of heroin addiction. This becomes historically interesting because Sigmund Freud's first dispersal of cocaine was to his friend, Dr. von Fleischl-Marxow, so he could get along without morphine. Doctors were allegedly prescribing AMP injections for the same reason. One Methedrine® user questioned Dr. Smith, a physician in the Haight-Ashbury district in San Francisco, California about this (Smith, 1969): "Then there was a doctor...who would write anything for anybody at anytime and he was making \$7 a visit and on the day we went down there he wrote almost 400 prescriptions at \$7 a head...He made \$2,800 that one day and they used to make caravans down there and even from [Los Angeles] to his place. You'd get within two blocks of his office and you'd start seeing people you knew all over."

During the late 1950's and 1960's in the Bay area, the use of Methedrine® as a substitute for heroin lead to the increasing levels of violence and the beginning of MA abuse in the United States (Smith, 1969). "Splash", AMP hydrochloride injected IV, emerged as a small self-contained drug culture in California. Intravenous use of amphetamines then spread across the U.S. and hit an all time high in the early 1960's, when Methedrine® became known as "speed".

In 1962, the federal, state, and local law enforcement agencies began to utilize the Drug Abuse Control Act of 1962 to crackdown on injectable AMP and MA sold in pharmacies. This increased enforcement, most likely due to the distribution of injectable amphetamines without prescriptions, with crudely forged prescriptions, or with bogus

telephone orders from people posing to be doctors. “Script-writers” were doctors who, for the price of an office visit, would prescribe people with their drug of choice: amphetamine. This rise in widespread abuse may have been responsible for the removal of Desoxyn (amphetamine) ampules (Abbott Labs) from the market. Additionally, in July of 1963, Burroughs Wellcome withdrew Methedrine® (methamphetamine) ampules from pharmacies but continued production for hospitals as adjuncts to surgical anesthesia. Some speculate this may have led to the rise in infection rates, believed to have arisen from non-sterile ampules of injectable amphetamines sold on the streets (Smith, 1969; Anglin et. al., 2000).

In 1963, the American Medical Association (AMA) Council on Drugs, while recognizing the great potential for abuse of amphetamines, stated “at this time, compulsive abuse of the amphetamines [constitutes] a small problem [in the United States]”.

Although many signs of abuse were being seen across the world, AMP was still looked upon as a useful and relatively safe agent, although toxic effects and dependence tendencies were being described (Kalant, 1966). In 1966, the AMA Committee on Alcoholism and Addiction and the AMA Council on Mental Health took note of the high quantities of AMP available throughout the United States. It was no longer being abused just on the West Coast. Studies showed AMP and MA traffic even in the more central portions of the United States, like Oklahoma City (Griffith, 1966). Researchers warned of the extent and dangers of using amphetamines (Griffith, 1966; Lemere, 1966) and in 1967 Kramer described the typical pattern of abuse (Kramer, 1967). Still, AMP prescriptions reached 31 million in 1967 (Anglin, 2000). An amendment in 1965

required more stringent record keeping, which the drug dealers were able to circumvent by having the amphetamines shipped to Mexico and then transported back to the U.S. This was similar to the diversion tactics observed with morphine and the Harrison Act of 1914. According to the Schaffer Library of Drug Policy (<http://www.druglibrary.org>), the Harrison Act of 1914 specifically provided that manufacturers, importers, pharmacists, and physicians prescribing narcotics should be licensed to do so, at a moderate fee.

Although MA ran rampant throughout California, probably the most famous usage of MA was in the Haight-Ashbury district in San Francisco, California. During the 1960's, substantial numbers of rebellious, mostly white middle-class young people, moved into this area of California and became known as "hippies". Illicit drug use, strange hair, and weird clothing appeared to come with this life-style. Speed, as all amphetamines were now referred to, began to take the place or was being used in combination with other drugs often abused, like LSD and mescaline. The conversion from the original drugs of choice, LSD and marijuana, to the amphetamines was facilitated by the anti-marijuana and anti-LSD campaigns at the time. Eventually, the speed users pushed the people looking for love and mind-expansion out of the area and many other drug-kick seeking people moved in (Smith, 1969). This prompted celebrity intervention, and Timothy Leary, the Beatles, and the Mothers of Invention warned against the dangers of speed (Le Dain Commission, 1970).

1.2.1 Illicit production of amphetamine and methamphetamine. Regardless of the bad publicity, the popularity of the AMP and MA grew exponentially. Before the Federal Drug Abuse Control Amendments of 1965, supplies of both AMP and MA were

plentiful. Enforcement of the 1965 amendment finally dried up the low-priced legal supplies; doing this inadvertently opened the door for profitable illicit manufacturing operations. Illicit AMP and MA producing laboratories, known as “speed labs”, no longer had to compete with diverted legal tablets that were priced at wholesale, which could be as low as thirteen to fourteen tablets for a dollar (Smith, 1969). Now that there was decreased pharmaceutical availability and increased enforcement of tablet diversion, it made it not only convenient but also necessary to clandestinely produce AMP and MA.

The many biker gangs in California that were accused of introducing the amphetamines into the psychedelic 60’s, and creating unwelcome violence, were now some of the number one suppliers (Miller, 1997; Lucas, 1997).

Illicit MA laboratories emerged in late 1962 (Anglin et. al., 2000). The Drug Abuse Control Act of 1962 crackdown no doubt triggered this onset and the 1965 reduction in pharmaceutical diversion just accelerated the rise of illicit speed producing labs.

The rise of speed labs during the 1960’s is well documented in “Love Needs Care” by David Smith and John Luce, published by Little and Brown (1970). This was documentation of the problems within the Haight-Ashbury Free Clinic, during the late 60’s. “The Speed Culture” by Lester Grinspoon and Peter Hedblom, and “Licit and Illicit Drugs” by Edward Brecher (1972) also helped document and discuss various aspects of drug abuse, especially MA. Brecher’s book gives one of the best historical looks at the common drugs of abuse.

In the beginning, clandestine manufacturing methods were very complex and did not yield good quality MA. By the 70s, the most popular method of MA synthesis

utilized phenyl-2-propanone, methylamine, mercuric chloride, and aluminum metal in alcohol (Anglin et al., 2000). Another method attempted to use an acetaldehyde/methylamine reaction that was refluxed with benzylmagnesium chloride. This was believed to have been a bad recipe taken from an underground publication titled “Whole Drug Manufacture’s Catalog Transmittal” by Chewbacca Darth in which the order of chemical addition varies from the correct synthesis procedure. Another popular and successful method used a Leuckart reaction, refluxing phenyl-2-propanone with either methylamine and formic acid or N-methylformamide to form the N-formylMA intermediate and then refluxing the intermediate with hydrochloric acid to form MA. Other methods included the combining of phenyl-2-propanone and methylamine and reducing the intermediate 1-phenyl-2-methyliminopropane to MA and reacting phenyl-2-propanone and methylamine in the presence of sodium cyanotrihydroborate at a slightly acidic pH, resulting in MA formation (Frank, 1983). The aforementioned techniques all yield a racemic mixture of MA, which contained both dextro (*d*) and levo (*l*) optical isomers.

Both AMP and MA laboratories used the Leuckart reaction, refluxing phenyl-2-propanone with ammonium formate or formamide then adding hydrochloric acid and refluxing again to produce a racemic product. In 1981, DEA reports 90% of the AMP and MA labs were using the Leuckart method (Frank, 1983). Large-scale production went on for years until the Controlled Substance Act of 1970 (Public Law 91-513) regulated the production of amphetamines in the United States. This did not seem to phase the large-scale operations that had been established in California. R.S. Frank gives

an excellent look into the various methods of MA synthesis in his publication titled, “The Clandestine Drug Laboratory Situation in the United States” (Frank, 1983).

A review of the various synthesis methods and essential chemicals employed in clandestine labs in the 1970's and 80's revealed that most manufacturing methods used the same precursor chemical, phenyl-2-propanone. Government officials postulated that regulation of this very essential chemical would help reduce the number of speed labs, a number that was continuing to rise every year throughout the 70's and 80's. So, on February 11, 1980, phenyl-2-propanone was regulated as a schedule II controlled substance under the U.S. Controlled Substance Act (Frank, 1983). This forced individuals to obtain the phenyl-2-propanone on the black market and served to be beneficial in the prosecution of many clandestine laboratories.

The control of phenyl-2-propanone and its complicated methods of MA synthesis led illicit MA producers to investigate additional synthesis techniques. By 1983, a new method utilizing *l*-ephedrine instead of phenyl-2-propanone as the precursor was used to produce MA (Frank, 1983). This method became so popular it was openly being advertised in magazines like “High Times” by 1983. During this time, the most commonly applied clandestine laboratory conversions of *l*-ephedrine to MA involved converting the *l*-ephedrine into its chloro analogue by a reaction with SOCl_2 , PCl_5 , POCl_3 , or PCl_3 , then the chloro analogue was reduced by catalytic hydrogenation resulting in MA production (Allen and Kiser, 1987). The *l*-ephedrine was shown as converted to the inverted stereoisomer configuration in the final product. *d*-MA could therefore be synthesized through an alternate anchimeric assistance mechanism via *l*-ephedrine (Allen and Kiser, 1987). This was a highly improved final product, no longer generating a

racemic mixture, but it was specifically producing the *d* isomer, resulting in a more pure and potent form of MA.

This may have been a step backwards from a public health perspective because the illicit phenyl-2-propanone methods produced a racemic mixture of MA that required IV administration to avoid severe adverse reactions, like tremors and stomach cramps (Potter and Kolbye, 1996). Even though the new methods reduced the need for needles and their associated health problems (hepatitis B, hepatitis C, HIV), it also made MA usage more appealing to several groups of individuals that may have been deterred by the previously required IV administration.

The years of 1979 and 1980 resulted in an explosion in the number of speed labs. The DEA and State level narcotics enforcers became proficient in tracking labs and showed an eleven-fold increase in lab busts (U.S. General Accounting Office Report GGD-82-8, 1981; Frank, 1983). Originally limited to outlaw motorcycle groups, the new and improved synthesis techniques expanded the typical MA user to include average college students, young professionals, minorities, and women (Potter and Kolbye, 1996; Lucas, 1997; Anglin et al., 2000).

As a result of the high demand for MA, large illicit laboratories were centered in Texas and California. These locations had easy access to Mexico for all of the necessary precursor chemicals, whether those were the illicit phenyl-2-propanone chemicals or the more recent ephedrine/pseudoephedrine methods. These large labs were becoming intimately associated with outlaw motorcycle gangs and appeared as no surprise that the big four gangs--Hells Angels, Bandidos, Pagans, and Outlaws--were known to finance and run large distribution operations (Parsons, personal communication). The synonym

“crank”, which is one of the most common slang terms for MA used today, originated with the motorcycle gangs, for they transported bulk MA in the crankcases of the motorcycles (Adair, personal communication).

Intensified enforcement efforts targeting bikers, along with the new simpler production methods (popularized in Southern California), caused the northern center of production to move south. MA was said to have then settled in the San Diego area, which increased Mexican trafficking (Morgan and Beck, 1997). Large amounts of MA, as well as precursors, were smuggled from Mexico to California and then moved east towards the southwest and Mid-western states.

The ephedrine method was further modified to use chemicals that could be obtained easily and legally. Common chemicals associated with the illicit production of MA include methanol (HEET), pseudoephedrine (Pseudo 60's), organic solvents (charcoal lighter fluid), iodine, red phosphorous, sodium hydroxide (Red Devil Lye), and hydrochloric acid (Muriatic acid).

The hydriodic acid (HI)/ red phosphorus method, more commonly referred to as the ephedrine reduction method, was originally popularized in the United States in Southern California (Anglin et al., 2000). By 1982, scientific literature (Fieser and Fieser, 1967; Buehler and Pearson, 1970) well documented the use of red phosphorus and HI to reduce carbonyl groups, nitrites, halides, and alcohols. Who knew that its inception into the world of illicit MA production would tote MA into becoming known as the illicit drug of the 90's and beyond? The new and improved clandestine synthesis techniques allowed a person to purchase all the essential chemicals to produce MA at the local hardware store and pharmacy. These methods utilized red phosphorus, which could be

legally obtained from chemical supply houses, extracted out of matchbook striker plates, or obtained from the striker plates on signal flares.

Red phosphorous is no longer available for purchase by average citizens from chemical supply houses. It is a listed precursor and requires specific paperwork for purchase. Iodine was also readily available at local farm and garden stores in its required crystalline form or could be purchased as the liquid tincture and easily converted to crystals with a simple method involving the addition of hydrogen peroxide. Iodine, although still available for purchase, has rapidly seen the effects of supply and demand. One would be astonished at the price of iodine crystals today, that is, if one were able to find them in stock.

The remainder of the synthesis involved the isolation of the final product via liquid-liquid extraction with any number of organic solvents. Gassing the product with hydrochloric acid gas to isolate the user-friendliest form, *d*-MA HCl, finally completes the synthesis process. Seemingly overnight, the precursors had switched from illicit P2P to either *l*-ephedrine or *d*-pseudoephedrine, both available in over-the-counter cold medications. The ease in obtaining chemicals made it tremendously easy for the average person to produce MA and this news spread fast across the United States.

Simultaneously, another surge of MA was occurring across the Pacific. The 1980's brought large quantities of highly pure *d*-MA hydrochloride from Far East sources like the Philippines, Japan, Korea, and Taiwan to Hawaii (Mack, 1990; Laidler and Morgan, 1997). This form of MA would rapidly dominate the Hawaiian Islands and Southern California. Almost simultaneously, the Mexican distributors would embrace this form of MA and begin full-scale production. This new smokable form of MA was

called “ice” and its domination of Hawaii, Southern California, and the Pacific Coast (Su’a, 1989) would only be the beginning.

In 1989, a clandestine laboratory was seized in Vacaville, California. Ephedrine, a known precursor used in the manufacturing of MA, was found on site. The other chemicals usually seen at ephedrine conversion laboratories were not found. Instead, found were tetrahydrofuran, ammonium chloride, lithium metal, and ammonia gas. A notebook with several methods of MA synthesis was also discovered along with these unusual chemicals.

One of the methods described a novel route to reduce ephedrine to MA using the above listed chemicals, a method that seemingly had not been reported in literature (Ely and McGrath, 1990). Further investigation into the methodology marked this lab as the first clandestine laboratory to employ the use of Birch reduction. As described in Ely and McGrath (1990), lithium metal, anhydrous ammonia, and ephedrine are combined in order to generate a reaction that will reduce ephedrine or pseudoephedrine to MA. This type of reaction was first described in literature in 1945 (Birch, 1945). Although none of the published articles (Birch, 1945; Augustine, 1968; Hall et al., 1971; Hall and Lipsky, 1971; Small et al., 1975) specifically mention the production of MA, the validity of this method in the production of MA has been well proven in society today.

The latest methods seen today at the Tulsa Police Department Forensic Laboratory for producing MA involve the combination of iodine with hypophosphorus acid, orthophosphorous acid flakes, or some have even tried phosphoric acid (unpublished data). The hypophosphorus acid method has been shown to produce approximately four times the amount of hydriodic acid, which as described before, is

responsible for the reduction of ephedrine and pseudoephedrine to MA (Ma et al., 2001). The use of the phosphoric acid derivative has become more popular today mainly due to the increased amount of enforcement that has been devoted to the diversion of red phosphorus. Some of these methods are new enough that the mechanism of action has yet to be published. One would postulate that the phosphorus within these acids is somehow associating with iodine to produce hydroiodic acid, a known chemical used in MA synthesis.

D-methamphetamine was the most prominent form of MA during the late 1980's and remains so today (Potter and Kolbye, 1996; Parsons, 2003; Adair, 2003). Methods of manufacturing continue to change; the one thing that has remained the same is that each new method continues to selectively produce the *d* isomer of MA.

1.3 Physical properties of methamphetamine

MA, a chiral drug, displays stereoselective differences in biological action. There are three types of MA: dextromethamphetamine (*d*-MA), levomethamphetamine (*l*-MA), and the racemic mixture of *d* and *l*-methamphetamine (*dl*-MA). *D*-MA is the most potent and widely used in the United States today (Potter and Kolbye, 1996). One should note that throughout the remainder of this dissertation, the abbreviation MA is to be considered the *d*-MA form, unless otherwise noted.

Pure *d*-MA hydrochloride is a white bitter tasting crystalline substance. Its molecular weight [C₁₀H₁₅N] is 149.24 with a pKa of 9.87 at 25 ° C (Baselt, 2000), and has a melting point of 170-175°C (Merek Index, 1989). Structural similarity to endogenous catecholamines, epinephrine, norepinephrine, and dopamine, give rise to the

pharmacological activity of MA and other AMP derivatives. The similarity in chemical structures between MA and the catecholamines is significant for understanding the sympathomimetic properties of MA (Figures 1-1 and 1-2). The mechanism of action of MA can be inferred from the actions of the catecholamines that are structural analogues (Biel, 1970; Miller et al., 1989). This will be discussed in more depth in the neurochemical sections of this dissertation.

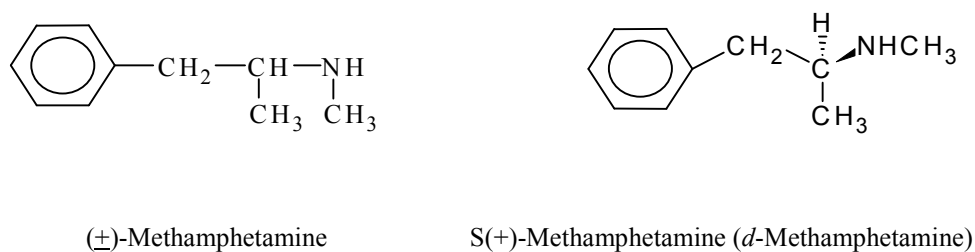
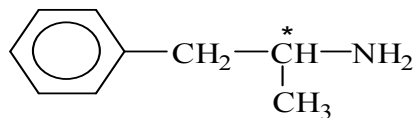
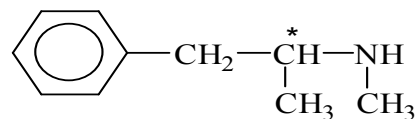


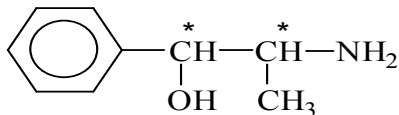
Figure 1-1. Comparison of racemic methamphetamine to *d*-methamphetamine.



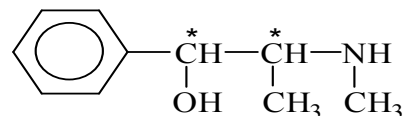
Amphetamine



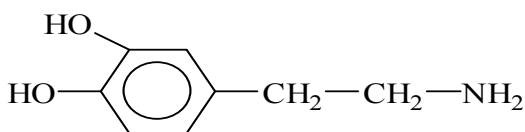
Methamphetamine



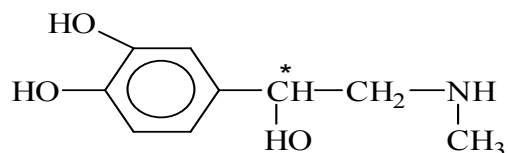
Phenylpropanolamine



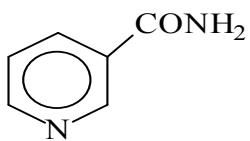
Ephedrine/Pseudoephedrine



Dopamine



Epinephrine



Nicotinamide

Figure 1-2. Structural comparison of various phenylethylamines, catecholamines, and nicotinamide. * denotes chiral carbon.

1.4 Administration

As described earlier, the most popular form of MA abused today is the dextrorotatory enantiomer, not the racemic mixture (Figure 1-1). Unlike racemic MA, *d*-MA can be ingested orally, snorted, smoked, or injected. *D*-MA has been shown to be absorbed easily through the gut (Linden et al., 1985). It has two distinct advantages over the racemic form. First, the preferred method of ingestion is not injection. Second, the more pure *d* form is the most potent and results in fewer adverse side effects (Potter and Kolbye, 1996).

Oral abuse of MA nowadays is almost nonexistent. Users today elect the speed and intensity of the “rush” that accompanies MA via smoking and injecting or “shooting”. Effects are almost immediate with the injection or smoking of the drug, whereas they occur approximately five minutes after snorting and twenty minutes after oral ingestion (Anglin et al., 2000). The kinetics associated with oral (Cook et al., 1992), smoking (Cook et al., 1993), and the injection of MA (Mendelson et al., 1995; Shappell et al., 1996; Hutchaleelaha et al., 1994; and Hutchaleelaha and Mayersohn, 1996), have been studied in a variety of models.

It is interesting to note that MA has greater CNS efficacy than *d*-amphetamine, presumably because of increased CNS penetration (Lake and Quirk, 1984; Derlet and Heischober, 1990; Beebe and Walley, 1995). MA is readily absorbed across surfaces of the nasopharynx, tracheobronchial tree, gastrointestinal tract, and vagina (Caldwell, 1980; Linden et al., 1985). Peak plasma concentrations occur within minutes of smoking or injecting versus about three hours with oral ingestion. Because of the high lipid solubility, extensive extravascular distribution is seen particularly in the brain, liver,

kidney, and lung. The peak effect is reached at approximately 18 minutes after being smoked, and 17 minutes post-injection, and is maintained for several hours (Perez-Reyes et al., 1991; Cook et al., 1993).

1.5 Clinical effects

The sympathomimetic and respiratory stimulant effects of the amphetamines were described as far back as the early 1930's (Alles, 1933a,b). Stimulant effects were found to be useful as early as 1935 with one of the first successful treatments of narcolepsy (Prinzmetal and Bloomberg, 1935). Treatments include obesity (Ersner, 1940), attention deficit disorder and Parkinson's (Whalen and Henker, 1980; Gainetdinov and Caron, 2001). None of these uses are nearly as recognized today as MA's ability to generate euphoria and pleasant stimulation in humans

MA is a sympathetic and CNS stimulant. Prominent CNS activity distinguishes it from other sympathomimetic agents and accounts for its high potential of abuse (Linden et al., 1985). MA is abused for its ability to induce many desired effects, which include euphoria, decreased fatigue, increased alertness, improved emotions, increased self-esteem, improved libido and sexuality, weight loss, improved self-confidence, increased initiative, motor, and speech activities (Gawin and Ellinwood Jr., 1988; Cho, 1990; Albertson et al., 1999). In addition to the many desired effects, MA has numerous unpleasant side effects (Table 1-1). In order to achieve the above desired effects, drug users will inevitably consume toxic levels of MA. The reason being, MA's toxic effects are merely an extension of its therapeutic action. Hypothermia, seizures, and muscular abnormalities, may occur from CNS-induced abnormalities or rhabdomyolysis (Allen and

Cantrell, 1998). Stereotypical movements that are fidgety, jerky, and random are common as is bruxism, teeth grinding (Miller et al., 1989). Increasing doses lead to anxiousness, slurred, rapid, and incoherent speech.

In toxic doses, MA induces agitation, anxiety, hallucinations, delirium, psychosis, seizures and death. CNS disorders induced by MA, which most likely lead to death, include cerebrovascular accidents due to hemorrhage or vasospasm and cardiac events (Kalant and Kalant, 1975). Previously, when the racemic MA injectors of the 1960's would overdose, they experienced shakes, tremors, cramps, and then heart attack or stroke. The current d-MA abusers that overdose do so without any warning signs.

A lethal dose of d-MA may have only one warning sign, high temperature (104°F or above) prior to heart attack or stroke (Potter and Kolbye, 1996). The rise in temperature is caused by the vasoconstricting properties of the drug, stimulation of the hepatic metabolism of fat and glucose, and the agitation and muscle rigidity produced by overdose (Mack, 1990). Cardiovascular problems, such as, chest pain, palpitations, and dyspnea, are even common with sub toxic doses of d-MA (Lam and Goldschlager, 1988; Bashour, 1994; Furst et al., 1990; Albertson et al., 1999). The most common presenting complaints are nausea, vomiting, headache, palpitations, anxiety, restlessness, or nervousness (Saxena and Kingston, 1982; Ekins and Spoerke, 1983).

Physiological actions that mimic adrenergic drug responses occur with every use. Rise in blood pressure, pulse rate, and pupillary dilation are seen with even the smallest doses (Miller et al., 1989). Hyperkalemia and hypokalemia have also been reported, most likely due to impaired or enhanced intracellular uptake of potassium caused by alpha- or beta-adrenergic stimulation, respectively (Williams et al., 1984). Hypertension

Table 1-1. Desired and undesired effects of methamphetamine usage.

Desired Effects	Undesired Effects
Euphoria	Aggressiveness
Improved emotions	Cardiac arrhythmias
Improved energy-decreased fatigue	Hallucinations- auditory*
Improved libido and sexuality	Hallucinations- visual*
Increased alertness	Insomnia
Increased initiative	Irritability
Increased self esteem	Psychosis
Self-confidence	Rhabdomyolysis
Weight loss	Seizures, Stroke, Tremors

*Although hallucinations are a desired effect of some drugs such as LSD, peyote, psilocybin, 3,4-methylenedioxyAMP and 3,4-methylenedioxymethamphetamine, the hallucinations associated with AMP and MA are generally due to ‘AMP psychosis’ and are not considered desirable.

and reflex bradycardia have also been attributed to alpha-adrenergic activity (Pentel, 1984). Cardiovascular effects manifest frequently as tachycardia and hypertension (Lam and Goldschlager, 1988; Lucas et al., 1986). Initially an increase in cardiac output is seen and then a reflexive drop from increased peripheral resistance of the vasoconstrictive effects.

Orthostatic hypertension (more severe in supine then sitting or standing) has also been observed (Miller et al., 1989), as well as acute and dramatic choreoarthetoid disorder triggered by MA (Rhee et al., 1988; Sperling and Horowitz, 1994). Many abusers experience respiratory complications. Although the exact incidence and prevalence have not been established, pulmonary hypertension has long been reported in MA users, as demonstrated by IV, intranasal, and oral MA users as far back as the 1960's (Derlet et al., 1990; Albertson et al., 1995; Arnett et al., 1976; Robertson et al., 1976; Lewman, 1972; Schaiberger et al., 1993).

Dehydration can usually be detected by the dry mucous membranes but confirmation lies with laboratory testing. Dry mouth, pale skin, and cool skin from peripheral vasoconstriction may occur also (Miller et al., 1989). Although metabolic hyperactivity usually leads to polyuria, increased urinary sphincter tone and inhibitory effect on sympathetic nerve supply to the bladder and bowel have resulted in urinary retention and constipation (Linden et al., 1985; Miller et al., 1989). Renal failure and hyperthermia have been associated with MA toxicity (Ginsberg et al., 1970; Foley et al., 1984) along with hepatocellular damage (Jones et al., 1994).

Susceptibility to the many toxic effects of MA varies greatly among individuals. Ingestion of as little as 1.5 mg/kg has proven fatal (Zalis and Parmley Jr., 1963; Potter

and Kolbye, 1996), whereas, tolerant individuals have been known to inject as much as 15,000 mg per day (Kramer, et al., 1967). Thus, toxicity is more accurately assessed by clinical symptomology than by drug dosage.

1.6 Pattern of abuse

Toxic effects are most commonly associated with the typical binge usage that most MA abusers follow. Upon initial administration (either smoking or intravenous), the person experiences an increase in heartbeat, pulse, and blood pressure. Associated with these physiological changes comes a feeling of euphoria, which may last from five to 30 minutes. This rush is followed by the period of being “high”, which usually lasts from four to 16 hours.

As the abuser begins to come down from the high, he/she will inject or smoke more MA to maintain the alertness and overall good feelings associated with the high. During subsequent administrations of the MA, the euphoria decreases until even IV administration does not result in any euphoria. At this point, nothing the person does will improve their mood or take away the feeling of neediness. This phase is the most characteristic of all and is referred to as “tweaking”. At this point, the subject becomes extremely paranoid, restless, and violent. This is the result of the user going without sleep for up to 10 or 15 days, while injecting upwards of 10 to 20 times per day.

Additionally, the user begins to display many of the psychological effects associated with toxic levels of MA, and many unpleasant CNS symptoms, such as, agitation, anxiety, hallucinations, delirium, and psychosis set in (Wilson et al., 1996;

Zalis and Parmley, 1963; Conci et al., 1988). “Tweakers” are usually high intensity abusers that can be recognized almost immediately by a trained eye.

Severe weight loss, central pallor (pale center of face), uncontrolled sweating, loss of personal hygiene with fairly characteristic body odor, bad teeth (usually gray and then eventually turning black), and numerous open sores on their skin are characteristic of a MA abuser. The sores are a result of excessive scratching due to the “crank bugs” that the users have experienced--a form of hallucination in which the individual feels bugs crawling on their skin. It is this phase of abuse that has brought so much attention to the high rate of MA abuse.

The extreme paranoia MA users experience is often associated with violence. As described by Potter (1996), one article taken from USA Today (Sept. 7, 1995) reads:

“‘Meth’ Use in the 90’s: A Growing ‘Epidemic’”

“Stimulant Fuels Many in the ‘Fast Lane’”

“When Eric Smith tossed his 14-year-old son’s head out his van window into a New Mexico highway he thought he was disposing of a demon. But Smith’s grisly act last July was just another bizarre outburst blamed on MA....”

“Experts are not surprised by the...brutality of the Smith case. ‘That is really pretty mild compared to the kind of cases we’re seeing’ in California, says Ron Siegel, a UCLA

psychotherapist who studies as many as five meth murders a week. We're seeing everything from serial killing to necrophilia.”

The above-mentioned is just one event that demonstrates the state of mind that is now referred to as “AMP psychosis”. This psychosis adds visual and auditory hallucinations to the already unbearable paranoia and agitation that is associated with MA abuse, thus usually ending in hysteria or possibly worse, as described.

After experiencing the euphoria, high, and tweaking phases, the MA user will inevitably crash--a one- to three-day period where the user sleeps. This period of sleep is reportedly without awakenings. During many Tulsa Police Department (TPD) search warrants, the suspects that are in this phase of the MA abuse cycle must be carried to the police car in order to be transported. The suspects are often unable to be awoken (Adair, Personal Communication). Eventually, after a period of time, the user will be back to a normal state—one slightly different than the normal self prior to the MA binge. Usually, this person will remain MA free for days before the cycle repeats itself. As the binge frequency increases, the time spent MA free in the normal phase decreases.

According to Dr. Alex Stalcup, Medical Director of the New Leaf Treatment Center in Concord, California, 93% of the abusers in treatment facilities will return to MA abuse (Potter and Kolbye, 1996). So what causes this addiction?

1.7 Neurochemistry of addiction

One of the most challenging problems in the neurobiology of drug addiction is to understand why individuals abuse drugs. Many have set out to identify certain brain chemistry that may predispose some people to becoming drug abusers. Although there is no conclusive evidence of any such chemistry, researchers have identified one very prospective system.

1.7.1 Dopamine hypothesis. By the early 1990's, evidence suggested many, if not all, drugs of abuse, including psychostimulants, act through mechanisms involving the brain neurotransmitter dopamine (DA) and the neural systems it regulates (Wise and Bozarth, 1987). Dopamine is a biogenic amine neurotransmitter that has been shown to mediate cardiovascular, renal, hormonal, and central nervous systems through the stimulation of alpha- adrenergic, beta-adrenergic, and dopaminergic receptors (Velasco and Luchsinger, 1998). A review by Michael Bardo (1998) summarized the multiple lines of research that have demonstrated the mesolimbic dopaminergic system's input in neurochemical drug reward.

To affect its target cells, dopamine interacts with specific receptors on the target-cell surfaces. Dopamine receptor subtypes fall into two families; the D₁-like family, which includes D₁ and D₅ receptor subtypes and the D₂-like family, which includes receptor subtypes D₂, D₃, and D₄ (Neve and Neve, 1997; Robinson, 1997). D₁- like receptors are coupled to G_s and thus stimulate adenylate cyclase to produce the intracellular second messenger cAMP. cAMP in turn activates cAMP -dependent protein kinase, which phosphorylates numerous substrates, including calcium channels and other

intracellular signaling components. D₂-like receptors are coupled to Gi/Go and thus inhibit adenylate cyclase and also activate inwardly rectifying potassium channels.

The amount of overall activation of the D₁-like and D₂-like receptors determines the state of the target cell. Additionally, there are D₂ autoreceptors on the dopaminergic terminals themselves (Le Moine and Bloch, Khan et al., 1998). D₂-like receptors have been suggested to be more essential for the enabling role of behavior (Drago et al., 1994; Xu et al., 1994). The Bardo review (1998) discusses the D₂-like receptors and possible D₁-like receptor role in drug reward. Evidence of the specific D₄ receptor in the reward system is still an open question, even though findings from Rubinstein et al. (1997) have indicated that it modulates normal, coordinated, and drug stimulated motor behaviors, as well as the activity of nigrostriatal dopamine neurons.

Dopaminergic receptors are not evenly distributed throughout the brain. The striatum (STR) has a high density of D₁ and D₂ receptors with localized D₃ in the ventral STR (also known as the nucleus accumbens), and lower levels of D₄ and D₅ (Mansour and Watson, 1995; Bordet et al., 1997). The nucleus accumbens (ACC), seems to be the key zone that mediates the rewarding effects (Robbins and Everitt, 1999). It too contains a high density of D₁-like and D₂-like dopaminergic receptors.

The dopamine hypothesis is evidenced by several sources (Volkow et al., 1999; Bardo, 1998; Rubinstein et al., 1997). Briefly, rats will self-administer tiny injections of AMP into their ACC by repetitively pushing a lever. The rats give themselves more of the AMP when their dopamine receptors are partially blocked pharmacologically, suggesting a drive to self-regulate the dopamine level. This has been evidenced with MA as well, by the systemic administration of DA antagonists increasing the self-

administration of MA (Woolverton, 1986). The repetitive behavioral acts that lead to the drug effects are assumed to be rewarding, this being an example of positive reinforcement.

If dopamine is massively depleted using a neurotoxin like 6-hydroxydopamine then, despite the recuperative capacity of the dopamine system (ability to synthesis DA once the stores are released), the rats no longer self-administer amphetamine. This is presumably because, in the absence of dopamine, these drugs lose their reinforcing properties (Wise and Bozarth, 1987). The dopaminergic system's reinforcing responses to psychostimulation have been successful in linking similar responses in rat to things seen in humans (Volkow et al., 1999; Bardo, 1998; Rubinstein et al., 1997).

1.7.2 Dopamine synthesis. Dopamine belongs to a group of neurotransmitters called catecholamines. These are single amine groups with a nucleus of a catechol (benzene ring with two adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives (Vallone et al., 2000). Dopamine is found in neurons of both the central and peripheral nervous systems. Since the pioneering studies of Blaschko (1957), dopamine has also been recognized as an important neurotransmitter in the mammalian brain.

Dopamine is mainly synthesized in the presynaptic area of the neuron from *l*-tyrosine. *L*-tyrosine is transported across the blood-brain barrier into the dopamine neuron where it is converted to dihydroxyphenylalanine (*l*-DOPA) by tyrosine hydroxylase--the rate-limiting step in the synthesis of dopamine. Once formed, *l*-aromatic amino acid decarboxylase (DOPA decarboxylase) very rapidly converts *l*-DOPA to dopamine (Cooper et al., 1996) where it can be stored until it is summoned for

release. Dopamine constitutes about 80% of the catecholamine content of the brain (Vallone et al., 2000).

Tyrosine $\xrightarrow{\text{-(tyrosine hydroxylase)}}$ *l*-DOPA

l-DOPA $\xrightarrow{\text{-(l-aromatic amino acid decarboxylase)}}$ Dopamine

1.7.3 Dopamine release. Dopamine release is a calcium-dependent mechanism. Calcium is used along with cyclic adenosine monophosphate (cAMP) to activate the tyrosine hydroxylase responsible for the dopamine production and for its subsequent release. Depolarizing stimuli evoke dopamine release mainly from vesicular pools but newly synthesized dopamine can also be released from cytoplasmic pools (McMillen and Shore, 1980). AMP has been used to demonstrate the release of both “stored” and “newly synthesized” dopamine (Chiueh and Moore, 1975) but the maintenance of the AMP induced release of dopamine is dependent upon the newly synthesized pool.

Once released, dopamine interacts with specific membrane receptors to produce its effects (Figure 1-3). Dopamine neurotransmission has been implicated in processes as diverse as muscle rigidity, hormonal regulation, thought disorder, and addiction (Jackson and Westlind-Danielsson, 1994; Missale et al., 1998; Emilien et al., 1999). Peripheral dopamine receptors mediate changes in blood flow, glomerular filtration rate, sodium excretion and catecholamine release (Missale et al., 1998; Emilien et al., 1999). Since peripheral receptors have not been linked to addiction or reinforcement properties, this dissertation will focus primarily on the central dopaminergic system in the brain.

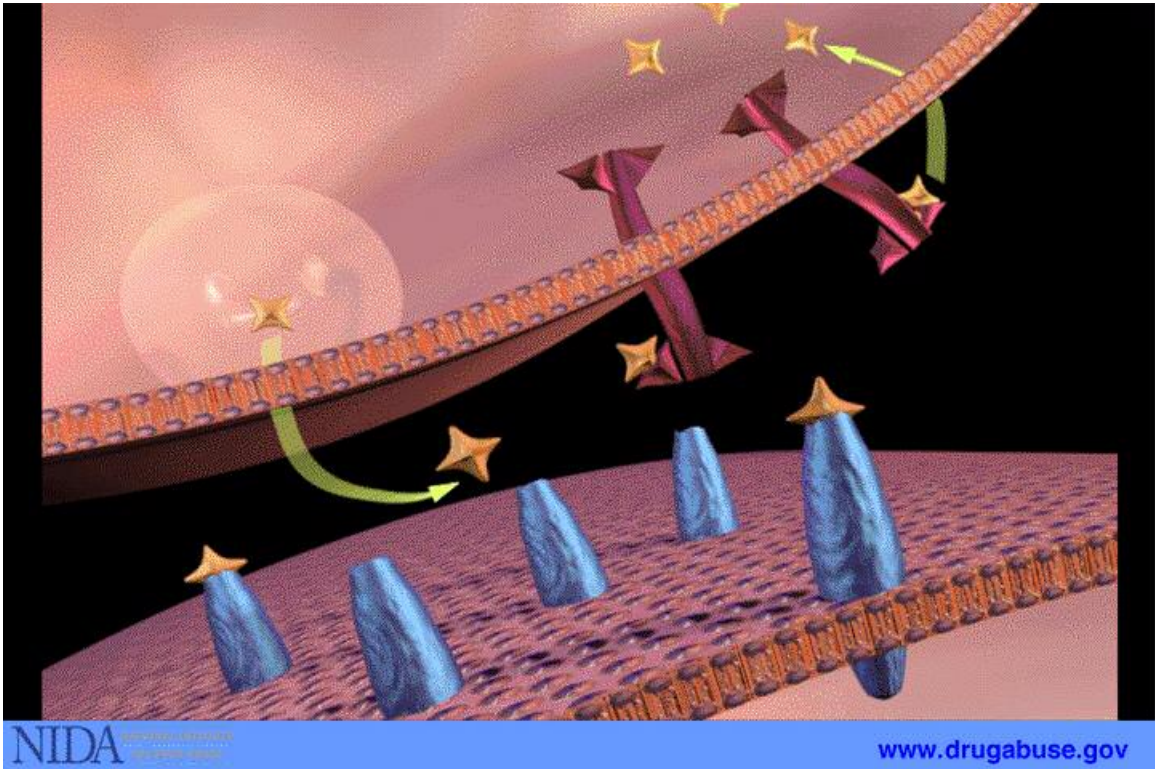


Figure 1-3. Representation of a dopaminergic synapse. During normal neuronal communication, dopamine is released by a neuron into the synapse (the small gap between neurons). The dopamine (orange star symbols) then binds with specialized proteins called "dopamine receptors" (blue cylinders) on the neighboring neuron, thereby, sending a signal to that neuron. The signal is terminated by the dopamine being taken back up into the original neuron by the dopamine transporter (red cylinders). Courtesy of <http://www.drugabuse.gov/Teaching>.

1.7.4 Central dopaminergic systems. There are three/four brain areas which synthesize dopamine and give rise to the central axonal pathways (Figure 1-4): (1) The nigrostriatal pathway, joining the substantia nigra to the striatum, involved in extrapyramidal motor functions (Carlsson 1988); (2) the mesolimbic and mesocortical pathways which are separate but generally discussed together. The mesolimbic originates in the ventral tegmental area (VTA) and terminates in the limbic area (nucleus accumbens and olfactory tubercle). The mesocortical pathway also originates in the VTA but terminates in the cortical structures. These systems together are responsible for the cognitive functions, motivation, and indirectly for motor activity (Missale et al., 1998; Emilien et al., 1999). (3) The tubular/infundibular pathway from the hypothalamus to the hypophysis, regulating neuroendocrine function (Missale et al., 1998; Emilien et al., 1999). In general, dopaminergic neurons join the frontal cortex with the septum, striatum, and nucleus accumbens (Hantraye, 1998).

1.7.5 Dopamine and addiction. By the early 1990's, converging evidence suggested that many (if not all) drugs of abuse act through mechanisms involving dopamine and the neural systems it regulates (Wise and Bozarth, 1987). It was acknowledged that such drugs could influence other neurochemical systems, but many of these primary responses lead to secondary effects involving dopamine (Robbins and Everitt, 1999).

A region at the base of the striatum, the ACC, is the key zone that mediates the rewarding effects of drugs such as AMP and cocaine (Figure 1-4). Evidence for this 'dopamine hypothesis' came from several sources as described by Wise and Bozarth (1987). Neuropharmacological studies have established the mesolimbic system (Figure

1-4) as a major neuronal substrate of the reinforcement processes involved in chronic psychostimulant self-administration in humans and rats (Hoebel et al., 1983; Carboni et al., 1989; Koob and Le Moal, 1997; Nesler and Aghajanian, 1997). This hypothesis is most certainly not universally accepted. For example, opiates also seem to have reinforcing effects mediated by dopamine-independent mechanisms in the ACC (Koob and Le Moal, 1997). Due to the fact that psychostimulants, like AMP and MA, have previously demonstrated their reinforcing abilities within the mesolimbic system, more specifically within the STR and ACC, the following studies will reside within those two areas of interest.

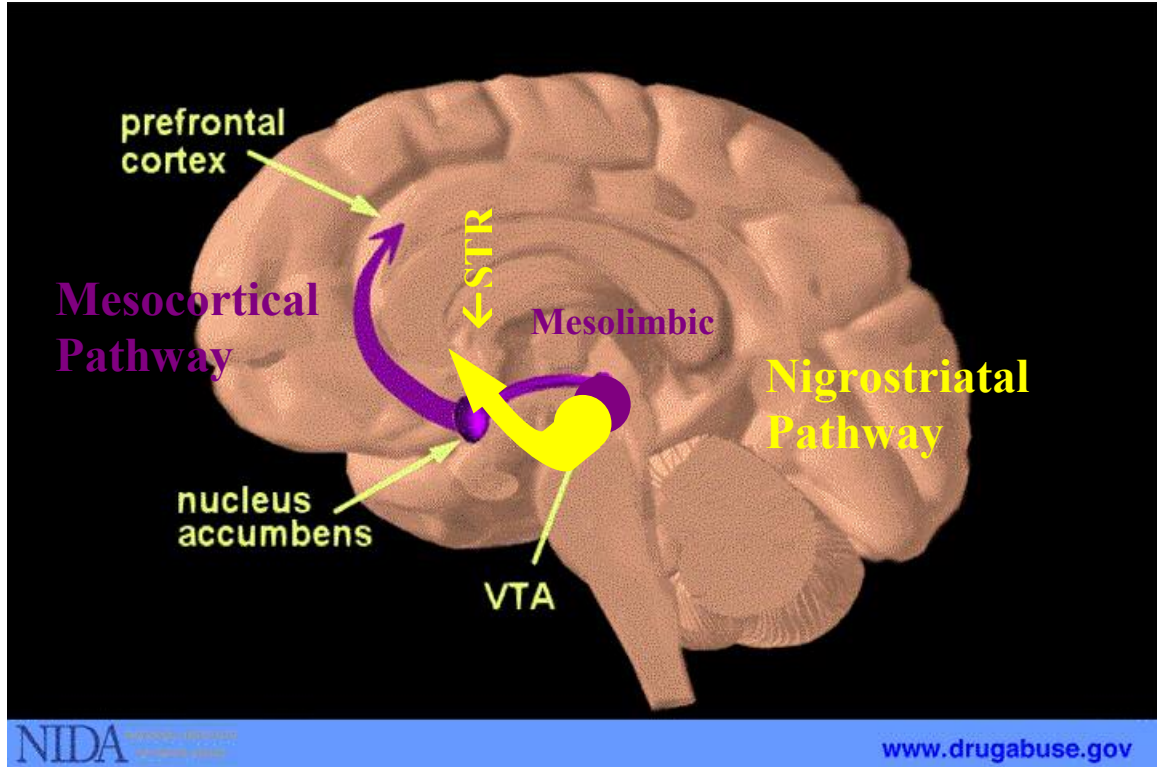


Figure 1-4. Representation of the major dopamine pathways (mesolimbic, mesocortical, and nigrostriatal) within the human brain. Striatum (STR); Ventral Tegmental Area (VTA). Courtesy of <http://www.drugabuse.gov/Teaching>.

1.7.6 Common properties of the dopaminergic receptor subtypes. Dopamine exerts its effects by binding to specific membrane receptors that belong to the superfamily of transmembrane domain G-protein coupled receptors (Cooper et al., 1996). It seems that each of the dopamine receptors conforms to the general structure of many G-protein coupled receptors. These are known to have seven membrane spanning-helices lined by protein loops with an extracellular amino terminus (Figure 1-5) (Vallone et al., 2000). The helices are bundled together in the membrane to form the ligand-binding site; information on the residues is available (Baldwin et al., 1997; Coley et al., 2000).

1.7.6.1 Dopamine receptor genes. Five distinct dopamine receptors have been isolated, characterized, and subdivided into two subfamilies, D₁-like and D₂-like, based on physiological or biochemical responses. The D₁-like subfamily includes the D₁ and D₅ receptors, while the D₂-like includes the D₂, D₃, and D₄ receptors. The difference in the two subfamilies homologies in the extracellular domain provides a structural basis for their pharmacological selectivity.

D₁-like receptors have short third intracellular loops and long carboxyl terminal tails, whereas, the D₂-like receptors have long third intracellular loops and short carboxyl terminal tails. The significance of this is that it appears to be functionally related to the receptor/G-protein interaction. It is the third intracellular loop of these receptors that is thought to be important in the interaction of receptor and G-proteins. For the D₂-like receptors, there are short and long variants (D₂S and D₂L) of the third intracellular loop, with the long form having a 29 amino acid insertion in the loop (Figure 1-5) (Giros et al., 1989).

D₂-like receptor variants have shown some differences in their abilities to couple with or activate G-proteins (Cravchik et al., 1996; Guiramand et al., 1995). Splice variants of the D₃ receptor encoding nonfunctional proteins have also been identified (Jackson et al., 1994). Furthermore, there are polymorphisms within the D₄ receptor loops; variants have yet to demonstrate differences in the binding of ligands or coupling to G-proteins (Kazmi et al., 2000).

1.7.6.2 Dopamine receptor expression. Dopaminergic ligands easily discriminate between the D₁-like and D₂-like receptor subfamilies. Subfamily receptors can be defined by using selective agonists, antagonists, or specific radioligands. D₁-like receptors are defined by SKF38393, SCH 23390, and [¹²⁵I]SCH23982 and D₂-like receptors are characterized by quinpirole, sulpiride, and [³H]raclopride, respectfully (<http://www.tocris.com>). Most of them do not clearly differentiate between members of the same subfamily. For example, the D₁-like receptor agonist and antagonist, SKF38393 and SCH 23390, have similar affinities for both D₁ and D₅ receptors (Vallone et al., 2000).

1.7.6.3 D₁-like receptor subfamily. The D₁ and D₅ receptors are classified as members of the D₁-like subfamily because of their similar amino acid sequences. The D₁ receptor differs structurally from the D₂ in several ways. The distribution of D₁ receptors corresponds to the projection regions of dopaminergic neurons. Thus, the highest amounts of D₁ receptors are found in the STR, ACC and olfactory tubercle (Cooper et al., 1996). The D₅ receptors are more restricted and expressed at much lower levels. The function of the D₅ receptor is still unknown; it is less abundant than the D₁ receptor and has a different distribution in the brain, being found in highest amounts in the

hippocampus and hypothalamus, with lower amounts in the STR and frontal cortex (<http://www.tocris.com>). The effects mediated by D₁ receptors in humans have been shown to control movement, cognitive function, and cardiovascular function. D₁-like receptors have been shown to be exclusively postsynaptic (Civelli et al., 1991).

D₁-like receptors are coupled to G_s and thus stimulate adenylyl cyclase--the enzyme that converts adenosine triphosphate (ATP) to the intracellular second messenger cAMP. The cAMP in turn activates cAMP-dependent protein kinase A, which phosphorylates numerous substrates, transcription factors, and other intracellular signaling components (Tiberi and Caron, 1994; Cooper et al., 1996; Berke and Hyman, 2000). D₁-like receptors have also been shown to modulate both directly and indirectly the level of intracellular Ca⁺² and inhibit K⁺ currents in striatal neurons (Cote et al., 1981; Onali et al., 1981; Kitai and Surmeier, 1993; Missale et al., 1998).

In the STR, D₁ receptor, stimulation leads to phosphorylation of ion channels (including calcium, sodium, and potassium and NMDA receptors), with complex effects on cell firing that depend in part on the activation state of the neuron (Surmeier and Kitai, 1993; Hernandez-Lopez et al., 1997; Cepeda et al., 1998; Cantrell et al., 1999). Although D₁ receptors have been shown to play a role in addiction, they are believed to play a greater role in learning (Beninger and Miller, 1998).

1.7.6.4 D₂-like receptor subfamily. Similarly, D₂, D₃ and D₄ receptors share substantial homology; therefore, they are classified as members of the D₂-like subfamily. Postsynaptic D₂-like receptors are predominantly expressed in the STR, limbic areas (ACC, olfactory tubercle), hypothalamus and pituitary (Cooper et al., 1996). This receptor is also expressed in the substantia nigra and in the ventral tegmental area (VTA),

which indicate that D₂ receptors also have a presynaptic location (Vallone et al., 2000). Since the pioneering research of Carlsson (1988), it has been clear that the activity of dopaminergic neurons in the midbrain can be modulated by the release or the exogenous application of dopamine. These presynaptic receptors were termed "autoreceptors" and are important in maintaining dopaminergic activity in the nigrostriatal and mesolimbic dopamine systems (Vallone et al., 2000; Leviel 2001; Glickstein and Schmauss 2001). According to Vallone et al. (2000), the D₂-like subtypes are predominantly found in the dopamine rich areas of the brain but also in the retina, kidney, vascular system, and pituitary gland. Additionally, the D_{2L} subtype has been shown to be more common than the D_{2S} subtype (Figure 1-5) (Vallone et al., 2000).

The D₃ and D₄ subtypes are much less abundant and have a different distribution. The D₃ receptors are located predominantly in the islands of Calleja, a few septal nuclei, hypothalamus, and distinct region of the thalamus and cerebellum (Jackson et al., 1994). In addition, the D₃ receptor location in the substantia nigra is also indicative of a presynaptic function. The D₄ appears to be highly expressed in the frontal cortex, amygdala, olfactory bulb, hippocampus, hypothalamus, and mesencephalon (Jackson et al., 1994; Oak et al., 2000; Rubinstein et al., 2001).

D₂-like receptors are coupled to Gi/Go and thus inhibit adenylate cyclase and also activate an inwardly rectifying potassium channel (Missale et al., 1998; Emilien et al., 1999; Berke and Hyman, 2000). Signaling through Gi leads to the inhibition of adenylate cyclase (Senogles, 1994; Ghahremani et al., 1999). D₂ and D₃ have been described as inhibitor of adenylate cyclase/cAMP, with D₃ showing less efficiency than D₂ and D₄ inhibiting cAMP accumulation in the retina (Missale et al., 1998). Signaling through Go

leads to the inhibition of calcium channels (Liu et al., 1994). The D₂ receptor modulation of intracellular calcium concentration is believed to play an important role in dopamine biosynthesis (Braun and Schulman, 1995).

As discussed previously, the D₂ receptor exists in two isoforms: D₂S and D₂L, generated by alternative splicing (Dal Toso et al., 1989; Giros et al., 1989). There are also indications about different functions and different G-protein coupling, but the selectivity has yet to be defined (Usiello et al., 2000; Montmayeur et al., 1993; Senogles et al., 1990). It has been demonstrated that the 29 aa insertion of the D₂L isoform confers a greater affinity for the binding of Gi₂ (Guiramand et al., 1995; Montmayeur et al., 1993) with respect to the D₂S isoform (Liu et al., 1994). A clear definition of the selectivity of the D₂ receptor has not yet emerged. It appears that D₂ receptors are tonically (continuously) stimulated by basal levels of dopamine and this tonic activity is important in normal motor activity (Berke and Hyman, 2000).

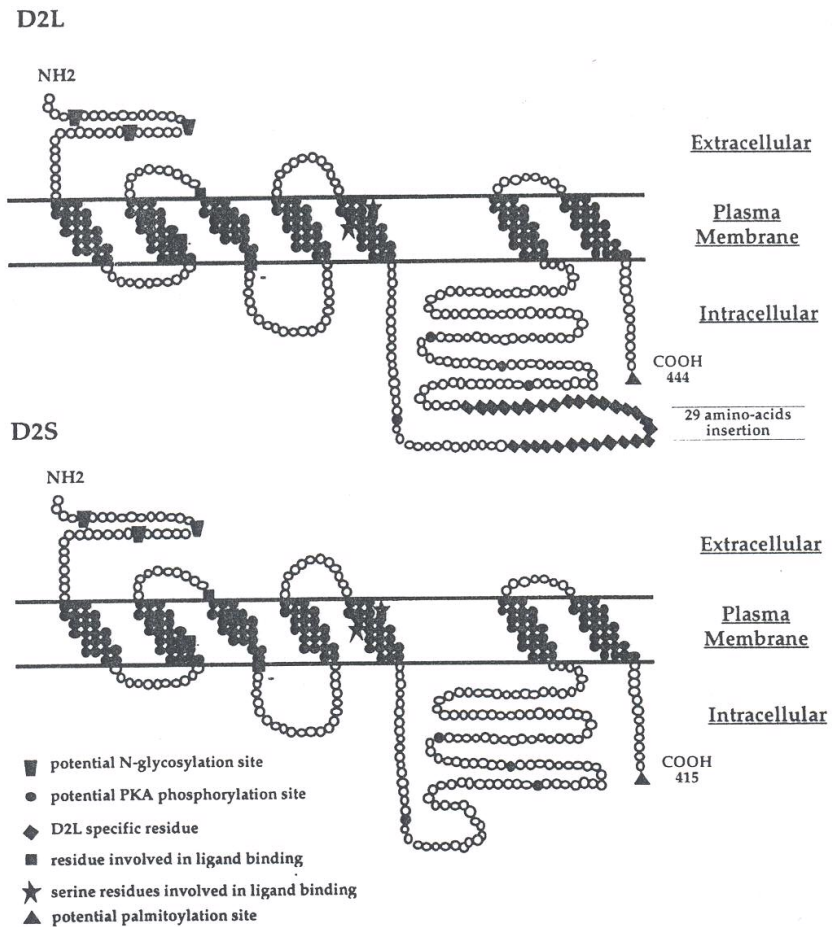


Figure 1-5. Scheme of the structures of the DA D_2 receptor isoforms. The D_2L specific amino acid insertion is indicated together with potential sites. Potential amino-acid residues involved in N-glycosylation, PKA phosphorylation, palmitoylation, and agonist binding is also shown. This figure was reproduced from Vallone et al. (2000).

1.7.7 Dopamine metabolism. The extracellularly released dopamine and its physiological effects are terminated by the re-uptake of dopamine into the pre-synaptic neuron by the Na⁺ and Cl⁻-dependent dopamine transporter (Reith et al., 1997; Drago et al., 1998; Kuhar, 1998), or by metabolic inactivation by monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT) (Kopin, 1985; Westerink and Spaan, 1982). Released dopamine is either converted to dihydroxyphenylacetic acid (DOPAC) by interneuronal MAO after reuptake or to homovanillic acid (HVA) extraneuronally by COMT and MAO (Kopin, 1985; Westerink and Spaan, 1982).

1.7.8 Dopamine transporter protein (DAT). The dopamine transporter, or carrier, is located on the plasma membrane of nerve terminals and is responsible for transporting dopamine across the membrane. By taking up synaptic DA into the neuron, it plays a critical role in terminating DA neurotransmission and in maintaining DA homeostasis in the CNS (Giros et al., 1996; Jones et al., 1998a,b).

Since the cloning of the DAT back in the early 1990s (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991), major advances have been made in its characterization. Though no X-ray crystallographic or high-resolution structural information is available for the topological assignments of the transporter, hydropathy analysis predicts a topology with 12 transmembrane segments connected by alternating extracellular and intracellular loops with the N- and C-terminals located in the cytosol (Chen et al., 1998). These 12 transmembrane domains have been arranged in a circular arrangement forming a pore (Edwardsen and Dahl, 1994).

The human and rat DAT, and many others, are functionally dependent on the presence of external sodium and chloride (Kilty et al., 1991; Giros et al., 1991,2,4;

Vandenbergh et al., 1992; Eshleman et al., 1995). Studies have demonstrated that the interactions of ions with the DAT are considerably more complex than the simple picture of 2 Na⁺ ions and one Cl⁻ ion being co-transported with one DA molecule (Chen et al., 1999; Earles and Schenk, 1999; Li and Reith, 1999). It has also been demonstrated that glycosylation of the DAT is important in plasma membrane expression (Lin et al., 1999) and DAT phosphorylation plays a role in transport protein sequestration (Zhu et al., 1997; Pristupa et al., 1998) and down-regulation of transport activity (Huff et al., 1997; Zhang et al., 1997).

Many substances such as AMP and MA structurally resemble DA and can be used as substrates for the DAT and can be transported by the DAT (Jones et al., 1998a,b; Miller et al., 1999). Studies show that the DAT requires molecules that possess a phenyl ring with a primary ethylamine side chain for optimal activity (Meiergerd and Schenk, 1994). It is proposed that the catechol appears to mediate the recognition of the substrate, whereas the amine side chain facilitates the conformational change of the transporter that results in movement of DA across the membrane. Yet, compounds without a phenolic hydroxyl group such as AMP and MA can bind to the carrier and be transported with the same V_{max} as DA (Chen and Justice, 2000). The phenethylamine structure, therefore, seems to be the most important structural element required by the DAT.

1.7.9 Effects of methamphetamine on the dopaminergic system. The ability of MA to alter the dopaminergic response within the STR and ACC has been extensively investigated (Brown et al., 2000; Krueger, 1990; Schmidt and Lovenberg, 1985; Wagner et al., 1980; Raiteri et al., 1979; Fumagalli et al., 1998; Fleckenstein et al., 1997; Bennett et al., 1997; Jones et al., 1986; Ricaurte et al., 1982,84). MA triggers the release of DA

from the cytosol to the extracellular space by means of reverse transport through the DAT, in addition to the normal release mechanism, exocytosis (Sulzer et al., 1995; Jones et al., 1998; Schmitz et al., 2002). Two models have been proposed to explain the role for dopamine in the actions of MA.

1.7.9.1 Exchange diffuse model. In the exchange diffusion model (Fisher and Cho, 1979; Mundorf et al., 1999), MA acts as a substrate for the DAT (Sonders et al., 1997) and reverses the transporter's conformation so that net dopamine efflux occurs (Jones et al., 1999). This model predicts cytosolic dopamine to be decreased by MA and MA administration induces extraneuronal dopamine oxidation (Seiden and Vosmer, 1984). Unfortunately, this model does not explain the oxidation of extraneuronal dopamine and its specificity for dopaminergic terminals rather than the expected nonspecific damage that should occur in all neighboring neurons, not just dopaminergic ones.

1.7.9.2 Weak base model. In the weak base model (Sulzer et al., 1995), MA acts via both the DAT and the vesicular monoamine transporter to promote the collapse of vesicular proton gradients, redistributing dopamine from synaptic vesicle to the cytosol (Mundorf et al., 1999), while preventing vesicular reuptake of cytosolic dopamine by destroying the driving force for vesicular dopamine accumulation (Maron et al., 1983), thus, predicting MA will increase cytosolic levels of dopamine, leading to dopamine oxidation within the neuronal cytosol (Cubells et al., 1994; Lavoie and Hastings, 1999; Lotharius and O'Malley, 2001). Regardless of the model, both agree that DAT plays an important role in MA neurotoxicity. Larson et al. (2002) demonstrated the ability of nomifensine and amfonelic acid, two DAT inhibitors, to offer neuroprotection.

Regardless of which model one chooses to accept, the mechanism of action of AMP and MA appears to be fairly complex. Amphetamine and MA enter the neuron either through plasma membrane monoamine transporters or by diffusion (Seiden et al., 1993). Once inside the neuron, the drug enters vesicles via the neuronal vesicular monoamine transporter and/or by diffusion, and reduces the vesicular pH gradient (Sulzer et al., 1995).

Amphetamine and MA are weak bases and, when transported into dopamine vesicles, they impair the proton gradient thereby inhibiting the sequestration of dopamine (Sulzer et al., 1995; Sulzer et al., 1993), thus, producing a redistribution of vesicular monoamines from vesicles to the cytoplasm, from where monoamines are transported to the extracellular space by reversal of the plasma membrane DAT (Sulzer et al., 1995). Furthermore, AMP and MA are both potent inhibitors of the intracellular enzyme MAO and thereby decrease dopamine metabolism (Seiden et al., 1993). Experiments in DAT knockout mice (Jones et al., 1998) clearly support this model of action and illustrate that although the DAT is pivotal, other processes are required.

Using animal models or pharmacological approaches, it has been possible to demonstrate that selective lesions of the ACC (Vallone et al., 2000) or blocking of dopamine receptors by D₁ or D₂-like antagonists, attenuated the reward effects caused by AMP in rats or mice (Feldman et al., 1997). Various studies utilizing genetically engineered mice have provided insights into the involvement of dopamine receptors and their link to abuse (Drago et al., 1996; Miner et al., 1995). It appears that the D₁ receptor is not as closely linked to the rewarding properties as previously believed; therefore, the

current studies will focus on the receptors most closely linked to addiction, the D₂ receptors.

A large body of evidence exists in looking at the effects of MA on the dopaminergic system, especially targeting the STR and ACC. It has been demonstrated in rats that MA administration can result in long-lasting decreases in DA levels, tyrosine hydroxylase activity, and DA uptake sites in the brain (Ricaurte et al., 1982; Seiden and Ricaurte, 1987; Gibb et al., 1994; Cass, 1997). Many studies have produced data that demonstrates a modification in the DAT function, which may be associated with both the altered uptake and release of dopamine (Ricaurte et al., 1982; Seiden and Ricaurte, 1987; Gibb et al., 1994; Cass, 1997; Bennett et al., 1997; Kokoshka et al., 1998; Kimmel et al., 2000).

Most studies will agree that that there is a decrease in the DAT binding in the STR and ACC with a decrease in affinity (K_m) without change in the maximal velocity of uptake/transporter density (V_{max}) (Kimmel et al., 2000; Kokoshka et al., 1998; Bennett et al., 1997). Kim et al. (2000) has reported that their group studies revealed a decrease in V_{max} without a change in K_m.

Previously, studies were unable to show similar effects in the ACC (Cass, 1997; Kokoshka et al., 1998). More recently, studies have been able to demonstrate the same decrease in DAT binding within the ACC that is seen in the STR (Kimmel et al., 2000).

These findings offer an excellent foundation of information to build upon. Not only is there a deluge of information within the brain areas of interest, these studies provide justification of an animal model, the Sprague-Dawley rat, and a series of neurochemical methodologies in which to study and characterize neurochemical changes

associated with MA administration (Ricaurte et al., 1982; Seiden and Ricaurte, 1987; Gibb et al., 1994; Cass, 1997; Bennett et al., 1997; Kokoshka et al., 1998; Kimmel et al., 2000). With this knowledge, the stage is set for additional neurochemical characterization of MA effects with NIC co-administration with respect to reward and addiction.

1.7.10 Other neurotransmitters. The neurotoxic and addictive effects of MA have been linked to several neurochemical factors, including serotonin (Schmidt and Lovenburg, 1985; Johnson, et al., 1994; Farfel and Seiden, 1995; Cass, 1996), oxygen radical formation (Fleckenstein, et al., 1997), and as discussed previously, the dopaminergic system (Schmidt and Lovenburg, 1985; Delle Donne, and Sonsalla, 1994; Cass, 1996; Fleckenstein, et al., 1997; Bennett, et al., 1997; Fumagalli, et al., 1998; Callahan, et al., 1998; Kim, et al., 2000). In contrast to the vital role associated with dopamine and addiction, Bardo (1998) also reviewed another catecholamine neurotransmitter, norepinephrine, but was unable to show any major role in addiction. Additionally, a review (Bardo, 1998) of serotonin, acetylcholine, amino acids, and other neuroactive peptides still supports the original hypothesis-- the use of dopamine to assess MA's neurochemical link to addiction and neurotoxicity.

1.8 Animal model

The role of dopamine and its neurochemical characteristics in response to MA has been well documented (Wagner, et al., 1979; Ricaurte, et al., 1980; Segal and Kuczenski, 1997a, 1997b; Schmidt et al., 1985; Fumagalli et al., 1998; Bardo, 1998). In the past, the study of the pharmacological and toxicological effects of MA utilized many animal

models. These models include guinea pigs (Lewander, 1971; Wagner, et al., 1979), mice (Fumagalli, et al., 1998; Jones, et al., 1998), cats (Chiueh and Moore, 1974), monkeys (Seiden et al., 1975), and humans (Volkow, et al., 2001a; Volkow, et al., 2001b), and, most commonly, rats (Wagner, et al., 1979; Ricaurte, et al., 1980; Ricaurte, et al., 1982; Ricaurte, et al., 1984; Peat, et al., 1983; Schmidt and Lovenberg, 1985; Zaczek, et al., 1989; Krueger, 1990; Green, et al., 1992; Melega, et al., 1995; Wagstaff, et al., 1996; Segal and Kuczenski, 1997a,b; Fleckenstein, et al., 1997; Bennett, et al., 1997; and Kim, et al., 2000). The use of numerous animal and human models has substantiated researchers' belief that MA addiction is somehow linked to and can be studied through the dopaminergic system.

1.9 Methamphetamine dosage

Multiple high-dose administrations of MA have been shown to cause persistent decreases in central dopamine uptake and dopamine transporter protein (DAT) binding in rodents, non-human primates, and perhaps humans (Wagner et al., 1980; Villemagne, 1998; Kim et al., 2000). Various dosing regimens have been used successfully in rats for studying the effects of MA on the dopaminergic system. Sandoval et al. (2000) compared four injections of 2 to 10 mg/kg s.c., given in 2-hour intervals to mice and rats, to compare similarities and differences. 10 mg/kg has been shown to cause a dose dependent decrease in [³H]dopamine uptake in striatal synaptosomes, prepared from mice and rats one hour after administration and within 24 hours after last MA treatment. The dopamine transport activity partially recovered, whereas ligand binding to the dopamine transporter was reduced similarly at one and 24 hours (Sandoval et al., 2000). Similar

results had previously been demonstrated with 5 mg/kg MA in the same dosing regimen (Cass, 1997), and a dose of 15 mg/kg was shown to produce similar effects (Fumagalli, et al., 1998).

One thing to consider is frequency of the administration. If AMP or MA is used repeatedly, some acute effects may diminish (tolerance), while others are enhanced (sensitization). Animals given several drug injections spaced out at intervals of a day or more tend to show sensitized locomotor activity and stereotypy, progressing with each injection, these being most appropriate for behavioral studies. Animals given the drug continuously through an osmotic pump or by closely spaced injections show a diminished locomotor response to a subsequent challenge (Post, 1980; Kuribara, 1996). Sensitized locomotor activity can persist in rats for over a year after the end of drug administration (Paulson, et al., 1991). One must, therefore, be careful to select the most appropriate dosing regimen for one's study.

The current studies utilized one day of either MA, NIC, COMBO, or saline injections (3 X 5 mg/kg i.p., 3 hours apart). On either one-hour post last injection (1hrPT) or seven-day post last injection (7dayPT), rats were sacrificed and tissue samples taken as described in chapter III. This dosing regimen is believed to model an overdose by a naïve drug user. The regimen produced visible behavioral effects indicating psychostimulant effects without causing any premature deaths in the experimental animals. Additionally, this model has been used in characterizing acute and chronic neurochemical consequences of MA administration (Davidson et al., 2004).

1.10 Enzymatic metabolism

1.10.1 Methamphetamine metabolism. Upon administration and distribution, MA is continually eliminated in an unchanged form. Under normal circumstances, up to 43% of a dose is eliminated unchanged in the 24-hour urine, with approximately 4-7% cleared as AMP (Basalt, 2000). Contrary to most belief, AMP is not the major metabolite of MA, primarily because it only accounts for less than 20% of the administered MA (Cho, 1990); however, it is the major active metabolite.

Metabolism generally occurs in the liver by enzymes whose activity and distribution will be discussed in the next section. About 15% of the MA is excreted as p-hydroxyMA (figure 1-6) and the remainder of the dose is accounted for as minor amounts of the same metabolites found after AMP administration (figure 1-7) (Caldwell et al., 1972). Amphetamine has demonstrated significant deactivation during metabolism, primarily by deamination to phenylacetone, which is subsequently oxidized to benzoic acid and excreted as glucuronide and glycine conjugates. A small amount of AMP is oxidized to norephedrine, which along with its parent compound, AMP, are p-hydroxylated to form p-hydroxynorephedrine and p-hydroxyamphetamine. Many of these metabolites are pharmacologically active and may contribute to the effects of the drug, especially during chronic usage.

Amphetamine is generally metabolized and excreted as 0.9% phenylacetone, 16-28% as hippuric acid, 4% as benzoylglucuronide, 2% as norephedrine, 0.3% as conjugated p-hydroxynorephedrine, and 2-4% as conjugated p-hydroxyAMP (Beckett and Rowland, 1965; Becket et al., 1969; Basalt 1996). MA has been shown to generate higher proportions of the pharmacologically active p-hydroxymetabolites than AMP

(10% in MA and less than 0.4% in amphetamine) (Yamada and Yoshimura, 1989; Cho, 1990).

In humans, the serum concentration peaks vary with differing amounts of oral administration. A maximum blood concentration was seen around one hour after a single oral dose of 10 mg (Lebish et al., 1970). A 12.5 gm oral dose resulted in a peak concentration at approximately 2.5 hours (Driscoll et al., 1971) and a 30 mg dose resulted in an average peak serum concentration between three to five hours and was maintained for up to 12 hours later (Shappell et al., 1996).

The fairly complex metabolism and excretion of MA in humans result in a fairly long half-life (approximately 10-12 hours) (Cho et al., 2001; Cho, 1990; Shappell et al., 1996). Urinary excretion of the unchanged drug is pH dependent. Urinary acidification decreases half-life and alkalinization increases the half-life. For every one-unit increase in urinary pH, there is an average seven-hour increase in plasma half-life (Drug Facts and Comparison, 1997). During urinary acidification, plasma elimination is shortened, and total clearance is increased as well (Beckett and Rowland, 1965). This has also been shown with AMP (Beckett et al., 1969) and is similar with MA (Beckett and Rowland, 1965). Though the elimination half-lives in humans and rats are quite different, they do share similarity and predictability, as discussed by Cho (1990). Furthermore, dosing regimens can be modified in rats to mimic human abuse patterns.

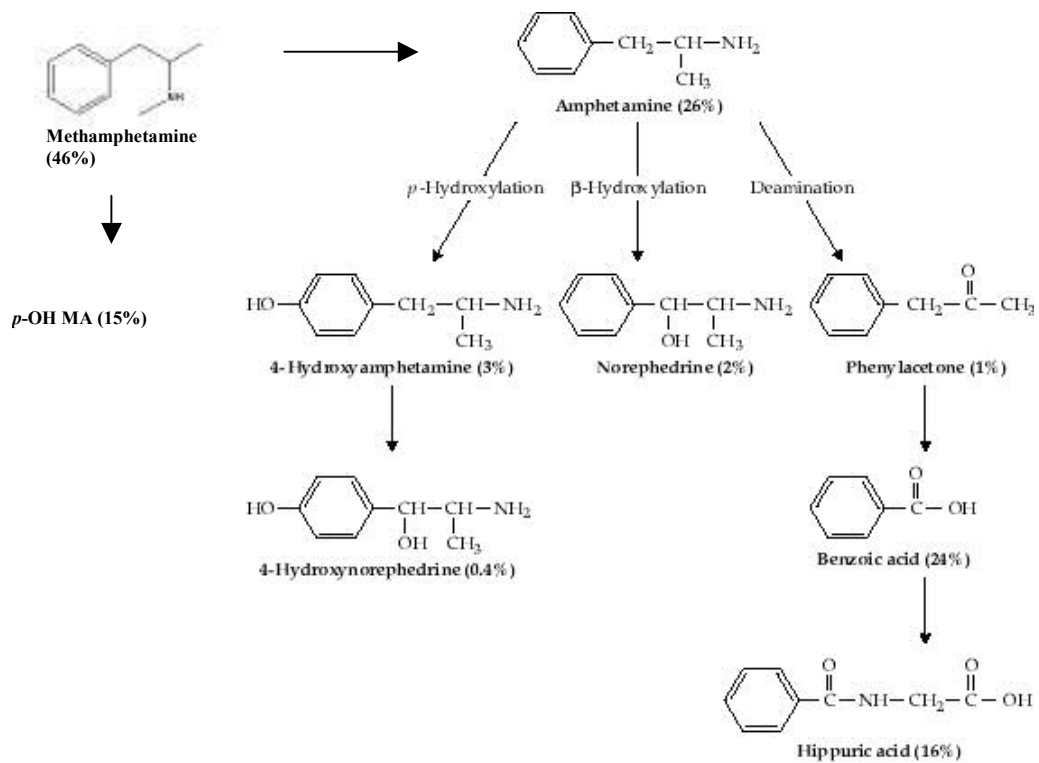


Figure 1-6. Metabolism of amphetamine schematic. Reproduced with modification from Feldman et al., *Fundamentals of Neuropsychopharmacology*, Sinauer Associates, Inc. (1999).

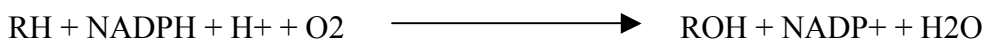
1.10.2 Enzymatic drug metabolism. Metabolism is the mechanism of elimination of drugs and foreign compounds from the body and controls the levels of other desirable compounds, such as vitamins. Metabolism of drugs is complex, with many drugs being metabolized by multiple enzyme systems. Enzymes involved in metabolism are present in many bodily tissues (gastrointestinal tract, lung, nasal mucosa, kidney, blood, skin, and blood cells) (Krishna and Klotz, 1994); however, the liver is the principal site of drug metabolism.

For many drugs, metabolism occurs in two phases. Phase I reactions are catalyzed by a super family of mixed function monooxygenase enzymes, known as the cytochrome P450 system. This occurs in liver microsomes, located in the endoplasmic reticulum of liver cells or hepatocytes, where the drug is made more hydrophilic by the formation of a new or modified functional group or a cleavage. Most of the time, these simple modifications are enough to increase solubility facilitating elimination through the kidneys (in urine) and the liver (in bile). Phase II metabolism involves conjugation, many times after their activation by Phase I enzymes. Phase I and Phase II enzymes acting in concert convert hydrophobic compounds to more hydrophilic compounds that can be readily eliminated.

1.10.3 Cytochrome P450 system. The most important enzyme system of Phase I metabolism is cytochrome P450, a microsomal superfamily of isoenzymes that transfer electrons and thereby catalyze the oxidation of many drugs. The electrons are supplied by NADPH-cytochrome P-450 reductase, a flavoprotein that transfers electrons from NADPH (the reduced form of NIC-adenine dinucleotide phosphate) to CYP. There are many good review articles on the Cytochrome P450 system (Kraemer and Maurer, 2002;

Snawder and Lipscomb, 2000; Meyer, 1996; Kivisto and Kroemer, 1997; Tucker, 1994; Wrighton and Stevens, 1992).

Briefly, CYP's are membrane bound proteins with an approximate weight of 50kD that contain a heme moiety. They are found along with other mixed function oxygenases in the endoplasmic reticulum of the liver. CYP enzymes are well known for their role in the metabolism of compounds of a rather non-polar nature (Azenbacher and Anzenbacherova, 2001). All the known CYP enzymes bind two atoms of oxygen, mainly O₂, but sometimes the -O-O- moiety of a peroxide structure. The binding of these to the heme portion of the enzyme provides the necessary properties for the splitting of the dioxygen molecule. Hence, in most cases, a hydroxylated product is formed, with the general monooxygenase reaction catalyzed by a CYP enzyme expressed as, where RH is the substrate:



The complete reaction scheme may be much more complex. The main alterations that occur involve a wide range of chemical reactions, including aromatic hydroxylation, aliphatic hydroxylation, oxidative N-dealkylation, oxidative O-dealkylation, S-oxidation, reduction, or hydrolysis (Gunaratna, 2000).

1.10.4 Drug interactions. CYP enzymes metabolize the majority of presently available pharmaceutical agents, including illicit MA (Lin et al., 1995, 1997). It is believed that only five of the hundreds of known CYP enzymes are responsible for approximately 95% of the hepatic CYP-mediated oxidations (Wrighton and Stevens,

1992). According to Guengerich (1996), human liver CYP2D6 and CYP3A4 participate in the oxidative biotransformation of ~80% of commercially available drugs.

Within the human population, genetic or hereditary factors are significant factors in drug metabolism (Van der Weide, 1999). Variability associated with the CYP enzyme system within each individual can cause marked responses. According to Gunaratna (2000), there are three general polymorphic groups of CYP enzyme individuals: a) Extensive metabolizers: Normal individuals;

b) Poor metabolizers (PM): Individuals that show complete absence of enzyme activity; and

c) Ultra extensive metabolizers: Individuals showing enhanced enzyme expression.

Also, it appears that metabolism decreases with age and clearances of certain drugs are different in men and women (Sotanieui, et al., 1997). Hormones have been shown to influence the activity of CYP enzymes (Tanaka, 1983). Environmental factors such as diet, smoking, alcohol consumption, and concomitant drug therapy, also influence the outcome of drug metabolism (Badyal and Dadhich, 2001).

1.10.5 Enzyme inhibitors. Enzyme inhibitors function in different ways. The competitive inhibitors compete with the substrate for the same active site and the non-competitive inhibitors bind to the enzyme substrate complex or to the heme-group. The third type, irreversible inhibitors, inactivate the enzyme either by heme or protein binding. Enzyme inhibition can lead to higher systemic levels of a drug causing enhanced efficacy or toxicity. When several CYP enzymes are responsible for metabolizing the same drug, administration of an enzyme inhibitor will not have a significant effect, since the drug has an alternate metabolic pathway.

1.10.6 Enzyme induction. Drug interactions involving enzyme induction are not as common as inhibition-based interactions. The most common mechanism is transcriptional activation leading to the increased synthesis of CYP proteins (Dossing et al., 1983). Metabolism of the affected drug is increased leading to decreased intensity and shorter duration of drug effects. The classic examples of induction are with chronic alcohol consumers and the decreased effectiveness of oral contraception, while women are taking certain antibiotics (i.e. macrolides).

1.11 Cytochrome P450

1.11.1 Cytochrome P450 nomenclature. Currently, there are about 30 human cytochrome P450 enzyme alleles present in humans (Chang and Kam, 1999), designated by a root symbol CYP, followed by an Arabic number for family, a letter for subfamily, and another Arabic number for the specific gene. For example CYP3A4 is a cytochrome P450 enzyme, belonging to family 3 and subfamily A. The last number 4 refers to the sequence of discovery. Enzymes in the 1A, 2B, 2C, 2D, and 3A subfamilies are important in mammalian metabolism; CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are considered the most important in human metabolism (Gunaratna, 2000).

1.11.2 CYP1A subfamily. The human CYP1A subfamily consists of two members, CYP1A1 and CYP1A2. CYP1A2 is mainly expressed in the liver, while CYP1A1 is primarily expressed in extrahepatic tissues. CYP1A1 is expressed at very low levels (Guengerich 1991; McKinnon et al., 1991; Raunio et al., 1995; Pelkonen et al., 1998) and participation in drug metabolism *in vivo* is not considered here. The expressed CYP1A2 represents about 15% of total CYP enzymes in the human liver (Pelkonen and

Breimer 1994; Shimada et al., 1994). There is some variability between individuals in the human liver CYP1A2 enzyme levels (Shimada et al., 1994). Because of the large interindividual variation in CYP1A2 activities, the *in vivo* testing of this enzyme has been quite difficult to evaluate (Kunze and Trager, 1993) and will not be considered for study here. Pelkonen et al. (1998) reviewed substrates and their enzyme kinetic parameters; CYP1A2 substrates included theophylline, caffeine, olanzapine, ondansetron, paracetamol, phenacetin and propranolol.

1.11.3 CYP2D subfamily. The human genome includes only one functional gene in the CYP2D subfamily, namely CYP2D6 (Nelson et al., 1996). Though two CYP2D7 pseudogenes and two pseudogenes of CYP2D8 (Heim & Meyer 1992; Nelson et al., 1996) exist, they will not be covered in this study.

CYP2D6 represents 1 to 5% of the total P450 enzymes (Pelkonen & Breimer 1994; Shimada et al., 1994; Pelkonen et al., 1998). About 7-10 % Caucasians and up to 30% of Chinese are PMs for this enzyme (Heim & Meyer 1992; Kroemer and Eichelbaum, 1995). One common feature of CYP2D6 substrates is that they contain at least one basic nitrogen atom at a distance of 5 or 7Å from the oxidation site. Secondly, there is a planar hydrophobic area near the oxidation site, and thirdly, the substrates exhibit a negative molecular electrostatic potential above the planar part of the molecule (Koymans et al., 1992; Strobl et al., 1993; de Groot et al., 1997).

The drugs known to be substrates for this enzyme include antiarrhythmic and other cardiovascular drugs, β -adrenergic blocking agents, tricyclic antidepressants, neuroleptics and many other commonly used therapeutical agents (Cholerton et al., 1992). Dextromethorphan O-demethylation, debrisoquine 4-hydroxylation, and bufuralol

1'-hydroxylation are the mostly used *in vitro* models. Of these substrates, debrisoquine is also employed in *in vivo* studies as a CYP2D6 model substance (Pelkonen et al., 1998, and references therein).

As a reference inhibitor for CYP2D6, quinidine is widely used in drug metabolism studies. It is a specific and potent inhibitor with an inhibitory constant, K_i , of 0.06 μM for CYP2D6, and the CYP next in sensitivity, CYP3A4, has a K_i value around 10 μM (Broly et al., 1989; Bourrie et al., 1996). CYP2D6's role in MA metabolism is reviewed in several articles (Lin 1995, 1997; Kraemer and Maurer, 2002). Due to the numerous drugs on the market that are metabolized by CYP2D6 and its proven involvement of MA metabolism, it will be examined for suspected drug-drug interactions.

1.11.4 CYP2C subfamily. The CYP2C subfamily is the second most abundant CYP protein in the human liver, representing about 20% of the total P450 (Shimada et al., 1994). This subfamily consists of three active members in the human liver, namely CYP2C8, CYP2C9, and CYP2C19. Of these, CYP2C9 and CYP2C19 are characterized as polymorphically expressed (Goldstein and de Morais, 1994; Gill et al., 1999). CYP2C8 will not be considered for drug metabolic importance, since it is expressed at very low levels in the human liver.

CYP2C9 is the major CYP2C isoform in the human liver (Goldstein & de Morais, 1994), and it has been shown to be genetically polymorphic with at least three different alleles that produce differently active protein. The functional consequences of these polymorphisms are not yet clear, although CYP2C9 has a major role in the metabolism of many clinically important, weakly acidic drugs, such as S-warfarin (Rettie et al., 1992),

tolbutamide, phenytoin (Doecke et al., 1991), sulphamethoxazole (Cribb et al., 1995), many of the non-steroidal anti-inflammatory compounds (Leeman et al., 1993), and celecoxib (Tang et al., 2000). The frequencies of the two variant alleles, CYP2C9*2 and CYP2C9*3, have been reported to range from 7 to 19% in Caucasian populations (Furuya et al., 1995; Sullivan-Klose et al., 1996; Stubbins et al., 1996).

With CYP2C19, the genetic polymorphism leads to the PM phenotypes exhibiting less active or completely inactive S-mephenytoin 4'-hydroxylase. This PM phenotype is produced by at least two major, and several minor, variant alleles of CYP2C19 (Goldstein & de Morais, 1994) and, consequently, CYP2C19 substrates are not metabolized as expected (Pelkonen et al., 1998). The deficiency of the 4'-hydroxylation pathway of S-mephenytoin occurs in 2 to 5% of the Caucasian population (Relling et al., 1990). This deficiency may lead to accumulation of certain drugs and to *in vivo* concentrations exceeding the therapeutic level and producing unexpected toxic effects. Since there are no selective CYP2C19 inhibitors and there has been no speculation to its activity with MA metabolism, CYP2D19 will not be used for these studies.

1.11.5 CYP3A subfamily. The CYP3A subfamily represents about 30% of the total P450 content in the human liver (Shimada et al. 1994; Pelkonen and Breimer, 1994), although the levels of the protein may vary 40-fold among individuals (Guengerich, 1995). This subfamily consists of three members (Nelson et al., 1996). CYP3A4, one of these three members, is the most abundant CYP enzyme in the human liver and it is expressed in several tissues, but the expression in the liver and in the small intestine is of major interest in view of the metabolism of drugs and other xenobiotic chemicals

(Guengerich 1999; Dresser et al., 2000). For this reason, CYP3A4 will be the only member of this subfamily used in these studies.

CYP3A4 participates in the metabolism of about half of the drugs in use today (Bertz and Granneman, 1997). For example, testosterone 6 β -hydroxylation, midazolam 1'- and 4-hydroxylations, nifedipine oxidation, and erythromycin N-demethylation are catalyzed by this enzyme. The known substrates of CYP3A4 vary in size from small molecules, such as acetaminophen to cyclosporin A (Guengerich, 1999). In addition to the substrates listed above, CYP3A4 participates in the metabolism of progesterone and andostenedione (Waxman et al., 1991), cortisol, quinidine, diltiazem, lidocaine, lovastatin, troleandomycin, warfarin, and triazolam (Guengerich and Shimada, 1991; Wrighton and Stevens, 1992).

CYP3A is inducible by many drugs, for example, rifampicin, dexamethasone, carbamazepine and phenobarbital type inducers (Pelkonen et al., 1998). The induction of CYP3A has an effect on interindividual variation and has been shown to affect both bioavailability and drug-drug interactions (Guengerich, 1999). The inhibitors of CYP3A have a wide variety of chemical structures; for example, azole-type fungicides, ketoconazole and itraconazole are potent inhibitors. Ketoconazole also inhibits other CYPs than CYP3A4, but at a concentration of 1 μ M it is relatively selective for CYP3A4 (Newton et al., 1994; Baldwin et al., 1995). Since CYP3A4 plays such an important role in xenobiotic metabolism, its activity may not be very simple in nature; conclusions drawn on the basis of only one substrate or inhibitor should be regarded as tentative. Although literature has not shown CYP3A4 to participate in MA metabolism, its

overwhelming presence in the liver and ability to metabolize so many other drugs, qualify it as a strong candidate for drug-drug interactions and will be examined.

1.12 Metabolism studies

1.12.1 Methods for studying metabolism *in vitro* and *in vivo*.

Pharmacological/toxicological metabolism studies generally consist of animal and/or human tissue-derived *in vitro* studies followed by animal studies. Studies are conducted in hope of establishing an appropriate animal model that can be used to extrapolate results to humans. Various *in vitro* methods have been developed by employing human tissue-derived systems (Wrighton et al., 1993), usually done by utilizing liver preparations from humans and trying to find the test species that most closely resembles human metabolism (Yuan et al., 1999). The two systems used in the present studies are liver microsomes and human cDNA-expressed enzymes in host cell microsomes, both of which will be discussed briefly here.

1.12.2 cDNA-expressed enzymes. Drug-metabolizing enzymes are available commercially as cDNA-expressed enzyme systems. In these preparations, an individual enzyme is produced within a variety of systems including: bacteria (Gillam et al., 1993), yeast (Guengerich et al., 1991a; Peyronneau et al., 1992), mammalian cell lines (Guengerich, 1995b), and baculovirus systems (<http://www.gentest.com>; Asseffa et al., 1989). The expression of human liver CYPs in a variety of artificial systems has become easier, due to the rapid development of recombinant DNA techniques (Gonzalez et al., 1991).

Since cDNA-expressed enzymes can be studied in isolation from other hepatic enzymes, they have become a valuable tool when initially assessing the metabolism of certain drugs and drug combinations. In the past, detection of multiple metabolites generally required novel, highly sensitive mass spectrometry tools, whereas cDNA-expressed systems can utilize the conventional measurement of fluorescence metabolite production for multiple enzymes (see, for example, <http://www.gentest.com>). cDNA-expressed CYPs can also be used in the high throughput screening kit (HTS) methodology when the goal is to screen large numbers of compounds in a short amount of time (White, 2000).

Still, the biotransformation of a drug or drugs by a single CYP does not necessarily accurately depict its participation within an *in vivo* situation. cDNA-expressed CYP enzymes lack the whole complement of hepatic enzymes, and the *in vivo* predictive value of the data obtained from expressed enzyme systems has been debated (Rodriguez 1999). cDNA-expressed enzymes have been used to study MA analogues *in vitro* (Ramamoorthy et al., 2002) and will be used in these studies because they are commercially available (<http://www.gentest.com>), rapid, inexpensive technique that can be used to determine the role CYP2D6 and CYP3A4 in the metabolism of a MA.

1.12.3 Liver microsomes. Liver microsomes are fractionated from subcellular organelles by differential ultracentrifugation. Microsomes are formed from smooth endoplasmic reticulum during tissue homogenization (Boobis, 1995). A microsomal fraction from human liver contains a full complement of P450 enzymes, which makes it a suitable tool for studying inhibitory CYP interactions (Kremers, 1999). Microsomes are relatively easy to prepare, and enzymatic activities are stable during prolonged storage

(Beaune et al., 1986; Yamazaki et al., 1997), if the original tissue is correctly handled and frozen immediately after excision (Kremers, 1999).

The validation of assay procedures between different laboratories in this respect has led to less inter-laboratory variation in the same assays (Boobis et al., 1998; Kremers, 1999). Liver microsomes can be easily and inexpensively produced from rats or obtained from commercially available sources, like the Gentest Corp. (<http://www.gentest.com>). Gentest even offers human liver microsomes. In rats, microsomes can be used to assess *in vivo* changes that may have occurred over a prescribed dosing regimen. Utilizing microsomes generally requires specific substrates and inhibitors, which will be discussed below.

1.12.4 Human hepatocytes. The utilization of human primary hepatocytes was not used in these studies because sufficient liver samples are quite difficult to obtain and hepatocytes are difficult to preserve for later use. Some successful attempts to cryopreserve primary hepatocytes have been described (Guillouzo et al., 1995). A prolonged culture method has also been published, in which hepatocytes are maintained for over 30 days (Kono et al., 1997), preserving some of their drug-metabolizing activities and inducibility, instead of the standard for up to one week. Although difficult to obtain and maintain, hepatocytes contain the full complement of Phase I and Phase II enzymes, and the whole metabolite pattern can be detected in incubations with hepatocytes, such that, induction of drug-metabolizing enzymes and possible toxic effects, can also be elucidated (Ferrini et al., 1997; Li et al., 1997; Maurel 1996; Morel et al., 1990).

1.13 High throughput inhibition assays

Animal studies are quite expensive, although necessary, and because of the marked differences in the drug-metabolizing enzymes between humans and animals, it is reasonable to perform *in vitro* testing to select the appropriate species best able to characterize human metabolism for any drug of interest. The use of human tissue-derived *in vitro* systems in affinity screening and metabolic studies gives fairly accurate information about the *in vivo* situation with the compound under study. Commercially available kits dedicated to the development and study of *in vitro* analysis of drug metabolism and toxicity can be purchased from BD Gentest Corp. (<http://www.gentest.com>). This company's kits are internationally recognized as leaders in the cytochrome P450 cDNA-expressed and *in vitro* drug metabolism techniques and supplies.

1.13.1 Selection of appropriate probes and inhibitors.

1.13.1.1 CYP2D6/CYP2D2 fluorescent probes. A non-fluorescent probe 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), which produces a fluorescent metabolite, 3-[2-(N,N-diethyl-N-methylamino) ethyl]-7-hydroxy-4-methylcoumarin (AHMC), has been used as a specific CYP2D6 probe in liver microsomes (Chauret et al., 2001) and cDNA expressed CYP2D6 enzymes (<http://www.gentest.com>). Furthermore, this same probe has successfully been used as a selective probe for the CYP2D2 enzyme in rat microsomes (Stresser et al., 2002),

providing a single probe that can be used to assess both human/cDNA expressed CYP2D6 and rat liver microsomes.

1.13.1.2 CYP2D6/CYP2D2 selective inhibitor. Quinidine has been proven to be a potent inhibitor for CYP2D6, even though it is also metabolized via CYP3A4 (Bourrie et al., 1996). Quinidine will be used in these studies because it is a portion of the commercially available high throughput inhibition kits to be used from BD Gentest Corp (<http://www.gentest.com>).

1.13.1.3 CYP3A4 fluorescent probe. A nonfluorescent 7-benzyloxy-4-trifluoromethylcoumarin (BFC), which produces a fluorescent metabolite, 7-benzyloxy-4-trifluoromethylcoumarin (HFC), has been used as a specific CYP3A4 probe in liver microsomes (Stresser et al., 2002) and cDNA expressed CYP3A4 enzymes (<http://www.gentest.com>), providing a single probe that can be used to assess both human/cDNA expressed CYP3A4 and rat liver microsomes.

1.13.1.4 CYP3A4 selective inhibitor. There are many selective inhibitors for CYP3A4 of which ketoconazole is the most widely used, even though it also affects other CYPs (Schmider et al., 1995; Bourrie et al., 1996). Ketoconazole will be used as the selective inhibitor for CYP3A4 in these studies because it is a portion of the commercially available high throughput inhibition kits to be used in this study available from BD Gentest Corp (<http://www.gentest.com>).

1.14 Animal studies

Even with cytochrome P450 cDNA-expressed enzymes and commercial kits, some testing has to be performed on animals. Human-derived *in vitro* systems are

increasingly reducing the number of animals needed for such testing. For the time being, animal tests are still necessary, but careful planning and effective use of the *in vitro* methodology can reduce the use of such, although a direct link between human CYP2D6, the primary human MA metabolizing CYP enzyme, and one of the various forms of the rat CYP2D, has not been established. Sprague-Dawley and Dark Agouti rats have both been successfully used as animal models in studying the metabolism of MA and other similar analogues in humans (Stresser et al., 2002; Ramamoorthy et al., 2002; Kraemer and Maurer, 2002; Chauret et al., 2001; Delaforge et al., 1999; Tyndale et al., 1999; Chow et al., 1999; Lin et al., 1995; Matsunaga et al., 1989).

1.15 Human abuse pattern

As discussed prior, MA has become the homemade drug of choice. Legal constraints have increased the cost and decreased the availability of substances used to make MA. Therefore, MA producers have found additional ways to increase their profit and the amount of product to be distributed. One way this is accomplished is by diluting the final product, illicit MA, with a cheaper legal substance. According to Sgt. Harold Adair, if a MA cook were to produce one ounce of essentially pure MA, it would have a street value of approximately \$1,500 in Tulsa, Oklahoma (Adair, 2003). If a person were to take that ounce and add four ounces of “Super B”, a vitamin supplement primarily consisting of vitamin B3, he would have a total of five ounces of substance that could be sold as MA.

The purity of this product is approximately 20% MA, which could be sold for approximately \$800 to \$1,500 an ounce, with a return of \$4,000 to \$7,500. This process

is referred to as “cutting” or “stepping on” the product and is routinely done. This method of boosting one’s profit is not new to the drug scene; it has been used for many years. The one thing that has become perplexing is that drugs, such as cocaine, are cut with numerous compounds such as baking powder, talc, benzocaine, procaine, lidocaine, caffeine, and many other white powders. In Tulsa, Oklahoma, MA is almost solely cut with NIC.

It is very rare for a sample of illicit MA to be identified in the Tulsa Police Department’s forensic laboratory that has anything other than NIC used as the cutting agent. Most samples contain what are considered to be synthesis by-products. Yet, it is extremely rare to find the presence of any diluents common to cocaine or heroin in a sample of MA. A one month series of studies conducted within the Tulsa Police Department forensic laboratory demonstrated the ratio of illicit MA samples to be anywhere from 3 to 97% MA (unpublished data). The average that was calculated from samples taken over that month was determined to be 55% MA, with the remainder being mainly NIC and a few other trace impurities.

When speaking to known MA abusers and discussing the reason they elect to use NIC to cut their MA, the answers inevitably vary. Some speak of NIC’s solubility in water and how everything goes into solution quickly (Bilyeu, personal communication). It has also been said, using NIC to cut MA prevents “sticky needle” (Doe, personal communication). This term generally refers to an increased amount of friction generated by the plunger in a syringe that has previously or repetitively been used to administer illicit MA. Others state that NIC changes to the same color of the illicit MA, preventing the detection of “cut dope” with the naked eye and that using a supplement with NIC

gives the dope “longer legs”, which means it made the effects last longer (Harp, personal communication). Harp compared the lasting effects to another cutting agent that is rarely used, which she referred to as “MSN”. She went on to say that on average a person would pay about \$15-25 for a bottle of Super B or Vitablend, which contain approximately 100 gm of NIC. The usage of vitamin B3 as the primary agent used to cut MA would not be so perplexing if it was a cheap substance. It is relatively expensive, compared to the other known cutting agents seen in cocaine and heroin. Additionally, the reports of altered solubility and enhanced effects have caused the lab to investigate the underlying mechanism it may possess in enhancing MA’s psychostimulant effects.

1.16 Vitamin B-3

Vitamin B-3 (B3) exists as the amide in nature, in NIC adenine dinucleotide (NAD). Vitamin B-3 comes in two basic forms: niacin (nicotinic acid) and niacinamide/nicotinamide (NIC). Pure NIC and niacin are synthetics. Originally, it was made from nicotine, a poison produced in tobacco plants, but when nicotine is simplified by chemically opening one of the six carbon rings, it becomes B3 (Hoffer 1989). Vitamin B-3 is made in the body from the amino acid tryptophan. Since the body makes Vitamin B-3, it does not meet the classic definition of a vitamin, which is defined as a substance that cannot be made. It really should have been classified with the amino acids, but the long usage of the term has given it permanent status as a vitamin.

One of the best-known vitamin deficiency diseases is pellagra. More accurately, it is a tryptophan deficiency disease, since tryptophan also can cure the early stages of the disease. Pellegra was an endemic in the southern United States until the beginning of

World War II, and can be described by the four D's: dermatitis, diarrhea, dementia, and death. In the early stages it resembles more of the schizophrenias, and it is very difficult to distinguish between the two. The dementia is a late stage phenomenon. The only method used by early doctors was to give their patients a small amount of niacin. If they recovered, they diagnosed them as having pellagra; if they did not, they referred to it as schizophrenia.

Vitamin B-3 has previously been used in lowering high cholesterol and in elevating high-density lipoprotein cholesterol levels (Brown, 1995), alleviating hypoglycemia (Shansky, 1981), treating anxiety (Vescovi et al., 1987; Bruce et al., 1992), and therapeutically for the treatment of schizophrenia (Hawkins et al., 1970) and other psychiatric disorders. Vitamin B-3 is needed to metabolize alcohol and has been shown to be of use to alcoholics (Cleary, 1985, 1986). It has been shown to be therapeutic and may help stop alcoholics from drinking, and with the treatment of alcohol-associated depression (O'Halloren, 1961; Smith, 1974; Replogle and Eicke, 1988).

NIC, the safer and a more selective form of B3, has also demonstrated its ability to improve alcohol metabolism in animals (Eriksson, 1974). Vitamin B-3 has demonstrated its ability to work in the brain similar to drugs, such as, benzodiazepines, which are used to treat anxiety (Bruce et al., 1992). Nicotinamide itself has also been recognized for its ability to improve the withdrawal from benzodiazepine abuse (Vescovi et al., 1987).

It is interesting to note, in regards to MA, B3 has been shown to improve tardive dyskinesia (Tkacz, 1984) and to benefit recovery rates of individuals with schizophrenia (Hawkins et al., 1970). Tardive dyskinesia and schizophrenia-like symptoms are two

common phenomena closely associated with MA abuse. Some theorize that B3 relieves the body of chronic stress and allows it to carry out routine repair functions more efficiently. The current recognition of hyperoxidation--the formation of free radicals--as one of the most damaging processes in the body leading to tissue damage at the cellular and tissue levels, has offered a more scientific explanation.

All living tissues, which depend upon oxygen for respiration, have to protect themselves against free radicals. Antioxidants, such as, vitamin C, vitamin E, beta-carotene, selenium, and others protect the body and rid it of the damage causing free radicals. NIC is thought to enhance the body's ability to deal with stress and oxidative damage, and has been demonstrated by the significant inhibition of oxidative damage induced by reactive oxygen species generated in rat brain mitochondria (Kamat and Devasagayam, 1999). More specifically, one may consider NIC a potent antioxidant capable of protecting the cellular membranes in the brain, which are highly susceptible to being oxidized, against oxidative damage induced by reactive oxygen species.

In addition to antioxidant activity, NIC's neuroprotective effects via energy repletion have been shown by the reduction of malonate, a mitochondrial respiratory chain blocker that depletes striatal ATP (induced lesions in rats), with additive effectiveness when combined with coenzyme Q (Schultz and Beal, 1995). Also, local administration of NIC after multiple doses of MA rescued dopamine neurons from damage, as evidenced by prevention/attenuation of MA induced tissue dopamine depletions (Stephans et al., 1998). The most interesting studies with respect to NIC and MA's combinational usage, perhaps, are the ones that demonstrate the attenuation of

AMP and MA induced STR DA depletion in rats when pretreated with NIC (Huang et al., 1997; Wan et al., 1999).

1.17 Significance

The historical abuse of MA, and more specifically *d*-MA, has been substantially documented in the previous sections, supported with ample literature citations. The physical properties, various routes of administration, dosages, purity, pharmacology, neurochemistry, and metabolism also have been discussed in detail. The typical pattern of abuse from MA's clandestine production to its sale on the street has been established, along with recent news that clearly demonstrates the violence and urgency to further understand MA abuse.

A review of MA's pharmacology and toxicology clearly demonstrates the drug's potent addictive and neurotoxic effects, as evidenced by long-lasting alterations in the dopaminergic system--the best-established and most widely agreed upon neurochemical evidence in the literature today. MA has been recognized as a potent psychostimulant drug for decades. Over the years it has been diluted with numerous chemicals in order to generate a higher profit margin in illicit sales. The most recent and almost exclusive substance used as an adulterant is NIC (vitamin B3). Knowing that using NIC is not the most economical way to dilute MA leads one to believe there are possibly other aspects influencing the choice of cutting agents. If it is not availability or economics dictating the cutting agent, there must be some improvement in desired effects.

It was such consideration that initiated the hypothesis that is to be tested: Vitamin B-3, more specifically NIC, while co-administered with MA, provides an enhancement in

psychostimulant effects, somehow providing a more desirable experience for the user. This study, therefore, will seek to identify any metabolic and/or neurochemical changes associated with the co-administration of NIC and MA that may provide enhancement of desirable psychostimulant effects.

Chapter II

PRELIMINARY STUDIES

2.1 Dose Ratio Determination

2.1.1 Introduction. In order to provide an understanding as to the composition of illicit MA, a total of 54 MA samples, previously identified by the Tulsa Police Department's forensic laboratory, were analyzed. MA seized from clandestine laboratories was excluded from this data under the assumption that it had not been processed for sale; it did not represent the typical street quality MA.

2.1.2 Chemicals. MA, NIC, sodium carbonate, and methanol were all purchased from the Sigma Chemical Corporation (St. Louis, MO).

2.1.3 Methods. A rapid and reliable method for determining the ratio of MA to NIC in illicit drug samples was used to analyze unknown samples. Stock solutions of MA (10 mg/ml) and NIC (10 mg/ml) were prepared in methanol and were serially diluted into five separate sample vials. These vials contained ratios of 10:0, 7.5:2.5, 5.0:5.0, 2.5:7.5, and 0:10 MA:NIC mixtures. Each vial was then made basic with one drop of sodium carbonate. For unknown samples, 10 mg of each unknown sample was dissolved in 1.5 ml of methanol and made basic with one drop of sodium carbonate.

Each sample was analyzed utilizing a Hewlett Packard (HP) 6890 gas chromatograph (GC) connected to a HP5973 mass spectrometer (MS). A split injection

(50:1) of 1 μ l was made onto an Agilent Ultra 1 capillary column (12 meter x 0.2 μ m x 0.33 μ m). Injection port temperature 250°C; He carrier gas, column temperature programmed to hold at 115°C for 1.5 minutes then ramped from 115°C to 280°C @ 35°C /min, and then held at 280°C for 4.21minutes.

The prepared concentration curve indicated an approximately equal ratio of chromatographic response between MA and NIC. Therefore, the area under the curve (AUC) of the MA peak was directly compared to the AUC of the NIC peak and used to determine the ratio of MA to NIC. Identification was made based on mass spectral data and the known retention times for MA and NIC standards (Figures 2-1 thru 2-3).

2.1.4 Results. Utilizing methods described above, 54 samples were run over the course of one month. Preliminary results indicate the percentage of MA in street quality MA ranges from 3 to 97% with the remainder of sample being attributed to NIC. The mean percentage of MA found was 57.14 \pm 29.22% and the median of samples studied was 54.50% MA and, thus, NIC was 45.50%. As evidenced by the large standard error, the ratio of MA to NIC varies greatly, as seen in figure 2-4. In order to simplify mathematical calculations, and because of the large variability, the following studies will utilize a 50:50 mixture of MA:NIC (COMBO).

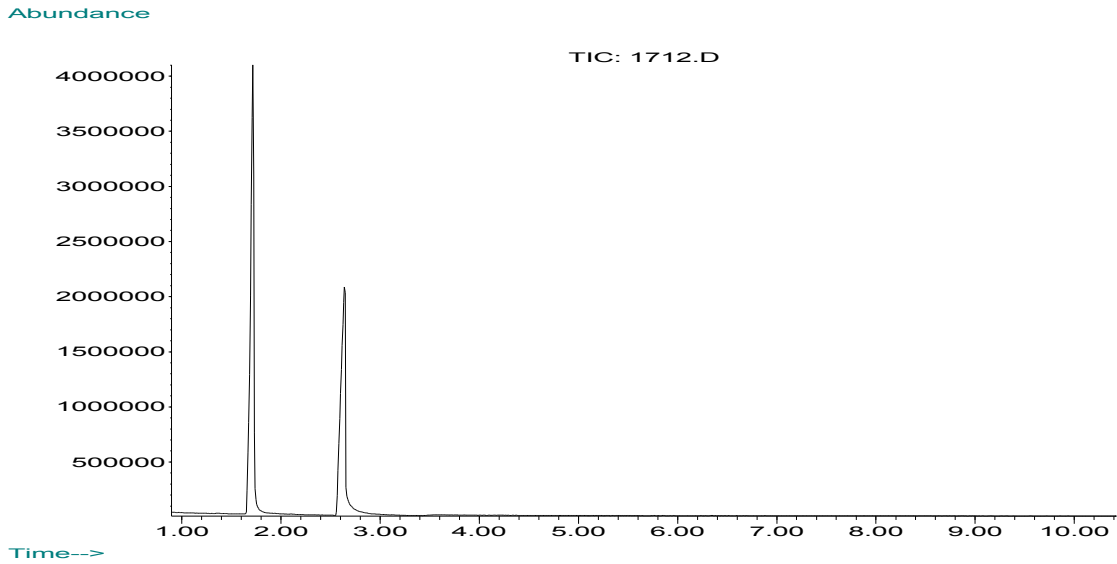


Figure 2-1. Gas Chromatograph demonstrating separation of methamphetamine (1.7 min) and nicotinamide (2.6 min) This chromatograph represents a 2:1 ratio of MA to NIC which would be reported as 66% MA and 33% NIC

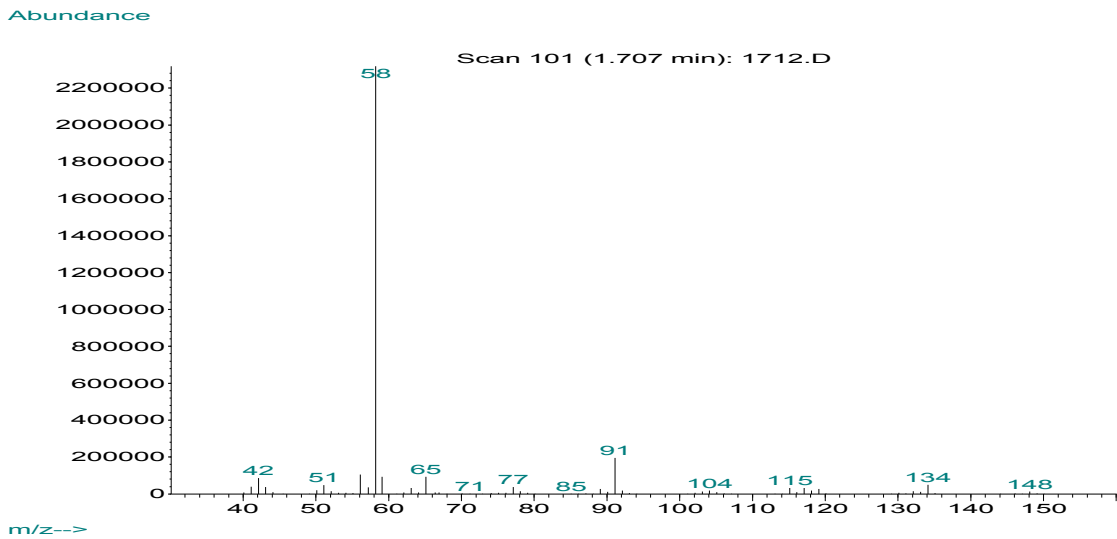


Figure 2-2. Mass spectrum of methamphetamine.

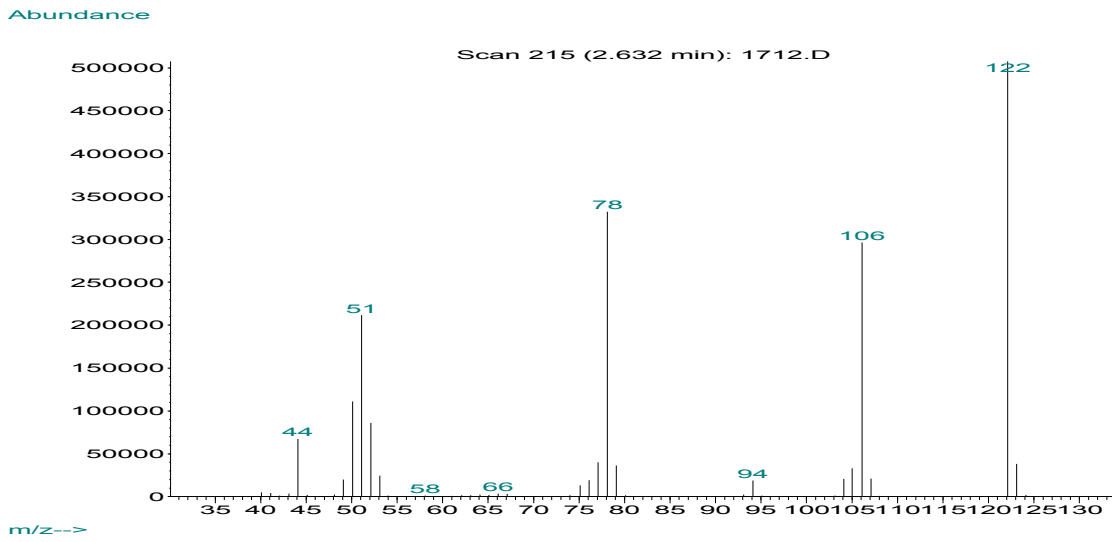


Figure 2-3. Mass spectrum of nicotinamide.

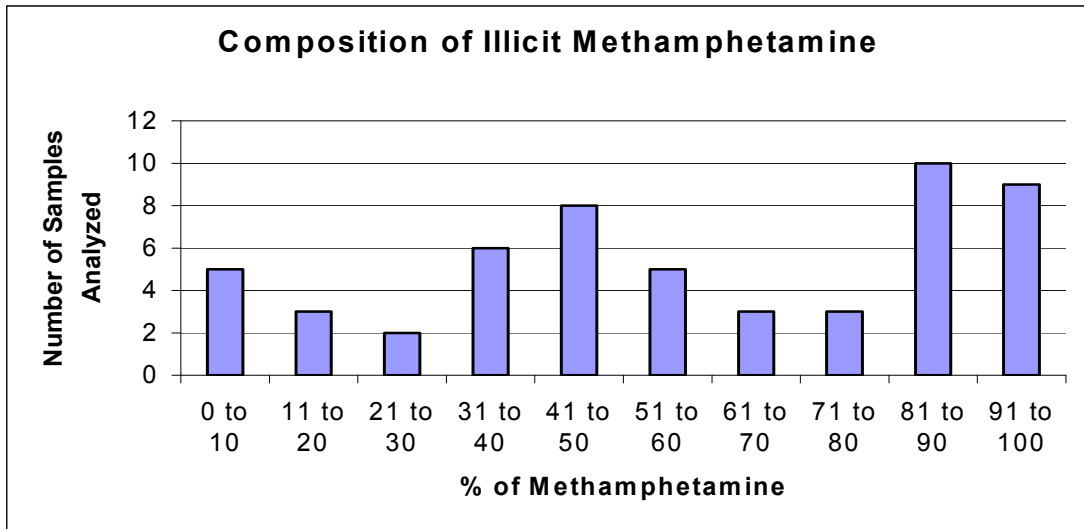


Figure 2-4. Composition and frequency distribution of illicit MA samples.

2.2 Phase solubility analysis

2.2.1 Introduction. It has been demonstrated that it is possible to increase the aqueous solubility of some lipid soluble drugs by the addition of NIC (Lim and Go, 2000). The following studies were established in order to determine if NIC could increase the aqueous solubility of MA. If so, further studies will be conducted in order to determine the effect on the kinetics and distribution of MA within the body.

2.2.2. Chemicals. MA, NIC, hydrochloric acid, ammonium hydroxide, n-butylchloride, and methanol were all purchased from the Sigma Chemical Corporation (St. Louis, MO). N-propylamphetamine was purchased from Alltech-Applied Science Laboratories (State College, PA).

2.2.3 Methods. Phase solubility analysis was carried out using a slight modification to a method published by Lim and Go (2000). Briefly, excess MA was added to a phosphate buffered saline (PBS) (pH=7.4) with a constant ionic strength or a solution of PBS that had various concentrations of NIC with an internal standard, 1 mg/ml n-propylamphetamine. Each tube was sealed, rotated for 15 minutes at room temperature, and then centrifuged for five minutes at 3000 rpm. A known amount of PBS (0.2 ml) supernatant was then drawn off and added to a tube. The pH was adjusted to 10.0 with ammonium hydroxide, 5 ml of n-butylchloride added, and the tube was rotated for 15 minutes at room temperature, and then centrifuged for 5 minutes at 3000 rpm. Finally, the organic layer was transferred to another tube containing 100 µl of methanol-HCl solution (99:1; v/v). The solution was vortexed and then evaporated to dryness under nitrogen. The residue was then reconstituted in 50 ul of methanol-

ammonia hydroxide (99:1/ v/v) and run on the GC-MS. At least five replicates were carried out for each determination at room temperature (23°C).

Each sample was analyzed utilizing a Hewlett Packard (HP) 6890 gas chromatograph (GC) connected to a HP5973 mass spectrometer (MS). A split injection (50:1) of 1 µl was made onto an Agilent Ultra 1 capillary column (12 meter x 0.2 µm x 0.33 µm). Injection port temperature 250 °C; He carrier gas, column temperature programmed to hold at 115 °C for 1.5 minutes then ramped from 115 °C to 280 °C @ 35°C/min, and then held at 280 °C for 4.21minutes

2.2.4 Results. No significant change in solubility was noted. Unlike other water/PBS insoluble substances, like halofantrine (Lim and Go, 2000), an increase in the solubility of MA in PBS by addition of NIC may not be possible. Unlike the other water insoluble drugs that have been made more soluble in aqueous solutions with NIC, MA is already extremely soluble in PBS, water, and other physiological solutions (Basalt, 1996). Since the solubility was not significantly altered, the studies herein will investigate alternate ways that NIC might alter the pharmacological profile of MA. One possible way this could be done is by either increasing or decreasing the rate of MA metabolism through the cytochrome P450 system. This will be discussed in detail in future sections.

2.3 Brain Permeability

2.3.1 Introduction. Initial *in vivo* studies were done to determine the effect NIC has on MA distribution within the rat brain. Studies will provide general information as to whether NIC increases, decreases, or does not change the amount of MA reaching the rat brain when administered i.p.

2.3.2 Chemicals. Certified 1mg/ml standards of (±)-amphetamine HCl and (±)-MA was purchased from Cerilliant (Cerilliant, Round Rock, TX). N-propylamphetamine HCl was purchased from Alltech-Applied Science Laboratories (State College, PA). Methanol, 1-chlorobutane, hydrochloric acid, sodium carbonate, ammonia hydroxide, and pentafluoropropionic anhydride (PFPA) were purchased from Sigma Chemical Corporation (St. Louis, MO).

2.3.3 Methods. Male Sprague-Dawley rats were injected i.p. with 10 mg/kg of either MA, NIC, or COMBO. After 30 minutes, they were decapitated and brains removed. The nucleus accumbens and striatum were dissected out, weighed, and added to a 10 ml screw cap glass tube. This material was homogenized with a tissue tearer and made basic (pH >10) with 1 ml of sodium hydroxide (6 M), after adding 125 ng of ISTD. The samples were then extracted with 3 ml of 1-chlorobutane on a rotary wheel for 15 minutes. The samples were then centrifuged at approximately 3000 rpm (900 g) and the organic layer transferred to a 5 ml conical screw top tube, which contained 100 µl of methanol-HCl mixture (99:1; v/v), vortexed for 15 seconds, and evaporated to dryness at 40°C under nitrogen. To the dry extract, 50 µl of PFPA was added, and then vortexed for 15 seconds, capped, and heated at 90°C for 30 minutes. After cooling, the derivatized extract was evaporated to dryness at 40°C under nitrogen. The residue was reconstituted with 50 µl of methanol-ammonium hydroxide mixture (99:1; v/v), of which 2 µl was injected into the GC-MS.

A HP6890 GC connected to a HP5973 MS was used with splitless injection of 2 µl onto an Agilent Ultra 1 capillary column (12m x 0.2 mm x 0.33 mm) injection port temperature 250 C; He carrier gas, column temperature programmed from 80-180 °C @

10/min, 180-280 °C @ 40 °C /min. The MS transfer line, 280 °C. The mass spectrometer was set up for selected ion monitoring (SIM) of 3 ions characteristic of MA at a specific retention time. The timing and selected ion components were established using full scanning mode of derivatized standards prior to optimizing the SIM and establishing the timed events (unpublished data). The amount of MA in each sample was quantified utilizing HP ChemStation Software on the GC-MS and a four point standard curve.

2.4.4 Results. The studies described above indicate that NIC decreases the amount of MA reaching the brain. As seen in figure 2-5, the amount of MA reaching the brain is significantly decreased with NIC co-administration. Utilizing an unpaired t test, statistical difference was determined between the two means with a $P = 0.0045$ (GraphPad Prism). Further studies isolating specific areas of the brain and looking at the metabolite distribution will be required to formulate a more reliable estimation about NIC's ability to alter MA distribution. Generally, kinetic studies are done on the drug of interest and its metabolites concurrently, in order to gain a better understanding as to the distribution and any significant changes that may occur, which is outside the scope of this project and will not be done herein.

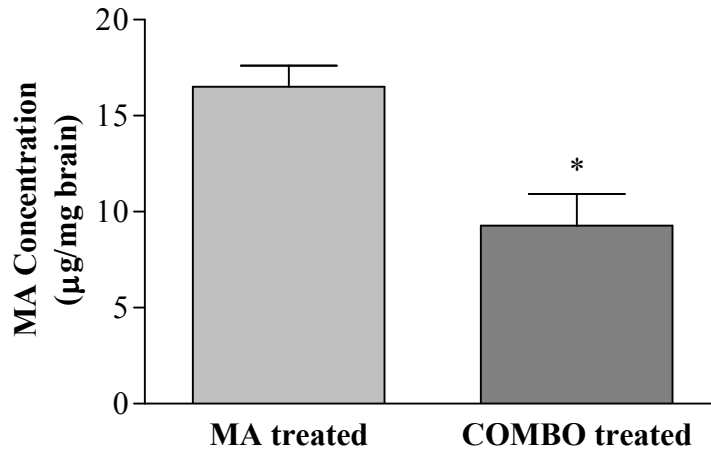


Figure 2-5. Effects of NIC on MA concentration within the brain 30 minutes post i.p. injection. Rats received one injection of either MA or COMBO and were decapitated 30 minutes post injection. Columns represent means ($\mu\text{g}/\text{mg}$ brain [original wet weight]) \pm SEM of determinations in six rats. * Values are means that differ significantly from MA treated rats ($p < 0.05$).

2.4 CYP assay development-instrument evaluation

2.4.1 Introduction. The sensitivity of the fluorescence plate scanner can profoundly affect high throughput assay performance. The fluorescent plate scanner sensitivity must, therefore, be evaluated in order to determine if the particular fluorescence plate scanner being used will provide adequate sensitivity to accurately determine the IC₅₀ values that are to be calculated from procedures proposed. An extended standard curve was performed for both fluorescent metabolite standards, CYP2D6 (AHMC) and CYP3A4 (HFC), in order to determine and confirm the linearity of the assay.

2.4.2 Chemicals. MA, NIC, and methanol were all purchased from the Sigma Chemical Corporation (St. Louis, MO). High throughput CYP2D6 and CYP3A4 inhibition assays were purchased from BD Biosciences Gentest™ (Woburn, MA).

2.4.3 Methods. The determination of the fluorescent plate scanner's sensitivity and the standard curves were generated according to the procedures described in literature contained within the high throughput enzymatic assays that were purchased from Gentest BD BioScience. These methods are also described online at <http://www.gentest.com>. Using the prepared standard curves, the excitation/emission filters were replaced one at a time and the curve rescanned. This allowed the researcher to determine the best combination of available filters to maximize the sensitivity and meet the signal to noise requirements. The high throughput assays will be discussed in detail in the materials and methods chapter.

Several assays were run according to the literature contained within the purchased high throughput enzymatic assays. This data were used to determine approximate IC₅₀

values for both the drugs of interest. Additional studies were carried out with serial dilutions of 1:2 rather than the suggested 1:3, in order to provide a more accurate measurement of the IC₅₀ values of interest.

2.4.4 Results. It was determined that the 405nm/535nm filters would be able to replace both the recommended 409nm/530nm excitation/emission wavelengths for the CYP3A4 and the 390nm/460nm for the CYP2D6 assay. Furthermore, the assay kits were run with success, evidenced by positive control curves seen in figures 2-6 and 2-7.

CYP3A4 Ketaconazole Positive Control Curve

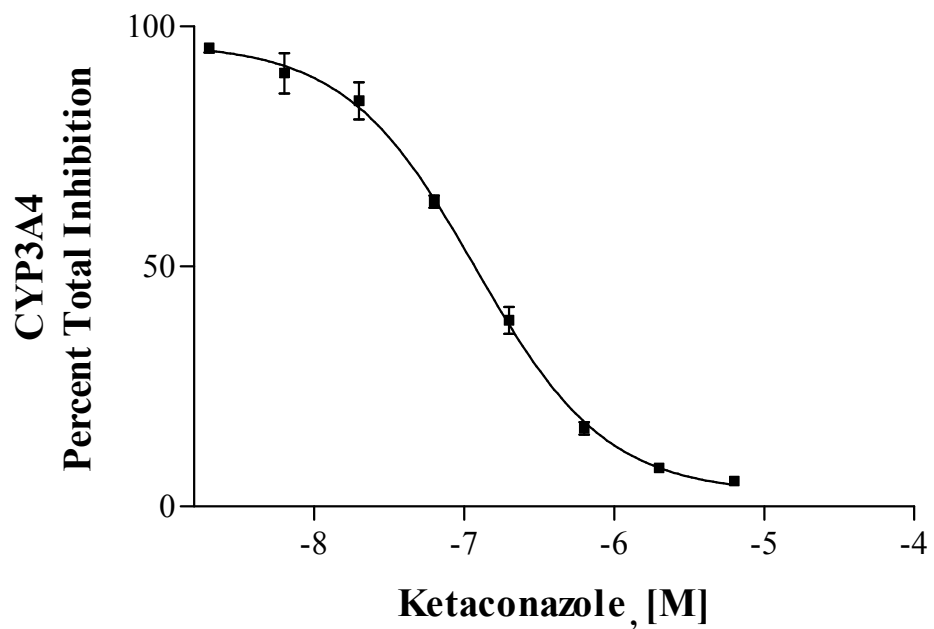


Figure 2-6. CYP3A4-Ketaconazole positive control curve. Calculated IC_{50} value of 100 nM.

CYP2D6 Quinidine Positive Control Curve

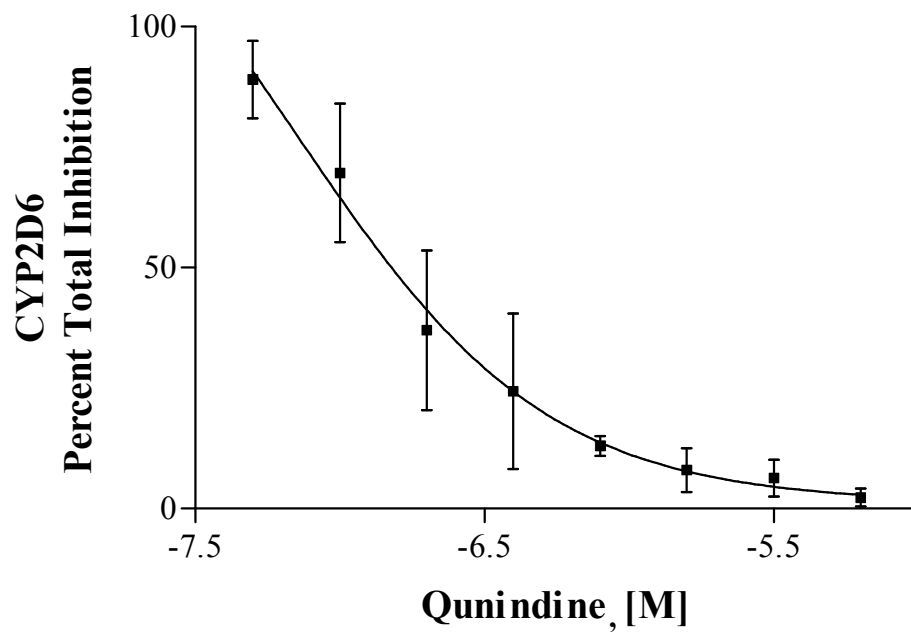


Figure 2-7. CYP2D6-Quinidine positive control curve. Calculated IC₅₀ value of 40 nM.

Chapter III

ALTERATION OF METHAMPHETAMINE METABOLISM BY CYTOCHROME P4502D ISOZYMES WHEN CO-ADMINISTERED WITH NICOTINAMIDE

3.1 Introduction.

Incidence of MA use has risen steadily from 1990 (164,000 new users) until 2000 (344,000 new users) (U.S. DHHS, 2001). Biotransformation of drugs is complex, with many drugs being metabolized by multiple enzyme systems. Generally, drug metabolism occurs in a two-step process.

The first step is Phase I biotransformation. Oxidation predominates in Phase I and is facilitated by a super family of mixed function mono-oxygenase enzymes termed cytochromes P450 or CYP. Phase II biotransformation involves some form of conjugation reaction which generally follows Phase I. CYP enzymes metabolize the majority of currently available pharmaceutical agents, including illicit MA (Lin et al. 1995; 1997). It is believed that only five of the hundreds of known CYP enzymes are responsible for approximately 95% of the hepatic CYP-mediated oxidations (Wrighton and Stevens, 1992). It is the significant involvement of CYP in that vast majority of drug metabolism that makes its mechanism of particular interest to researchers. Inhibition of CYP mediated metabolism is often of major concern when dealing with new drugs or

other drugs with narrow therapeutic ranges. Additionally, alterations within the CYP enzymes give rise to yet another mechanism to alter a drug's pharmacological response.

Previous characterization of the enzymatic nature of MA metabolism has demonstrated the urinary excretion products of this compound to include amphetamine (AMP), the demethylated product, and the 4-hydroxy derivative (4-OH MA) (Caldwell et al., 1972). These types of reactions have been shown to be catalyzed by CYP2D enzymes (Billings et al., 1978; Cho et. al., 1975; Smith, 1986). Wright et al. (1977) and Florence et al. (1982) have further documented the metabolism of AMP and MA by liver microsomes. More recently, the specific metabolism of MA in rats by CYP2D2 (Lin et al., 1995) and in humans by CYP2D6 (Lin et al., 1997) has been elucidated.

In this study, the effects of MA, NIC, and COMBO on human CYP3A4 and CYP2D6 Supersomes® and isolated rat liver microsomes at 1hrPT and 7dayPT were investigated. The metabolism of HFC or AMMC to their fluorescent metabolites was used as index reactions of CYP3A4 and CYP2D6, respectfully. The drugs screened were MA, NIC, and their COMBO.

Given the importance of drug metabolism in determining the magnitude of the effect(s) of a drug, reduced MA metabolism, due to NIC co-administration, may favorably impact the abused psychostimulant properties seen with individuals using MA. Our main objective was first to use commercially available high-throughput P450 assays to test the effects of MA, NIC, and/or COMBO exposure on the activities of the isozymes responsible for MA metabolism. Successful *in vitro* studies lead the investigators to modify the commercially available kits in order to assess these compounds utilizing

RLM. The use of RLM allowed investigators to conduct *in vivo* metabolism studies and to look at speculated liver function changes after drug administration.

3.2 Materials and methods

3.2.1 Drugs and chemicals. MA hydrochloride, NIC, and the other chemicals utilized in the buffer solutions were purchased from either Sigma Chemical Corporation (St. Louis, MO) or Fisher Chemical Company (Denver, CO). High throughput inhibitor screening (HTS) kits, CYP2D6/AMMC and CYP3A4/BFC, were purchased from Gentest Corporation BD Biosciences (<http://www.gentest.com>). Each P450 HTS kit provides all the necessary components including: insect cell microsomes (Supersomes®) prepared from human baculovirus insect cell expressing CYP3A4 or CYP2D6 individual cytochromes, fluorescent P450 substrate, positive control inhibitor, metabolite standard, NADPH regenerating system, reaction buffer, stop reagent, control membrane protein, and instructions for measuring IC₅₀, which can be calculated and used as a basis for comparison between a series of tested components.

3.2.1.1 HTS Kit Substrates. A nonfluorescent probe 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), which produces a fluorescent metabolite, 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC), was used with human baculovirus cell expressed CYP2D6 Supersomes® and rat liver microsomes.

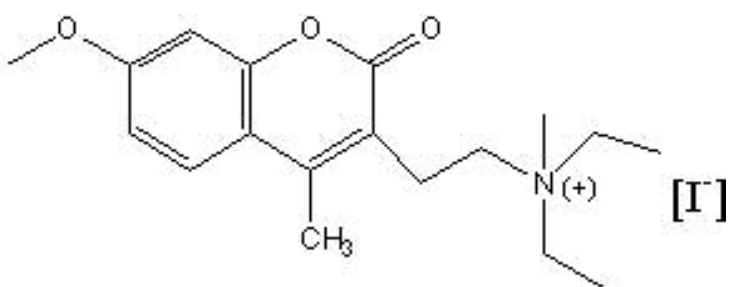


Figure 3-1. AMMC structure. AMMC is demethylated by human CYP2D6 to the fluorescent product AHMC.

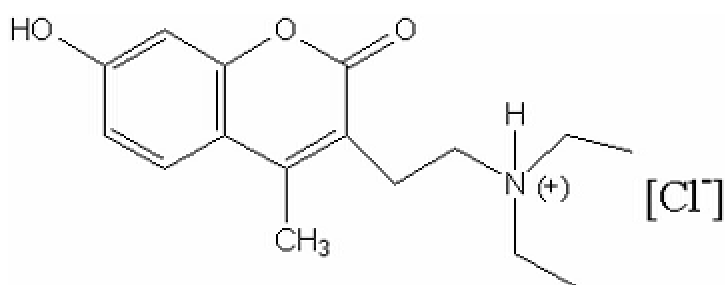


Figure 3-2. AHMC structure. AHMC is the fluorescent product of AMMC demethylation by CYP2D6.

The nonfluorescent 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC) is dealkylated by CYP3A to 7-Hydroxy-4-(trifluoromethyl)-coumarin (HFC) and was used with human baculovirus cell expressed CYP3A4 Supersomes®. Both AMMC and BFC were included in the purchased HTS kits from GENTEST Corp. and BD Biosciences (<http://www.gentest.com>).

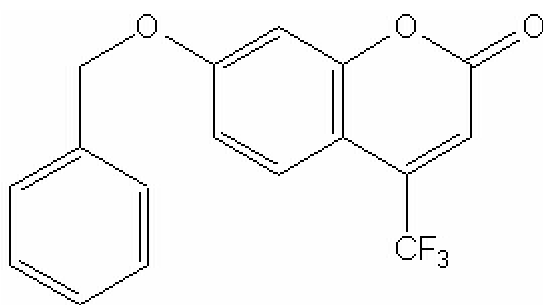


Figure 3-3. BFC structure. BFC is dealkylated by human CYP3A to the fluorescent product 7-HFC.

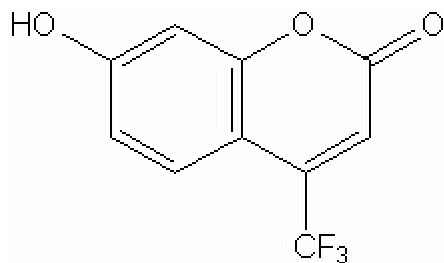


Figure 3-4. 7-HFC structure. 7-HFC is the fluorescent product of BFC demethylation by CYP3A4.

3.2.2 *In vitro* inhibition study. Methanol was used to make stock solutions of MA, NIC, and the COMBO. CYP2D6/AMMC and CYP3A4/BFC high throughput inhibitor screening kits were purchased from BD Gentest (<http://www.gentest.com>). These commercially available kits utilize Supersomes[®], which have baculovirus-insect-cell-expressed human cytochromes P450.

The CYP3A4/BFC experiments were carried out as described in the kit's instruction sheet. The CYP2D6/AMMC assay followed the kit instructions with only a few modifications. Briefly, assays were conducted in black 96-well top reading plates (Corning Costar, Cambridge, MA). Each set of 12 wells in a row was used for one inhibition curve. NADPH-Cofactor mix (0.19 ml) was added to well 1 of each row and 0.1 ml to each of the remaining wells 2-12. Ten microliters of one of the drug stock solutions or the positive control solution was added to well 1 of each row. Rows 1 and 2 were positive control, 3 and 4 MA, 5 and 6 NIC, and 7 and 8 were the COMBO drug combination. Serial dilution of 0.1 ml was made starting in column 1 and carried out through column 8 for each of the rows. The excess 0.1 from well 8 was removed and discarded. Wells 9 and 10 contained no inhibitor and rows 11 and 12 were blanks for fluorescence (stop solution was added before the enzyme).

After the substrate and inhibitor addition, the plates were prewarmed to 37°C. Incubations were initiated by the addition of 0.1 ml prewarmed enzyme/substrate mixes to all wells but rows 11 and 12 (final incubation volume of 0.2 ml). Incubations were carried out for 30 minutes (CYP3A4) and for 60 minutes (CYP2D6) at which point a stop solution was added.

After addition of stop solution, prewarmed enzyme/substrate mix was added to wells 11 and 12 in all rows. Crespi et al. (1997) previously demonstrated linear metabolite formation for these two assay procedures. Fluorescence in each well was measured using a Bio tek Synergy HT plate reader (Bio tek Instruments, Winooski, Vermont, U.S.A). The CYP3A4 metabolite, HFC, was measured at excitation/emission wavelengths of 405nm/530nm and the CYP2D6 metabolite, AHMC, at 405nm/535nm.

3.2.3 *In vivo* inhibition study.

3.2.3.1 Animals. Male Sprague-Dawley rats (Harlan Sprague-Dawley Laboratories, Madison, WI: 200-300 g) were group housed and allowed access to food and water ad libitum in a room with temperature control ($23\pm 2^{\circ}\text{C}$) and a 12-hour light:dark cycle. The rats were transferred to individual cages for the initial injection regimens and remained individually housed for the duration of the experiment, one hour or seven days post last injection (1hrPT or 7dayPT, respectfully). Over the course of the experiment, all animals experienced the same environmental conditions.

3.2.3.2 Injections. Rats received one intraperitoneal (i.p.) injection every two hours for a total of three injections. Each injection was made in the lower left quadrant of the rat abdomen. This area was chosen because other than the small intestines there are no vital organs present in the vicinity. In contrast, the lower right abdomen additionally contains the caecum, and the upper abdominal area houses the liver, spleen, and stomach. A commercially available restraining device was used in order to prevent the unnecessary stress of prolonged handling.

3.2.3.3 Drug treatment. Rats were randomly assigned to one of four treatment groups: The first group received three injections of MA (5 mg/kg/injection, i.p.); the second group received three injections of NIC (5 mg/kg/injection, i.p.); the third group received three injections of a (50:50) MA:NIC combination (5 mg of MA and 5 mg NIC/kg/injection, i.p.); and the control group received three 0.1 ml saline injections (i.p.) All drug concentrations were calculated as the free base. Additionally, the concentration of MA in the MA and COMBO treatment groups was the same, 5mg/kg/injection.

Drug solutions were prepared in isotonic saline at a concentration of 10 mg/ml; rats would receive between 0.1 and 0.15 ml of solution per injection, depending upon their weight. In order to simplify the dosage calculation, rats weighing from 200-233 gm received 0.1 ml, 234-266 g received 0.12 ml, and 267-300 gm received 0.15 ml of the prescribed drug solution. This resulted in the administration of approximately 5mg/kg \pm 0.7mg/kg of rat per injection. Upon completion of drug treatment, 1hrPT or 7dayPT, rats were euthanized utilizing carbon dioxide, and decapitated with the brains removed for dissection (see chapter 4) and the livers removed as described below.

3.2.3.4 Liver dissection. Upon completion of drug treatment, 1hrPT and 7dayPT rats were euthanized, decapitated after brief exposure to a CO₂ chamber, and a partial hepatectomy was performed. The rat liver is composed of four lobes with the median and left lateral lobes comprising approximately 70% of the liver. The third lobe is the right lateral lobe that partially overlaps the median lobe on the right side. The smallest lobe is the caudate lobe, which surrounds the abdominal portion of the esophagus. The liver lobes are bound together by folds of the peritoneum that constitute the suspensory ligaments of the lobes (Waynforth and Flecknell, 1992). The two largest lobes of the

liver (median and left lateral lobes) were harvested from each rat. These weighed on average approximately 10 gm together.

In order to obtain the described liver lobes, a midline ventral abdominal skin incision was made extending from the xiphoid cartilage about half the distance towards the base of the tail. A similar incision was made in the abdominal muscles. The rat was positioned with a small bolster under the thorax causing the liver to fall away from the diaphragm. The suspensory ligament attaching the liver to the diaphragm--the falciform ligament--was cut toward the posterior vena cava with blunt-ended scissors. At this point, the median and left lateral lobes were retracted from the rats' abdominal cavity with two gloved fingers and severed with a pair of blunt-ended scissors. The two lobes were placed in a 50 ml plastic tube and stored frozen (-80°C) until liver enzyme studies were performed at a later date.

3.2.3.5 Microsomal preparation. Microsomes were prepared from excised Sprague-Dawley rat livers at 1hrPT or 7dayPT. Microsomal fractions were prepared as described in Nelson et al. (2001) with slight modifications. Briefly, frozen (-80°C) rat livers weighing approximately 10 g were thawed in homogenizing buffer (0.1 M potassium phosphate (pH 7.4) and 0.25 M sucrose), minced with scissors, and then placed in 30 ml of homogenization buffer. Homogenization was completed with 10 strokes at 900 rpm using a mechanically driven Teflon pestle in a glass homogenizer.

Nuclei and mitochondria were removed by centrifugation at 9,000 g for 20 min in a Beckman Avanti J-25 refrigerated centrifuge at 4°C using a Beckman JA-14 rotor. The homogenate was transferred to a 50 ml centrifuge tube and spun at 100,000 g for 60 min in a Beckmen L7-55 refrigerated centrifuge using a Beckman Ti-45 rotor. The

microsomal pellet was resuspended in 20 ml of incubation buffer (0.1 M potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 5% glycerol) and used immediately, or stored frozen (-80°C) until time of assay. Florence et al. (1982) demonstrated that the activity of washed microsomes could be stored at – 80°C for up to 30 days without loss of activity.

3.2.3.6 Protein analysis. Protein analysis was completed with the commercially available BioRad Protein Assay kit from BioRad (BioRad Life Science Group, Richmond, CA). The BioRad Protein Assay is based on the method of Bradford (1976) and is a simple and accurate procedure for determining the concentration of solubilized protein. It involves the addition of an acidic dye (Coomassie® Brilliant Blue G-250) to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve of bovine serum albumin (BSA) provides a relative measurement of protein concentration. Each synaptosomal and microsomal solution used in experimentation was assayed for protein concentration for standardization.

3.2.3.7 Inhibition studies. The 96-well plate assay, as described above, was used to evaluate the inhibitory potential of MA, NIC, COMBO, or saline on drug regimen treated rats using the inhibition of a very well characterized CYP2D6 inhibitor and AMMC as a probe. Livers from 4 rats in each group were pooled to form a rat liver microsomal (RLM) preparation. Pooled RLM were used in order to test all components under identical and reproducible conditions. The above prepared RLM from 1hrPT and 7dayPT were utilized in the high throughput inhibitor screening kits instead of the human

CYP2D6 Supersomes®. Quinidine, a well-characterized CYP2D6-selective inhibitor, was used as the appropriate inhibitor for enzyme investigation.

In addition, the CYP2D6-selective fluorescent probe AMMC was used to quantify enzyme metabolism. This probe displayed high selectivity in rat CYP2D2 isoform for AMMC demethylation (a substrate selective for CYP2D6 in human liver microsomes) (Stresser et al., 2002). By utilizing the CYP2D6 selective probe and inhibitor (AMMC and quinidine, respectfully), the investigators did not feel it necessary to isolate and quantify the amount of specific CYP isoforms present in the RLM.

Multiple inhibitor concentrations were tested to generate quantitative inhibition parameters. Inhibition curves were generated by the amount of CYP3A4, CYP2D6, or RLM enzyme mediated fluorescent product generated (RFU) per microgram of protein expressed as a percentage of the control sample plotted against the log concentration of inhibitor. Human baculovirus cell expressed CYP3A4 and CYP2D6 Supersomes data represents the average of samples run in duplicate with an N = 6. Each value in the rat microsomal studies represents an N = 4 RLM pooled and run in quadruplicate.

3.2.3.8 Statistical Analyses. The enzyme parameters used to evaluate inhibition are the IC_{50} values (inhibitor concentration that causes a reduction in enzyme activity by 50%). Acquired data from the HTS assays was exported as Excel spreadsheets and analyzed using nonlinear regression to determine IC_{50} values. One-way ANOVA with Bonferroni post tests, or Dunnetts post test was performed on the calculated IC_{50} values in the CYP3A4 and CYP2D6 Supersome® studies, and two-way ANOVA with Bonferroni post tests were used to compare the rat liver microsome studies IC_{50} values.

All statistical analysis was done using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, <http://www.graphpad.com>.

3.3 Results

3.3.1 Inhibition of human CYP3A4 Supersomes®. CYP3A4 high throughput inhibition assay kits were used to rapidly screen for potential inhibition of CYP3A4 catalytic activity. The 96 well plate assay, as described above, was used to evaluate the inhibition potential (4 μ M to 10 mM) of MA, NIC, and their COMBO using BFC as a probe. The human CYP3A4 Supersomes studies yielded a net fluorescence signal in the range of 3000-4000 relative fluorescence units (RFU). The effects of MA, NIC, or the COMBO on BFC dealkylase activity studied using human baculovirus insect cell, expressed CYP3A4 isozymes yielded the following calculated IC_{50} values 6 mM, >10 mM, 5 mM, respectfully (figure 3-5). These values compared to the ketoconazole IC_{50} (0.1 μ M) suggest little to no inhibition within physiologically relevant drug concentration ranges. The observed IC_{50} for ketoconazole is in agreement with other reported IC_{50} values [0.08 μ M] in similar high throughput studies (Crespi et al., 1997) and human liver microsomal studies [0.2 μ M] (Baldwin et al., 1995).

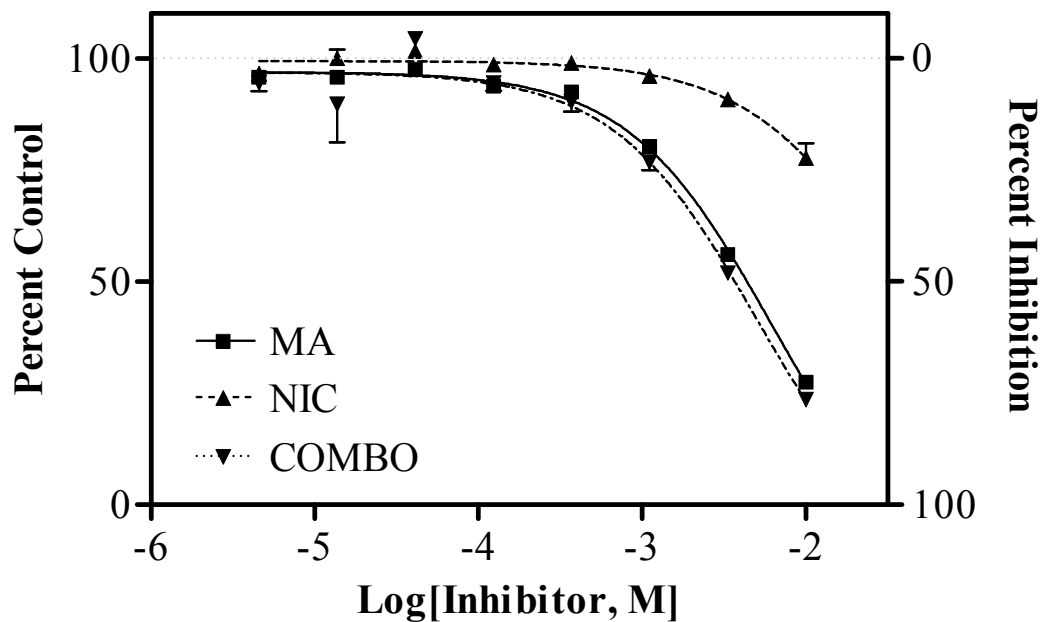


Figure 3-5. CYP3A4 Supersome inhibition curves. Representative inhibition curves (4 μ M to 10 mM) for MA, NIC, and COMBO using BFC as a probe. Results are expressed as Percent Control (RFU/mg protein) and Percent Inhibition (RFU/mg protein) with the mean \pm S.E.M of six experiments performed in duplicate. Curves corresponding to the MA and COMBO inhibition curves were found to be significantly different from the NIC inhibition curve ($P < 0.001$); however, there was no difference seen between the MA and COMBO inhibition curves.

3.3.2 Inhibition of human CYP2D6 Supersomes®. CYP2D6 high throughput inhibition assay kits were used to rapidly screen for potential inhibition of CYP2D6 catalytic activity. The 96 well plate assay, as described above, was used to evaluate the inhibition potential (1.4 μM to 180 μM) of MA, NIC, and their COMBO using AMMC as a probe. The human CYP2D6 Supersomes® studies yielded a net fluorescence signal in the range of 3000-4000 relative fluorescence units (RFU). The results yielded the following calculated IC_{50} values 31 μM , > 180 μM , 40 μM , respectfully (figure 3-6). These values compared to quinidine IC_{50} (40 nM), a potent CYP2D6 inhibitor, suggest the possibility that MA and COMBO treatments may possess an inhibitory potential within a physiologically relevant drug concentration range which is in the nanomolar to micromolar range (Basalt, 2000). The observed IC_{50} of quinidine is in agreement with other reported IC_{50} values utilizing high throughput studies for quinidine inhibition of CYP2D6 [10 nM] (Crespi et al., 1997).

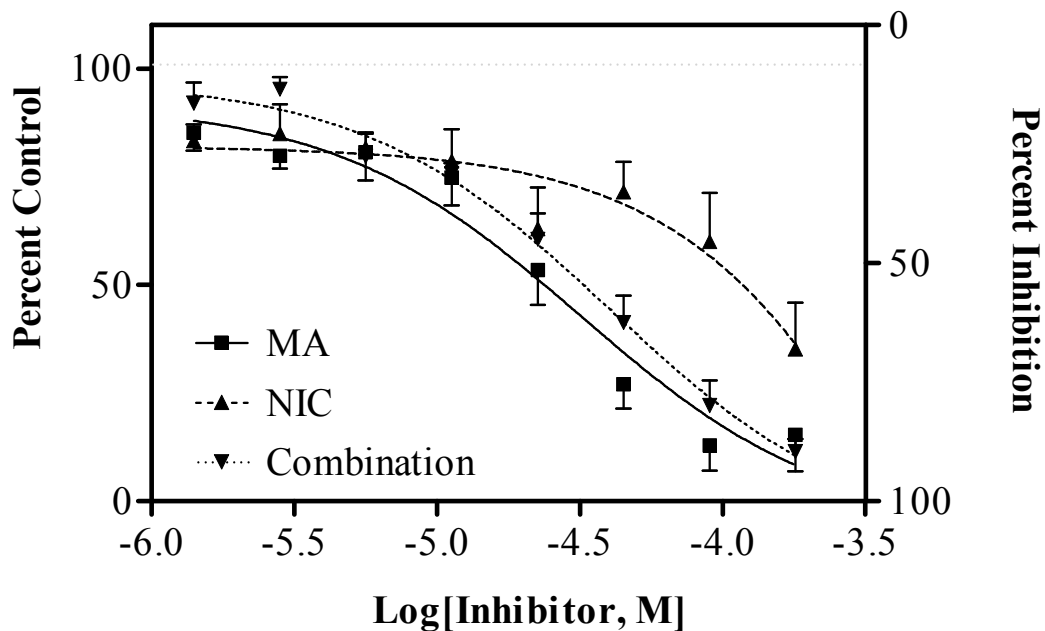


Figure 3-6. CYP2D6 Supersome inhibition curves. Representative inhibition curves (1.4 to 180 μ M) for MA, NIC, and COMBO using AMMC as a probe. Results are expressed as Percent Control (RFU/mg protein) and Percent Inhibition (RFU/mg protein) with the mean \pm S.E.M of six experiments performed in duplicate. Curves corresponding to the MA and COMBO inhibition curves were found to be significantly different from the NIC inhibition curve ($P < 0.001$); however, there was no difference seen between the MA and COMBO inhibition curves.

3.3.3 Inhibition of rat microsomes 1 hour and 7 days post treatment regimen.

CYP2D6 high throughput inhibition assay kits were modified as described above and used to determine the inhibition of CYP2D6 catalytic activity in RLM. The 96 well plate assays were used to evaluate the inhibitory potential (3.9 to 500 nM) of MA, NIC, and COMBO treated rat livers using AMMC as a probe. The liver microsome studies yielded a net fluorescence signal in the range of 5000-6200 relative fluorescence units (RFU). The results of the 1hrPT rat liver microsomes yielded the following calculated IC_{50} values for saline, MA, NIC, and COMBO: 103 μ M, 0.8 μ M, 86 μ M, and 32 μ M, respectively (figure 3-7). The results of the 7dayPT rat liver microsomes yielded the following calculated IC_{50} values for saline, MA, NIC, and COMBO: 110 μ M, 11 μ M, 75 μ M, and 57 μ M respectively (figure 3-8). The findings from both of the RLM studies can be seen in figure 3-9. A summary of these IC_{50} values along with the previous sections IC_{50} values are summarized in table 3-1.

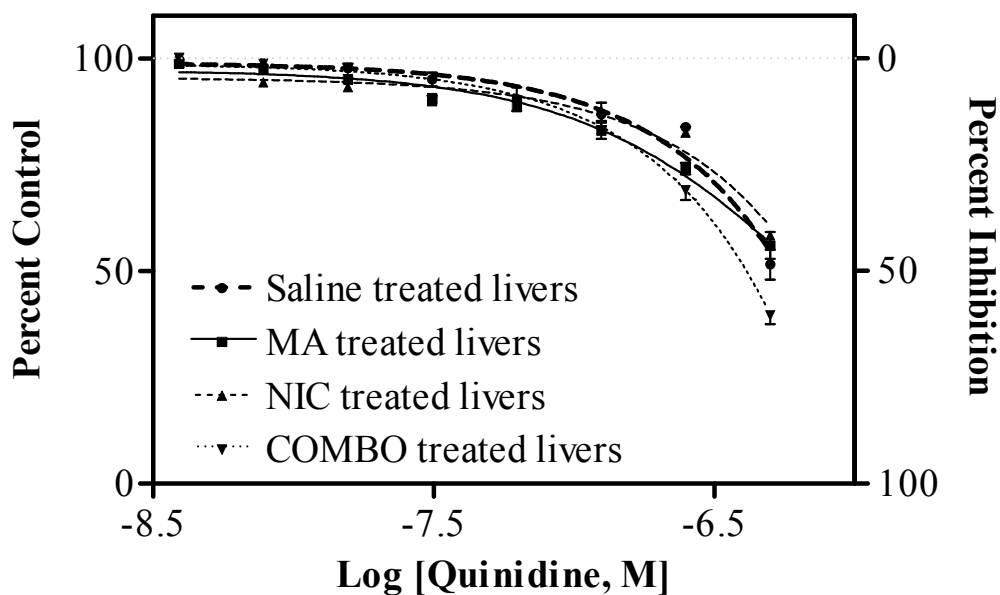


Figure 3-7. Rat Liver Microsomes 1 Hour PT inhibition curves. Representative quinidine inhibition curves (3.9 to 500 nM) were used to evaluate the metabolic potential of MA, NIC, and COMBO treated rat livers using AMMC as a probe. Results are expressed as Percent Control (RFU/mg protein) and Percent Inhibition (RFU/mg protein) with the mean \pm S.E.M of four experiments performed in duplicate. Curves corresponding to the MA ($P < 0.001$) and COMBO ($P < 0.05$) inhibition curves were found to be significantly different from the saline treatment group's inhibition curve. There was no difference seen between the NIC and Saline or between the MA and the COMBO treatment groups.

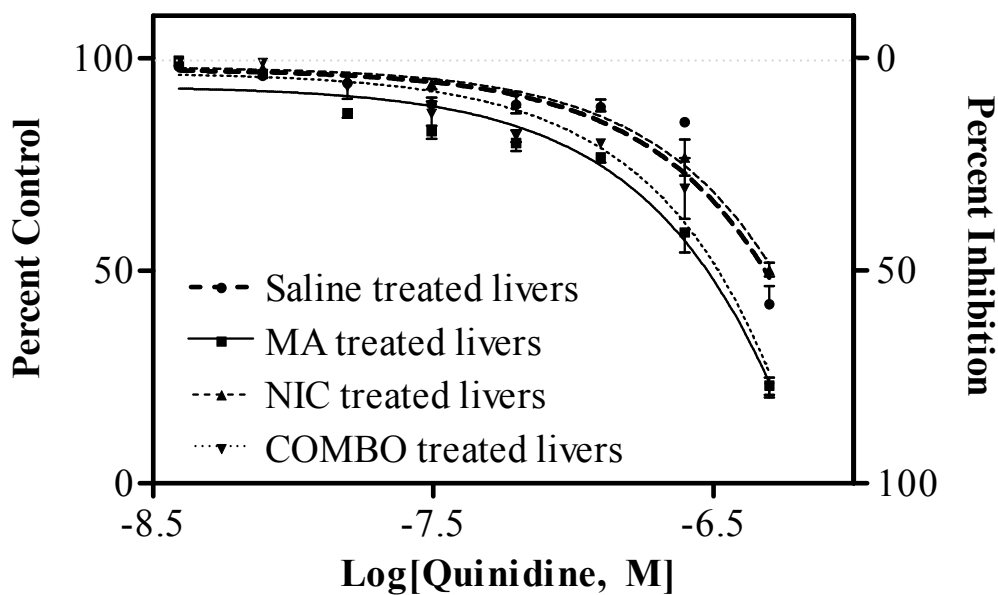


Figure 3-8. Rat Liver Microsomes 7 days PT inhibition curves. Representative quinidine inhibition curves (3.9 to 500 nM) were used to evaluate the metabolic potential of MA, NIC, and COMBO treated rat livers using AMMC as a probe. Results are expressed as Percent Control (RFU/mg protein) and Percent Inhibition (RFU/mg protein) with the mean \pm S.E.M of four experiments performed in duplicate. The curve corresponding to the MA ($P < 0.01$) was found to be significantly different from the saline treatment group's inhibition curve. Additionally, the MA and COMBO treatment groups were found to be significantly different ($P < 0.01$) as the COMBO group returned to levels similar to the Saline control values. There was no difference seen between the COMBO or NIC groups and Saline.

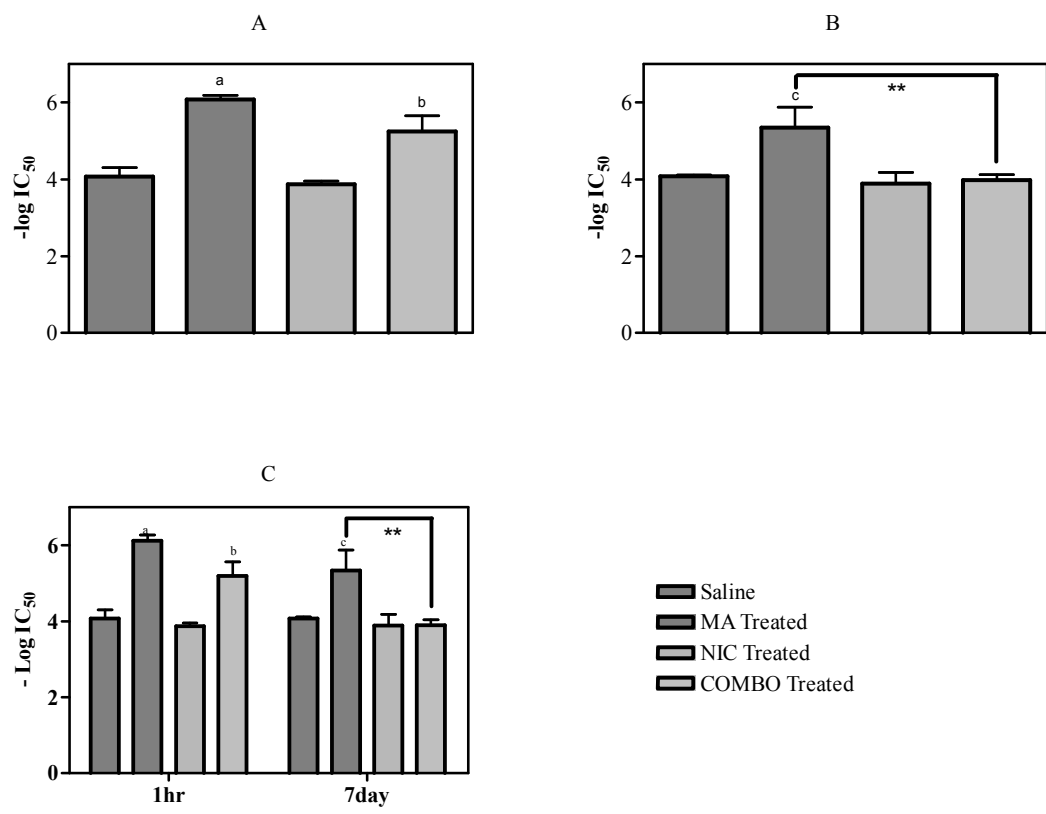


Figure 3-9. IC_{50} values ($-\log IC_{50}$) from rat liver microsome studies. A) 1hrPT RLM studies (B) 7dayPT RLM studies (C) Both 1hrPT and 7dayPT values. The above-calculated IC_{50} values were determined from inhibition curves seen in figures 3 and 4. IC_{50} values were significantly different than saline control value (a $p < 0.001$; b $p < 0.01$; c $p < 0.05$) and from each other as indicated (** $p < 0.01$) and evaluated by ANOVA (n=4).

Table 3-1. 50% inhibitory concentrations (IC₅₀). The listed IC₅₀ values were determined from inhibition curves seen in figures 1-4. The IC₅₀ values of MA and COMBO were significantly different than NIC (* p < 0.001) as evaluated by ANOVA (n=6). The IC₅₀ values were significantly different than saline control values (^a p < 0.001; ^b p < 0.01; ^c p < 0.05) and each other as indicated (** p < 0.01) and evaluated by ANOVA (n=4).

CYP3A4- Supersomes

Methamphetamine	6 ± 0.5 mM*
Nicotinamide	>10 mM
Combination	5 ± 0.6 mM*

CYP2D6- Supersomes

Methamphetamine	31 ± 4.1 μM*
Nicotinamide	>180 μM
Combination	40 ± 6.0 μM*

Rat Liver Microsomes-1 Hour Post Treatment

Saline	103 ± 20.0 μM
Methamphetamine	0.8 ± 0.25 μM ^a
Nicotinamide	86 ± 8.70 μM
Combination	32 ± 28.0 μM ^b

Rat Liver Microsomes-7 Days Post Treatment

Saline	110 ± 10.0 μM
Methamphetamine	11 ± 8.40 μM ^{c, **}
Nicotinamide	75 ± 10.0 μM
Combination	97 ± 3.70 μM ^{**}

3.4 Discussion

The elimination of foreign compounds from the body and the control of levels of other compounds in the body, such as drugs, is known as metabolism. Metabolism of drugs is complex, with many drugs being metabolized by multiple enzyme systems. Enzymes involved in metabolism are present in many tissues (gastrointestinal tract, lung, nasal mucosa, kidney, blood, skin, and blood cells) within the body; however, the liver is the principal site of drug metabolism.

For many drugs, metabolism occurs in two stages. Phase I reactions are catalyzed by a super family of mixed function monooxygenase enzymes, known as the cytochrome P450 system. This occurs in liver microsomes, located in the endoplasmic reticulum of liver cells or hepatocytes, where the drug is made more hydrophilic by the formation of a new or modified functional group or a cleavage. Most of the time, these simple modifications are enough to increase solubility facilitating elimination through the kidneys (in urine) and the liver (in bile). Phase II metabolism involves conjugation and many times occurs after the alteration by Phase I enzymes. Acting in concert, Phase I and Phase II enzymes convert hydrophobic compounds to more hydrophilic compounds that can be readily eliminated.

The Phase I metabolism of MA by CYP-mediated enzymes generates two pharmacologically active metabolites, amphetamine (Basalt, 2000) and the para-hydroxy derivative (p-OH MA) (Caldwell et al., 1972). CYP2D enzymes have been shown to catalyze these types of reactions (Billings et al., 1978; Cho et. al., 1975; Smith, 1986). N-Demethylation of MA has been reported to occur primarily by CYP enzymes (Baba et

al., 1988), but may also occur via flavin-containing monooxygenases (Yamamda et al., 1984; Lawton et al., 1994).

Upon administration, MA is continually eliminated in an unchanged form. Under normal circumstances, up to 43% of a dose is eliminated unchanged in the 24-hour urine, with less than 20% cleared as amphetamine (Basalt, 2000). Contrary to most belief, amphetamine is not the major metabolite of MA, primarily, because it accounts for less than 20% of the administered MA (Cho, 1990); it is however the major active metabolite. About 15% of the MA is excreted as p-OH MA and the remainder of the dose is accounted for as minor amounts of the same metabolites found after amphetamine administration, such as phenylacetone, norephedrine, benzoic acid, hippuric acid, benzoylglucuronide, p-hydroxynorephedrine, and p-hydroxyamphetamine (Caldwell et al., 1972; Cho, 1990).

The fairly complex metabolism and excretion of MA in humans result in a fairly long half-life (approximately 10-12 hours) (Cho, 1990; Cho et al., 2001; Shappell et al., 1996). Urinary excretion of the unchanged drug is pH dependent. Urinary acidification decreases half-life and alkalinization increases the half-life. For every one-unit increase in urinary pH, there is an average seven-hour increase in plasma half-life (Drug Facts and Comparison, 1997). During urinary acidification, plasma elimination is shortened, and total clearance is increased as well with amphetamine and MA (Beckett and Rowland, 1965). Though the elimination half-lives in humans and rats are quite different, they do share similarity and predictability as discussed by Cho (1990). Furthermore, dosing regimens can be modified in rats to mimic human abuse patterns, which have been utilized in these studies.

The aim of this study was to evaluate the inhibitory effects of MA, NIC, and COMBO utilizing a novel and selective high throughput assay which utilized recombinant human CYP3A4 Supersomes®. The human CYP3A4 Supersomes® inhibition curves can be seen in figure 3-5 and the results of the calculated IC₅₀ in table 3-1. Our findings indicate that neither MA, NIC, nor the COMBO drug group affect the metabolism of BFC a specific probe used to assess CYP3A4 metabolism.

The second set of studies was a repeat of the first, except the assays utilized recombinant human CYP2D6 Supersomes®. The 96 well plate assay, as described above, was used to evaluate the inhibitory potential of MA, NIC, and COMBO using AMMC, a highly selective probe toward CYP2D6 enzymes (Crespi et al., 1999). It has been demonstrated by Chauret et al. (2001) that CYP2D6 is the only observable human P450 enzyme involved in the metabolism of AMMC. All other human CYP450 enzymes tested yielded <1% 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC) fluorescent metabolite relative to CYP2D6. These observations imply minimal contribution of other enzymes to AMMC demethylation to AHMC under similar conditions. The human CYP2D6 Supersomes® inhibition curves can be seen in figure 3-6 and the results of the calculated IC₅₀ values in table 3-1. The results yielded the following calculated IC₅₀ values 31 μM, > 180 μM, 40 μM, respectfully.

Statistical analysis utilizing a one-way ANOVA with Bonferonni post tests indicated significant drug effects ($F_{(2,43)} = 0.3108$; $p < 0.001$) for NIC vs. MA and COMBO. In summary, MA and COMBO were significantly different from NIC, which is as expected because it appears CYP2D6 is not a primary metabolizing enzyme of NIC. Additionally, there was no significant difference demonstrated between MA and

COMBO. At this time, there is no indication of NIC altering the metabolism of MA via the human CYP2D6 isozyme.

It has been hypothesized that there is an added or synergistic inhibitory interaction with MA and NIC co-administration. If the metabolism of the affected drug were decreased, this would indicate an increase in intensity and increased duration of drug effects. These interactions have been studied and will be discussed. Levy hypothesized that all drugs that are metabolized to a significant degree by the same enzyme are inhibited by inhibitors of that enzyme and display the same spectrum of interactions (Levy, 1995). According to Levy's hypothesis, if NIC is metabolized by the CYP2D6 enzyme system it could potentially interact with other CYP-catalyzed metabolism.

The recombinant human CYP2D6 Supersomes® studies did not identify any inhibitory interaction between NIC and CYP2D6. Therefore, one would not speculate any combined effects. IC₅₀ values of >10 mM (CYP3A4 Supersomes) and > 180 μM (CYP2D6 Supersomes) indicate no direct inhibitory effect on CYP3A4 or CYP2D6 activity. These results clearly demonstrate that direct inhibition of CYP3A4 or CYP2D6 by NIC is not the source of an interaction. Additional studies are necessary to determine if an alteration of CYP expression may be responsible for their interaction. Drug interactions involving enzyme induction are not as common as inhibition-based interactions; the most common enzyme induction mechanism is transcriptional activation leading the increased synthesis of CYP proteins (Dossing et al., 1983).

During the final portion of these studies, rats were injected with one of four drug treatment regimens, as described above, and 1hrPT or 7dayPT the rat livers were harvested and microsomes were isolated for the present studies. Unlike humans, which

express a single CYP2D enzyme (CYP2D6), six CYP2D isoforms (CYP1/2/3/4/5/18) have been identified in rats (Gonzalez et al., 1987; Matsunaga et al., 1989,00; Kawashima et al., 1996).

Stressor et al. (2002) investigated human and rat isoform catalytic selectivity among a panel of fluorescent substrates. In these studies, they concluded that rat CYP2D2 and human CYP2D6 displayed complete selectivity with low concentrations of AMMC with little or no activity contributed from CYP3A isoforms, CYP1A2, or extrahepatic enzymes, thereby, making AMMC an appropriate probe to investigate human CYP2D6 mediated reactions in rats via the rat CYP2D2 isozyme. Additionally, Chauret et al. (2001) has published AMMC as a selective probe for CYP2D6 in human liver microsomes.

The 1hrPT inhibition curves can be seen in figure 3-7 and the results of the *in vivo* calculated IC₅₀ values in table 3-1. A two-way ANOVA (time by treatment group) of the rat liver microsomal studies demonstrated significant time and treatment affect, ($F_{(1,24)} = 6.14$; $p = 0.02$) and ($F_{(3,24)} = 16.99$; $p < 0.0001$) respectfully; however, they failed to show an overall interaction within the ANOVA, ($F_{(3,24)} = 2.43$; $p = 0.09$).

Rat liver microsomal studies demonstrated significant inhibition as compared to saline control values in the 1hrPT MA ($p < 0.001$) and 1hrPT COMBO ($p < 0.05$). Additional analysis with a one-way ANOVA and Newman-Kuels (NK) post hoc testing demonstrated a significant difference between the MA and COMBO treated groups ($p < 0.05$). However, there was no statistical difference observed between the 1hrPT MA and COMBO treated RLM groups utilizing the two-way ANOVA with Bonferroni post

hoc testing. This most likely is due to the conservative nature of the Bonferroni test when used in a two-way ANOVA.

The 7dayPT inhibition curves can be seen in figure 3-8 and the results of the *in vivo* calculated IC₅₀ values in table 3-1. Rat liver microsomal studies demonstrated significant inhibition in the 7dayPT MA group (p<0.05); no difference was seen between the COMBO and the saline control group RLM as seen in the 1hrPT studies. There was in fact a significant difference demonstrated between the MA and COMBO treated groups (p<0.01) illustrating the differential effects 7dayPT when MA is co-administered with NIC.

Graphical representation of the IC₅₀ values (-logIC₅₀ vs. time) can be seen in figure 3-9. The 1hrPT studies demonstrated a 50% inhibition of log IC₅₀ values, which is represented by 100-fold difference between the MA and saline IC₅₀ values and a 30% inhibition of log IC₅₀ values, which is represented by 3 fold difference between the COMBO and saline IC₅₀ values. The 7dayPT studies demonstrated a 35% inhibition of log IC₅₀ values, which is represented by 10-fold difference between the MA and saline IC₅₀ values.

The observation of the COMBO treated group liver functions returning to control level indicate that the CYP2D-mediated liver functions had returned to the normal saline control level. Based upon these observations, one can infer that the NIC treatment may enhance the return of CYP2D-mediated metabolic function to normal. One should note that although statistical significance was not demonstrated between the MA and COMBO group in the 1hrPT studies when using the two-way ANOVA with Bonferroni post hoc analysis. There was an apparent difference in their inhibitory ability represented by a

15% inhibition of log IC₅₀ values, which is represented by a 30 fold difference between the two treatments IC₅₀ values. This difference was statistically significant when analyzed with a more liberal test. One could speculate that the inhibitory effect of MA *in vivo* is blunted when co-administered with NIC and substantiated statistically with the more appropriate test. A comparison of the two statistical methods utilized about would demonstrate that the Bonferroni is a much more conservative test especially when utilized in a two-way ANOVA. Utilizing the one-way ANOVA with a more liberal post hoc test and finding a significant difference between the MA and COMBO treated groups at the 1hrPT time point would bring up two questions; one, statistical vs. clinical significance and two, liberal vs. conservative testing. To be conservative one must infer that there is no significant difference seen with the combination of MA and NIC at 1hrPT; however, additional testing could be done to establish the clinical relevance. A 30 fold difference in IC₅₀ value is a relatively large difference and may possess some clinical significance.

In order to explain these finding one may consider the possibility that NIC serves to increase transcriptional activation of the CYP2D6 (human) and/or CYP2D2 (rat) enzymes leading to the increased synthesis of CYP proteins leading to decreased intensity and shorten the duration of drug effects as described by Dossing et al. (1983). This type of alteration could explain the return of CYP2D function seen at 7dayPT as compared to the MA treatment group, which is still experiencing inhibition.

NIC has been shown to block lesions produced by the mitochondrial toxin malonate (Beal et al., 1994). NIC has also been shown to attenuate MA toxicity (Huang et al., 1997; Stephans et al., 1998). Because NIC is the precursor molecule for the electron carrier NAD, its administration would be expected to increase the number of

reducing equivalents available for mitochondrial oxidative phosphorylation, improving mitochondrial energy production. If the inhibition of MA metabolism is a consequence of energy depletion, improving mitochondrial energy production, NIC should at least attenuate such deficits. This is clearly demonstrated at the 7dayPT time point where the COMBO group has returned to saline control values. Additionally, this can be speculated at the 1hrPT time point although the reliability as to the statistical vs. clinical significance may still be debated.

One should note that the CYP2D6 Supersome data IC₅₀ values of MA and COMBO are very similar to those observed in the RLM studies. Stresser et al. (2002) demonstrated that the AMMC probe displayed high selectivity in rat CYP2D2 isoform for AMMC demethylation (a substrate selective for CYP2D6 in human liver microsomes) and, thus, is an appropriate choice for the comparison of two different isoforms in two different species.

Previous research indicates that the two different isoforms described have very similar functions in the metabolism of MA (Lin et al., 1997). AMMC, therefore, was utilized in order to quickly examine liver enzyme function after a drug treatment regimen. Clinical studies utilizing human liver microsomes after a standardized drug treatment regimen would produce the best *in vivo* data; the invasive procedures necessary to complete such an experiment would most likely prevent any such studies.

Surprisingly, the RLM studies did not demonstrate alterations in CYP activity in the presence of NIC. This data, coupled with the social pattern of abuse currently observed in the United States, does not explain why Americans adulterate illicit MA with NIC and not other less expensive more available alternatives. There must be some

underlying reason for this pattern of specific adulteration. Based upon the current pharmacokinetic studies, there is no overwhelming evidence that would indicate that NIC co-administration enhances MA's pharmacological effects significantly enough to drive the population to solely use NIC as the adulterant of choice. Additionally, recombinant human CYP2D6 enzyme studies, compared to RLM studies, failed to show any significant difference in the metabolic inhibition between MA and the COMBO.

The completion of the current studies has lead the investigator to consider that NIC may enhance the transcriptional activation of the CYP2D6 (human) and/or CYP2D2 (rat) enzymes leading to the increased synthesis of CYP proteins, thus, leading to decreased intensity and shorter duration of drug effects. A decreased intensity may allow the user to maintain a more steady state of alteration without the extreme ups and downs associated with typical MA. Another possibility is that the decrease in metabolic inhibition prevents as much MA from making it to the brain and therefore increasing the users desire to administer more MA in order to maintain the state of euphoria. The shorter duration of drug effect would also explain the increased frequency of administration. However, this theory was not able to be evaluated at this time because this set of studies utilized the same amount on RLM CYP protein in each assay. A more specific method of quantifying the amount of human CYP2D6 or rat CYP2D2 needs to be employed in order to make this determination.

Additional studies may address the possibility that the addition of NIC may somehow prevent the buildup of toxic metabolites or alter the metabolic cascade, allowing increased production of the pharmacologically active molecules and/or more

rapid elimination of inactive intermediates or toxic compounds. Yet, in order to further understand the possible interaction between MA and NIC, future studies will be required.

In conclusion, NIC by itself has little or no inhibitory effect on the CYP isoforms tested within this study. The mechanism that is speculated to cause drug interactions between co-administered MA and NIC is not related to direct CYP inhibition by NIC but is speculated to be by the alteration in the amount or activity of the CYP proteins that are present or perhaps related to the increased number of reducing equivalents available for the production of mitochondrial energy production. Further evaluation is needed to reveal the mechanisms of NIC-induced drug interaction and to offer a better understanding as to the common illicit combination of the two.

Chapter IV

DOPAMINERGIC CHANGES ASSOCIATED WITH ILLICIT METHAMPHETAMINE ABUSE WHEN CO-ADMINISTERED WITH THE COMMON ADULTERANT, NICOTINAMIDE.

4.1 Introduction

MA's popularity has continued to rise since 1990 (U.S. DHHS, 2001). Adulteration of illicit MA with NIC has become so prevalent in the United States, researchers must now consider the possibility that NIC may possess some ability to enhance MA's psychostimulant effects. One of the most challenging problems in studying the neurobiology of drug addiction is the difficulty in scientific quantification of perceived drug reward.

There is evidence that high or repeated doses of MA induce long-term deficits in basal ganglia neurotransmission, both in rodents and humans, mainly affecting the dopaminergic system (Seiden et al 1976; Ricaurte et al 1980; Marek et al. 1990). It has been shown that MA has the ability to alter DA synthesis (Gibb and Kogan, 1979; Schmidt et al., 1985), DA transport (Schmidt and Gibb, 1985; Marek, 1990) and DA receptors (O'Dell et al., 1993). Substantial animal data now implicate the ACC within the striatum as a critical target in the mechanism of action in all drugs of abuse, including MA (Robinson and Berridge, 1993; White and Kalivas, 1998; McLeman et al., 2000). Additional studies conducted have indicated that MA initiation of pleasure and reward is controlled in the mesolimbic and striatal dopaminergic systems (Roberts and Vickers

1984; Woolverton 1986; Bardo 1998). Knowing this, along with studies that demonstrated D₂ receptors helped to predict response to psychostimulants in humans by favoring pleasant response (Volkow et al., 1999), allowed us to focus our studies on two brain regions (STR and ACC) and on a specific receptor subclass, the D₂ receptors.

In the present study, we utilized neuropharmacological studies to investigate the effects of NIC co-administered with MA (vs. MA only) in rats to determine (1) if there were any alterations in the release of DA, (2) if the treatment modified DA D₂ receptors, and (3) if there were any concomitant alterations in DA transport or transporter density/number. These studies compared two separate time points: 1hrPT and 7dayPT. We believe that the illicit adulteration of MA with NIC enhances the psychostimulant effects of MA by either enhancing DA release, attenuating DA uptake, or altering the number of DA receptors or their binding affinity. We feel the results will reveal differential dopaminergic changes between MA and the co-administration of MA with NIC.

4.2 Materials and methods

4.2.1 Animals and treatments. Adult male Sprague-Dawley rats (200-300 g; bred in house) were housed at 23°C with a 12 hour alternating light /dark cycle. Food and water were provided *ad libitum*. On the day of the experiment, rats were randomly assigned to one of four treatment groups. Each group would receive one injection every 2 hours for a total of three injections. The first group received MA (5 mg/kg/injection, i.p.), the second group received NIC (5 mg/kg/injection, i.p.), the third group received a

(50:50) MA:NIC combination (5 mg of MA and NIC/kg/injection, i.p.) (COMBO), and the control group received three 0.1 ml saline injections, i.p. All drug concentrations were calculated as the free base.

Drug solutions were prepared in sterile isotonic saline at a concentration of 10 mg/ml; therefore, rats would receive between 0.1 and 0.15 ml of solution per injection, depending upon their weight. One hour, or seven days, post last injection (1hrPT and 7dayPT, respectfully), the rats were sacrificed by rapid decapitation. The brains were removed immediately and STR and ACC dissected out on an ice-cooled dish. Upon completion of the dissection, the STR and ACC were either used immediately for functional assays or stored frozen (-80°C) until used in the binding assays.

4.2.1.1 Dissection. Immediately after decapitation, the brains were removed from the skull by cutting the skin and head muscles to expose the skull. Then, one tine from a set of sharp scissors was held on a vertical plane and inserted into the foramen magnum at the rear base of the skull cavity just below the midsagittal suture. The bone was cut along the suture above the dura mater. The temporal and occipital bones of the skull were lifted and removed utilizing a stainless steel straight cranial rongeur. The brain was lifted with an ice-cold spatula to expose the optic nerves, which were then severed. The brain was removed with a portion of the spinal cord still intact.

Once removed, the brain was immediately placed ventral side up in an ice-cold metal (zinc:nickel alloy) brain matrix dissecting mold with 1 mm increment cutting slots (Harvard Instruments, Holliston, MA). In order to remove the section of interest, one ice-cold razor blade was inserted 4 mm posterior to the most anterior portion of the brain and the other blade inserted 4 mm posterior to the first blade (figure 4-1). Once the section of

interest was removed, the striatum (consisting of the caudate and putamen nuclei) and nucleus accumbens were dissected out using a set of ice-cold razor blades and a set of finely curved forceps (figure 4-2). The regions of interest were either used immediately for functional assays or frozen and stored at -80° C for future binding studies. Bardsley and Bachelard (1981) provide a review of the rat brain anatomy needed for this specific technique.

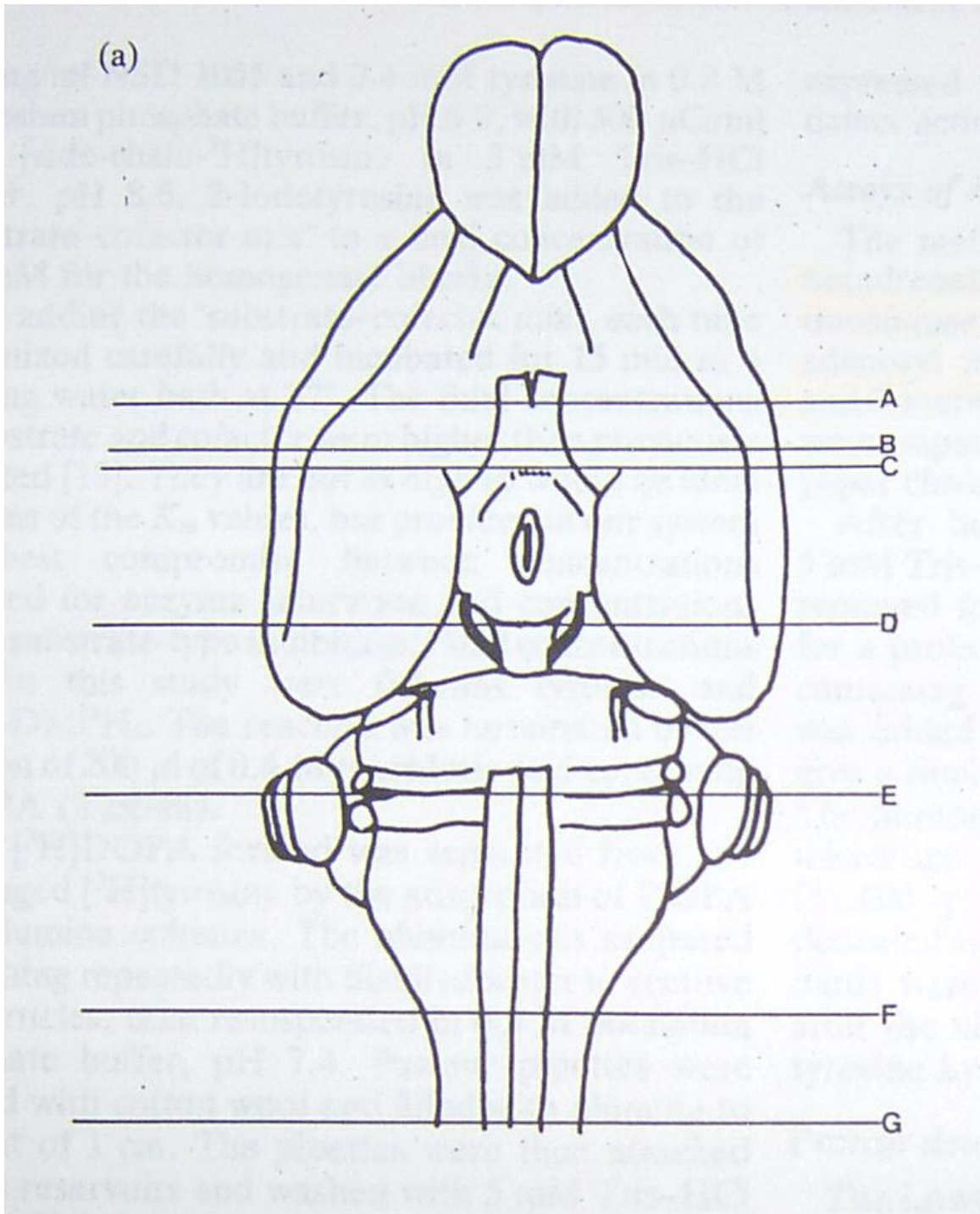


Figure 4-1. Ventral view of the rat brain with examples of vertical section locations. Reproduced with slight modification from Bardsley and Bachelard (1981).

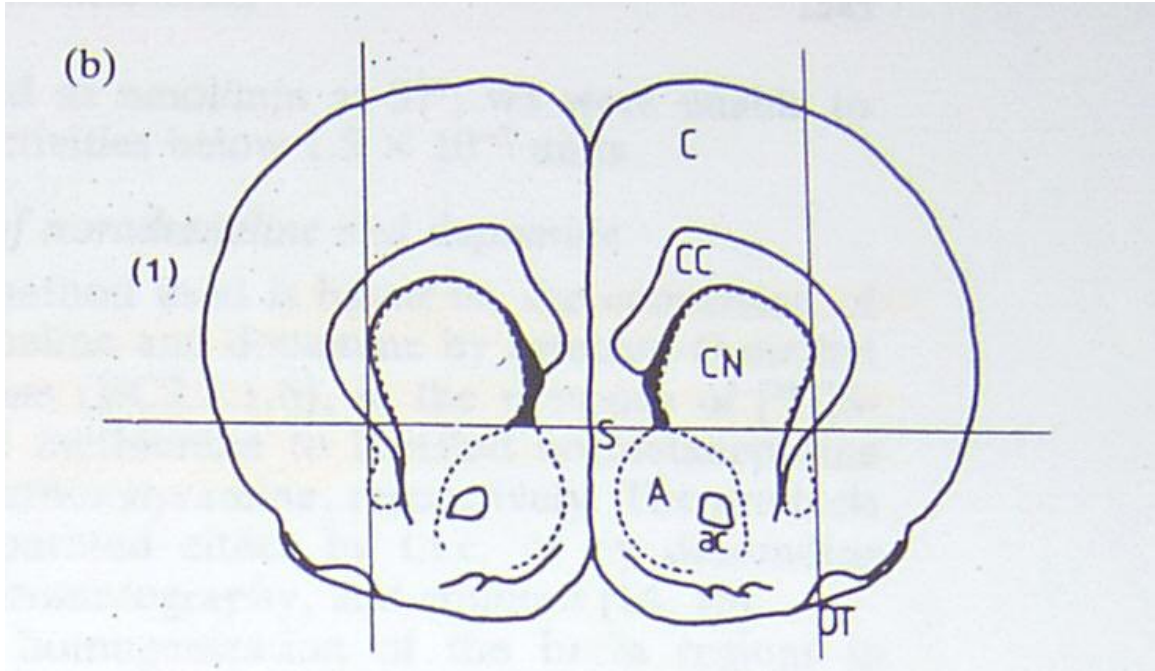


Figure 4-2. Cross section of rat brain thru section A of figure 3-1. Dissection included a brain section from cut A (figure 3-1) where once identified the accumbens and striatum (caudate and putamen nuclei) could be removed. Key: C-Cortex, CC-corpora callosa, CN-striatum (caudate and putamen nuclei), S-Septal nuclei, A-Accumbens, ac-anterior commissure. Reproduced with slight modification from Bardsley and Bachelard (1981).

4.2.2 Drugs. MA hydrochloride, NIC, dopamine, 1-(2-Diphenylmethoxyethyl)-4(3-phenylpropyl) piperazine (GBR-12935), 1-(2-(Bis-4-fluorophenyl) methoxy)ethyl)-4-(3-phenyl-2-propenyl) piperazine (GBR-12909), and the other chemicals utilized in the buffer solutions were purchased from either Sigma Chemical Corporation (St. Louis, MO) or Fisher Chemical Company (Denver, CO). Pargyline hydrochloride and nomifensine maleate were purchased from Research Biochemicals International (Natick, MA). [7,8-³H]Dopamine (spec. act. 50.0 Ci/mmol) was purchased from Amersham Bioscience. [³H]GBR12935 (spec. act. 45.0 Ci/mmol) and raclopride, [methoxy-³H] (spec. act. 80.3 Ci/mmol) were purchased from Perkin Elmer Life Science (Boston, MA).

4.2.3 Synaptosomal preparation. Isolated nerve terminals, synaptosomes were prepared as described by Fleckenstein et al. (1999) with minor modifications. Once the STR and ACC were separated, each was separately homogenized in 20 volumes of 0.32 M sucrose using 10 up and down strokes of a pre-chilled Teflon-glass homogenizer at 900 rpm. Homogenate was then centrifuged at 1,000 X *g* for 11 minutes at 4°C to remove cellular debris. The supernatant obtained was centrifuged again at 22,000 X *g* for 11 minutes at 4°C. The pellet obtained in the latter centrifugation contains the synaptosomal fraction because apart from synaptosomes, it has also been shown to contain myelin and free mitochondria (Garcia-Sanz et al., 2001). This pellet was suspended in the appropriate assay buffer (see text) for the day's functional experiments.

Once the appropriate amount of fresh synaptosomes were acquired for the day, the remainder of solution containing the excess synaptosomes was centrifuged again at 22,000 X *g* for 11 minutes at 4°C and that pellet was stored in 0.32 M sucrose at -80°C until binding studies could be completed. Protein analysis was completed with the

commercially available Bio-Rad Protein Assay kit from BioRad (BioRad Life Science Group, Richmond, CA). The Bio-Rad Protein Assay is based on the method of Bradford (1976) and is a simple and accurate procedure for determining the concentration of solubilized protein.

4.2.4 [³H]Dopamine uptake. Synaptosomes were prepared as described above. The fresh synaptosomal pellet was resuspended in uptake assay buffer by gentle homogenization. Uptake assay buffer consisted of 25 mM Hepes, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl, 1.2 mM MgSO₄, 10 mM glucose, 1 mM ascorbic acid, and 5 μM pargyline (pH 7.4). Cold saturation studies were prepared by adding a 400 μl aliquot of the synaptosomal tissue with either 50 μl of assay buffer (total binding), 50 μl of 10 μM manzindol (non-specific binding), or 50 μl of dopamine ranging from 0.34 to 17,000 nM. All concentrations reported are the final reaction vial concentrations and all reaction vials were run in duplicate.

Uptake reactions were initiated by addition of 50 μl of [³H]dopamine (0.5 nM final concentration) to each of the tubes. Once the reaction was initiated, samples were allowed to go to equilibrium by incubating at room temperature (22°C) for 15 minutes. Dopamine uptake was terminated by rapid filtration with the Brandel Tissue Harvester (Gaithersburg, MD) and three separate 5 ml washing (approximately 15 seconds total) with ice-cold 0.9 % NaCl solution onto Whatman GF/B glass-fiber filter paper previously soaked for 1 hour in 0.03% polyethyleneimine solution.

The remaining radioactivity trapped in filters was determined using a Beckman LS1801 scintillation counter (40-50% efficiency) with Scintiverse scintillation fluid (Fisher, Pittsburg, PA). Protein analysis was completed with the commercially available

Bio-Rad Protein Assay kit from BioRad (BioRad Life Science Group, Richmond, CA). Total uptake was considered the amount of uptake without any unlabelled dopamine present and nonspecific binding was determined with mazindol (10 μ M). Specific uptake was defined as total (or test sample) binding – nonspecific binding. The resulting cold saturation data was analyzed with a nonlinear computer-fitting program to estimate apparent Vmax (transporter density) and IC₅₀ values. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way analysis of variance (ANOVA) (Winer, 1971), followed by Bonferroni post hoc comparison where appropriate. Results were considered significant when P was < 0.05. All data analysis was performed using GraphPad Prism version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

4.2.5 [³H]GBR12935 binding assay. Previously stored frozen (-80°C) synaptosomes from the dopamine uptake studies were reconstituted in binding buffer. Binding buffer consisted of 50mM Tris-HCl, 120 mM NaCl, and 0.01% BSA at pH 7.4. Cold saturation studies were prepared by adding a 400 μ l aliquot of the synaptosomal tissue with either 50 μ l of binding buffer (total binding), 50 μ l of 5 μ M GBR 12909 (non-specific binding), or 50 μ l of GBR12935 ranging from 0.01nM to 5 μ M. All concentrations reported are the final reaction vial concentrations and all reaction vials were run in duplicate.

Binding was initiated by addition of 50 μ l of [³H]GBR12935 (5 nM) to each of the tubes and allowing them to achieve equilibrium by incubating at room temperature (22°C) for 60 minutes. Incubation was terminated by rapid filtration with the Brandel Tissue Harvester (Gaithersburg, MD), and three separate 5 ml washing (approximately 5

seconds per wash) with ice-cold wash buffer solution onto Whatman GF/B glass-fiber filter paper previously soaked for 1 hour in 0.03% polyethylenimine solution. The wash buffer used in the binding experiments was ice-cold 50 mM Tris-HCl buffer (pH=7.4). Radioactivity trapped in filters was counted using a liquid scintillation counter, as described above. Protein content was measured using a purchased BioRad Protein assay (BioRad Life Science Group, Richmond, CA).

The resulting cold saturation data was analyzed with a nonlinear computer-fitting program to estimate Bmax (transporter density) and IC₅₀ values. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way ANOVA (Winer, 1971), followed by Bonferroni post hoc comparison, where appropriate. Results were considered significant when P was < 0.05. All data analysis was performed using GraphPad Prism version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

4.2.6 [³H]Raclopride binding. Due to the lower concentration of D2-like receptors in the tissue of interest, crude synaptosomes were prepared as described above. Briefly, previously frozen tissue (-80°C) from the release studies was thawed to room temperature on ice. Once the tissue was at room temperature, the tissue was homogenized in 5 ml of binding buffer (50 mM Tris-HCl, 120 mM NaCl at pH=7.4), using 10 up and down strokes of a pre-chilled Teflon-glass homogenizer at 900 rpm. The homogenate was centrifuged at 1,000 x g for 10 min. in a Beckman Avanti J-25 refrigerated centrifuge at 4° C using a Beckman JA-25.15 rotor. The supernatant obtained was diluted to 12 ml with binding buffer and stored on ice, until use.

Cold saturation studies were prepared by adding a 400 μ l aliquot of the synaptosomal tissue with either 50 μ l of binding buffer (total binding), raclopride (10 μ M) (non-specific binding), or 50 μ l of unlabelled raclopride ranging from 0.01nM to 5 μ M. All concentrations reported are the final reaction vial concentrations, and all reaction vials were run in duplicate. Binding was initiated by addition of 50 μ l of 5 nM raclopride, [methoxy-3H] (spec. act. 80.3 Ci/mmol) to each of the tubes, allowing them to achieve equilibrium by incubating at room temperature (22° C) for 60 minutes.

The binding was terminated by filtration under reduced pressure with a Brandel Tissue Harvester (Gaithersburg, MD) and three separate 5 ml washing (approximately 5 seconds per wash) with ice-cold 50 mM Tris-HCl buffer (pH=7.4) solution onto Whatman GF/B glass-fiber filter paper previously soaked for 1 hour in 0.03% polyethylenimine solution. Analysis of the radioactivity trapped in the filters was accomplished by Beckman LS1801 scintillation counter (40-50% efficiency). A portion of the synaptosomal solution was assayed for protein concentration using the commercially available Bio-Rad Protein Assay kit from BioRad (BioRad Life Science Group, Richmond, CA).

Total binding was considered the amount of uptake without any unlabelled raclopride present, and nonspecific binding was determined with raclopride (10 μ M). Specific binding was defined as total (or test sample) binding – nonspecific binding. The resulting cold saturation data was analyzed with a nonlinear computer-fitting program to estimate Bmax (receptor density) and IC₅₀. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way ANOVA (Winer, 1971), followed by Bonferroni post hoc comparison where appropriate. Results were

considered significant when P was < 0.05 . All data analysis was performed with GraphPad Prism version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

4.2.7 [^3H]Dopamine release. Brains were removed rapidly and STR and ACC dissected out on an ice-cooled dish from male Sprague-Dawley rats, after designated treatment regimen and decapitation, as described above. STR and ACC were dissected and sliced into 400 μm slices with a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey). Slices were suspended in a 50 nM [^3H]DA solution of modified Krebs–HEPES uptake buffer consisting of 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM HEPES, 10 mM glucose, 1 mM ascorbic acid, 0.1 mM pargyline; pH adjusted to 7.4 with NaOH) for 30 minutes at 37° C.

Approximately 5-10 slices in a 200 μl aliquot were deposited between two Whatman GF/B glass-fiber filter discs within a chamber of a Brandel (Gaithersburg, MD) superfusion apparatus. Tissue slices were perfused for 30 minutes at a flow rate of 0.6 ml/min. in a wash out stage using release buffer. Release buffer is uptake buffer containing an additional 10 μM of nomifensine and 1 μM domperidone. These drugs were included in all subsequent steps to prevent reuptake of, and feedback inhibition, by released [^3H]DA.

A low, stable baseline of basal release (approximately 1%/min.) was established. After 8 minutes (fraction 4), tissue was stimulated to release [^3H]DA by a 2 minute exposure to 30 mM KCl. The perfusion solution was returned to the non-stimulating releasing buffer for an additional 16 minutes, allowing the [^3H]DA release to return to the basal level. Fractions of release buffer containing [^3H]DA were collected at 2 minute

intervals in scintillation vials and, finally, the filter discs and tissue were collected from each of the chambers.

The remaining [³H]DA content in tissue and discs was assessed at the end of each experiment by treating the remaining tissue and discs with 1 ml of 0.2 M HCl and incubation for one hour at room temperature to extract the remaining radioactivity. Under similar experimental conditions, the released radioactivity has been shown to be primarily dopamine (Werling et al., 1988). Released radioactivity was determined by liquid scintillation spectroscopy. Release was calculated as fractional release as calculated as described in Bennett et al. (1997) and defined as:

Fractional Release (%):

$$\frac{\text{c.p.m. in each fraction}}{\text{c.p.m. in all collected fractions} + \text{c.p.m. in remaining tissue and discs}} \times 100$$

The peak analysis focused on the highest amount of fractional release being seen in fraction number 6; the peak release of [³H]DA (fraction 6) was used to compare treatment groups for the statistical analysis. The AUC or area under the curve analysis was done utilizing the sum of fractions 5-9 which would approximate an (AUC for the total amount of [³H]DA released in response to the stimulus. Finally, the basal release analysis took the average amount of basal release calculated as the sum of fractions 1-4 and compared it for statistical difference. Data were expressed as fractional release (%) in all three of the analysis (peak, AUC, and basal release studies).

The highest amount of fractional release was seen in fraction number 6. The peak release of [³H]DA (fraction 6) was used to compare treatment groups for the statistical analysis. Additional data analysis was done utilizing the sum of fractions 5-9, which would approximate an area under the curve (AUC) for the total amount of [³H]DA released in response to the stimulus.

Finally, each of the treatment groups' AUC was compared to the average saline (control) treated groups AUC and reported as percent of control. Statistical significance was determined by utilizing a two-way ANOVA (Winer, 1971), followed by Bonferroni post-hoc comparison. Statistical significance was assumed at P values less than 0.05. All data analysis was performed using GraphPad Prism version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA), and all data were analyzed prior to transformation to percent control for graphical representation.

4.2.8 Competition analysis. Nonlinear regression analysis was used to fit the data to equations that minimize the sum of squares of the relative distances of the data points to the curve in order to obtain binding parameters. Relative distances are used rather than absolute distances largely due to the bulk of the error being attributed to pipetting, which means the standard deviation of replicate measurements will be on average a constant fraction of the amount of binding. In contrast, other experiments which may include scatter or where the standard deviation is not related to the amount of binding would preferably utilize equations that minimize the sum of squares of the absolute distance. One should, therefore, only utilize weighting by relative distances when analyzing total binding data (Motulsky, 1999).

Competition analysis data was entered into GraphPad Prism version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA). The F-test was run to determine the best-fit value, which determined if the data best matched a one or two-site model. Our data was best fit to the one-site model. The one-site model states that if the radioligand and competitor both bind reversibly to the same binding site, binding at equilibrium follows this equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{X - \text{LogEC}_{50}}}$$

X is the logarithm of unlabelled drug concentration and Y is the response. BOTTOM is the Y value at the bottom of the plateau; TOP is the Y value at the top of the plateau, and LogEC₅₀ is the X value when the response is halfway between TOP and BOTTOM. LogEC₅₀ is the logarithm of the EC₅₀, the concentration that gives a response halfway between TOP and BOTTOM. The variable LogEC₅₀ is the concentration of

competitor required to compete for half the specific binding. EC₅₀ (effective concentration producing a response halfway between the top and bottom) is generally used with sigmoidal curves, whereas, IC₅₀ (inhibitory concentration producing a response halfway between the top and bottom) is a more specific term used in competition analysis. In this case either can be used, though IC₅₀ is more descriptive of the studies and thus more appropriate.

In the specific instance of cold saturation experiments, the bottom plateau of the binding curve is equal to nonspecific binding. As described above, the concentration of unlabeled drug that produces radioligand binding halfway between the upper and lower plateaus is called the IC₅₀ (inhibitory concentration 50%). The top plateau of the binding curve is equal to radioligand binding in the absence of the competing unlabeled drug.

The Top value was converted from cpm to fmol using the GraphPad prism radioligand calculator (GraphPad Software, San Diego California USA, (<http://www.graphpad.com>)). The conversion of cpm to molar units is done using the following equations:

$$\text{cpm/ml} \times \text{dpm/} \frac{\text{cpm}}{\text{Ci}} \times 10^{12} \text{dpm} \times \text{mmol/} \frac{\text{Ci}}{1000} \times 1000 \text{mL/L} \times 1 \text{ mol/1000mmol} = \frac{\text{cpm}}{\text{Ci}} \text{mol/L}$$

The determined molar value (i.e. fmol) was then divided by the concentration of protein in the synaptosomal preparation (mg of protein), in order to estimate the total number of DA D₂ receptors (Raclopride studies) or dopamine transporter protein (GBR12935 studies) sites in the tissue being studied. The B_{max} occurs when all of the receptors/transporters are occupied by radioactive drug. The B_{max} values in our studies are reported as fmol/mg of protein. Generally, the B_{max} cannot be determined from

competition studies. Bmax values can be determined if the labeled and unlabeled ligand are the same; the term apparent Bmax is used in the text to identify that the Bmax was not directly determined from saturation analysis.

Saturation analysis is used to determine the dissociation constant (K_d) and Bmax. K_d is the equilibrium dissociation constant. It is equal to the concentration of radioactive ligand required to occupy 50% of the receptors. With a known K_d , one can determine the K_i , Bmax, and fractional occupancy. K_i is the equilibrium dissociation constant for a competitive inhibitor of the receptor. Note: The competitive inhibitor can be an agonists or an antagonist. It is referred to as a competitive inhibitor because its value is determined by measuring the ability of the unlabeled drug to compete with a radiolabeled drug for the receptor. The K_i value for an unlabeled drug should be the same as the K_i value obtained if the drug is labeled. The K_i can be calculated from the EC_{50} using the K_d value determined in saturation studies using the Cheng-Prusoff equation (1973) where:

$$K_i = EC_{50} / (1 + [ligand]/K_d)$$

In order to confirm that the completed studies targeted a representative sample of the receptor of interest's total population, the fractional occupancy should be calculated and reported for each experiment where appropriate. The law of mass action predicts the fractional receptor occupancy at equilibrium as a function of ligand concentration. Fractional occupancy is considered the fraction of all receptors that are bound to ligand and is defined as:

$$\begin{aligned} \text{Fractional Occupancy} &= [\text{Ligand} \times \text{Receptor}] / \text{Total Receptor} \\ &= [\text{Ligand} \times \text{Receptor}] / [\text{Receptor}] + [\text{Ligand} \times \text{Receptor}] \end{aligned}$$

This equation is not useful, because the concentration of unoccupied receptor, [Receptor], is usually not known. One can rearrange the equation to be useful.

$$\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}] + K_d}$$

Since our studies were looking to determine a difference between several drug treatment regimens, instead of characterizing a specific receptor or transporter, saturation analysis was not used. We used IC₅₀ values as an approximation of K_d.

The Prism software determined the EC₅₀ which is equivalent to IC₅₀ under our conditions and apparent Bmax values for each of the curves. The software was then used to average the IC₅₀ values and determined and presented as the mean ± the standard error of the mean (S.E.M). Data are considered significant at P < 0.05. All statistical analysis were done using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, <http://www.graphpad.com>. Upon completion of the nonlinear regression, the appropriate experimental values were statistically analyzed utilizing the two-way ANOVA (treatment vs. time) with Bonferonni post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA. All statistical analysis were performed on untransformed data.

4.3 Results

4.3.1 [³H]Dopamine uptake. It has previously been reported that a single administration of MA decreased striatal [³H]DA uptake to 63-72% of control values in striatal synaptosomes prepared one hour after drug administration (10mg/kg) (Metzger et al., 1998a). Similar to a single administration, multiple high-dose injections (i.e. four

10mg/kg injections at 2 hr intervals) of MA decreased [³H]DA uptake to 30% of control values.

The competitive inhibition of [³H]DA uptake into STR and ACC synaptosomes was examined over a concentration range of 0.34 to 17,000 nM of unlabelled dopamine. Results presented in figures 4-3 and 4-4 demonstrate the competitive inhibition curves generated with synaptosomes from each of the treatment groups at 1hrPT and 7dayPT. Cold saturation studies failed to demonstrate an alteration in the IC₅₀ values between any of the treatment groups or time points (figure 4-5).

Results presented in figure 4-6 and table 4-1 demonstrate that our 1hrPT regimen reduced MA and COMBO treated STR and ACC [³H]DA uptake to 60%, 58%, 71% and 74% of control values, (MA-STR, COMBO-STR, MA-ACC, COMBO-ACC) respectfully. [³H]DA uptake returned to control values at the 7dayPT time point as demonstrated by the values of control 96%, 83%, 92%, and 85%, respectfully (figure 4-6 and table 4-1). One should note that the COMBO treated groups in both STR and ACC did not return to the 90+% control values, as seen in the MA treated groups. A significant difference was determined to be present between the MA and COMBO treated groups at the 7dayPT time point ($p < 0.05$). This difference was not observed in the ACC brain region. A comparison of the apparent V_{max} values can be seen in figure 4-7. Table 4-1 compares the maximum specific [³H]DA uptake (apparent V_{max}) and the percent of control values in the STR and ACC at 1hrPT and 7dayPT.

Our studies most closely resembled the results of Metzger et al. (1998a), where they observed an IC₅₀ value of 63±8 nM for the single administration of MA compared to our results, which yielded 78±15 nM in the STR and 85±13 nM in the ACC. No

correlation was observed between the calculated IC₅₀ values and the ability of the agents to alter dopamine uptake after administration *in vivo*. Furthermore, no difference was observed between the MA and COMBO treated values in either brain region or time points

Specific uptake was defined as total (or test sample) binding–nonspecific binding. The resulting cold saturation data was analyzed with a nonlinear computer-fitting program to estimate apparent V_{max} (transporter density) and IC₅₀ values. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way analysis of variance (ANOVA) (Winer, 1971), followed by Bonferroni post hoc comparison, where appropriate. Results were considered significant when P was < 0.05. The apparent V_{max} and IC₅₀ values were determined and statistically analyzed from original data prior to being transformed to percent of control for graphical representation. All data analysis was performed using GraphPad Prism Version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

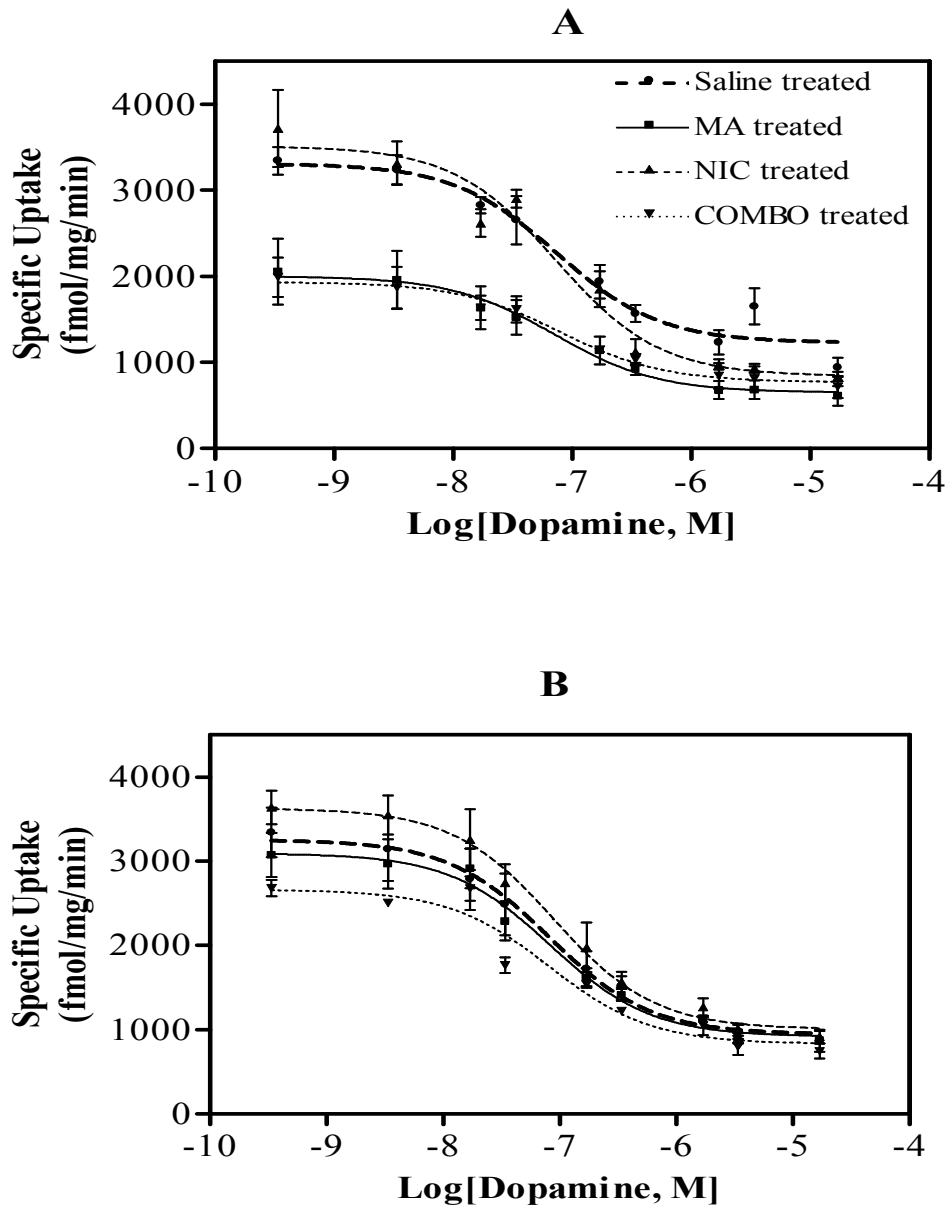


Figure 4-3. Dopamine uptake in the STR. Dopamine uptake studies were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh STR synaptosomal preparations over a concentration range of 0.34 to 17,000 nM of unlabelled dopamine. Data represents the mean (\pm SEM) of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

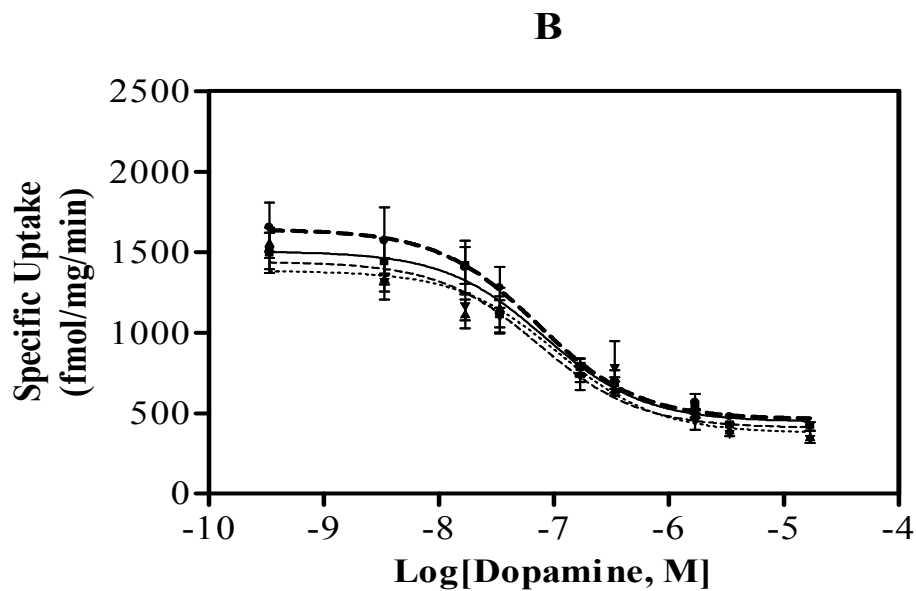
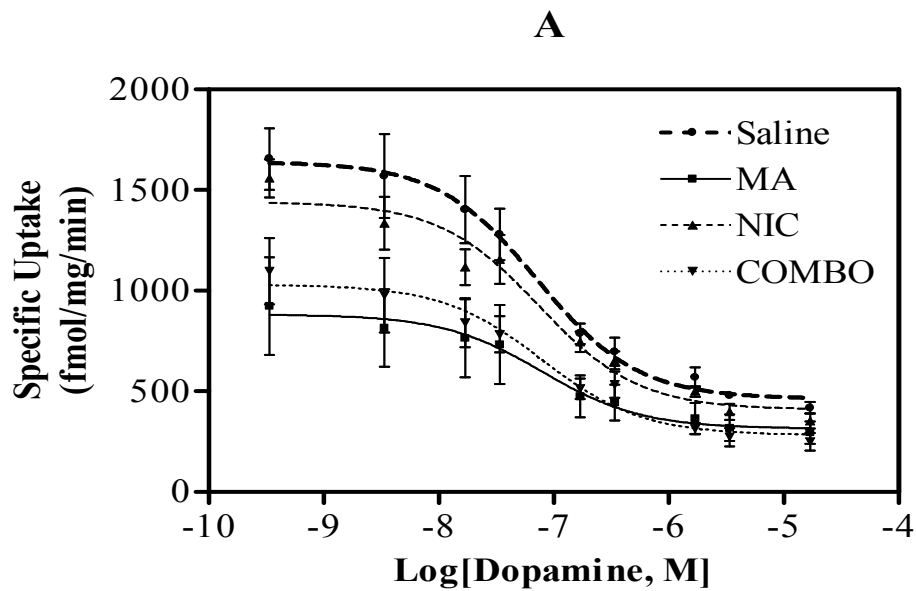


Figure 4-4. Dopamine uptake in the ACC. Dopamine uptake studies were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh synaptosomal ACC preparations over a concentration range of 0.34 to 17,000 nM of unlabelled dopamine. Data represents the mean (\pm SEM) of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

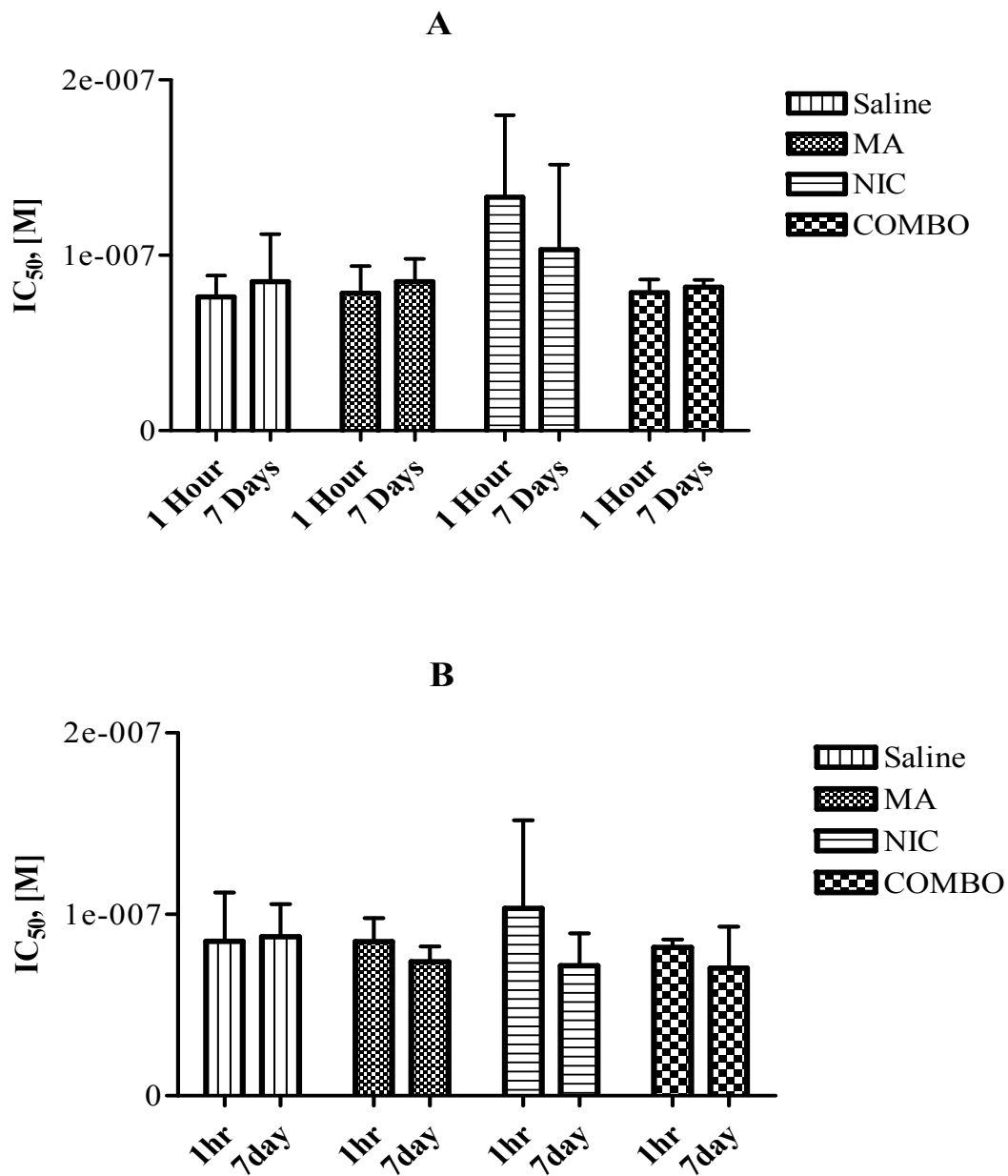


Figure 4-5. [³H]Dopamine uptake IC₅₀ values. [³H]Dopamine uptake IC₅₀ values as determined from nonlinear regression analysis expressed as molar equivalent at the 1hrPT and 7dayPT. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

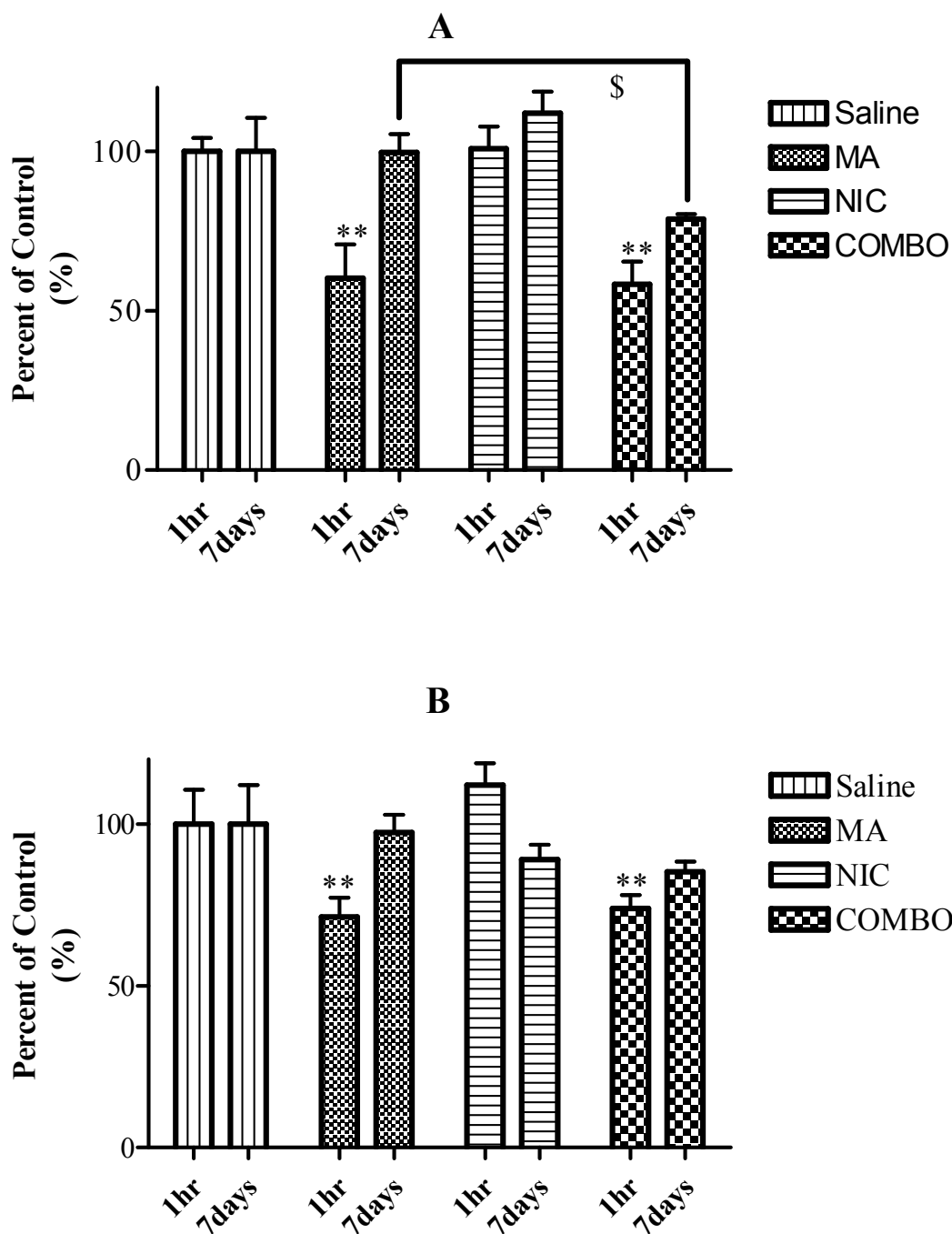


Figure 4-6. [³H]Dopamine uptake as percent of control (%) at the 1hrPT and 7dayPT. (** p < 0.01; * p < 0.05) \$ denotes difference between the MA and COMBO treated groups (p < 0.05) at the 7dayPT STR time point. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

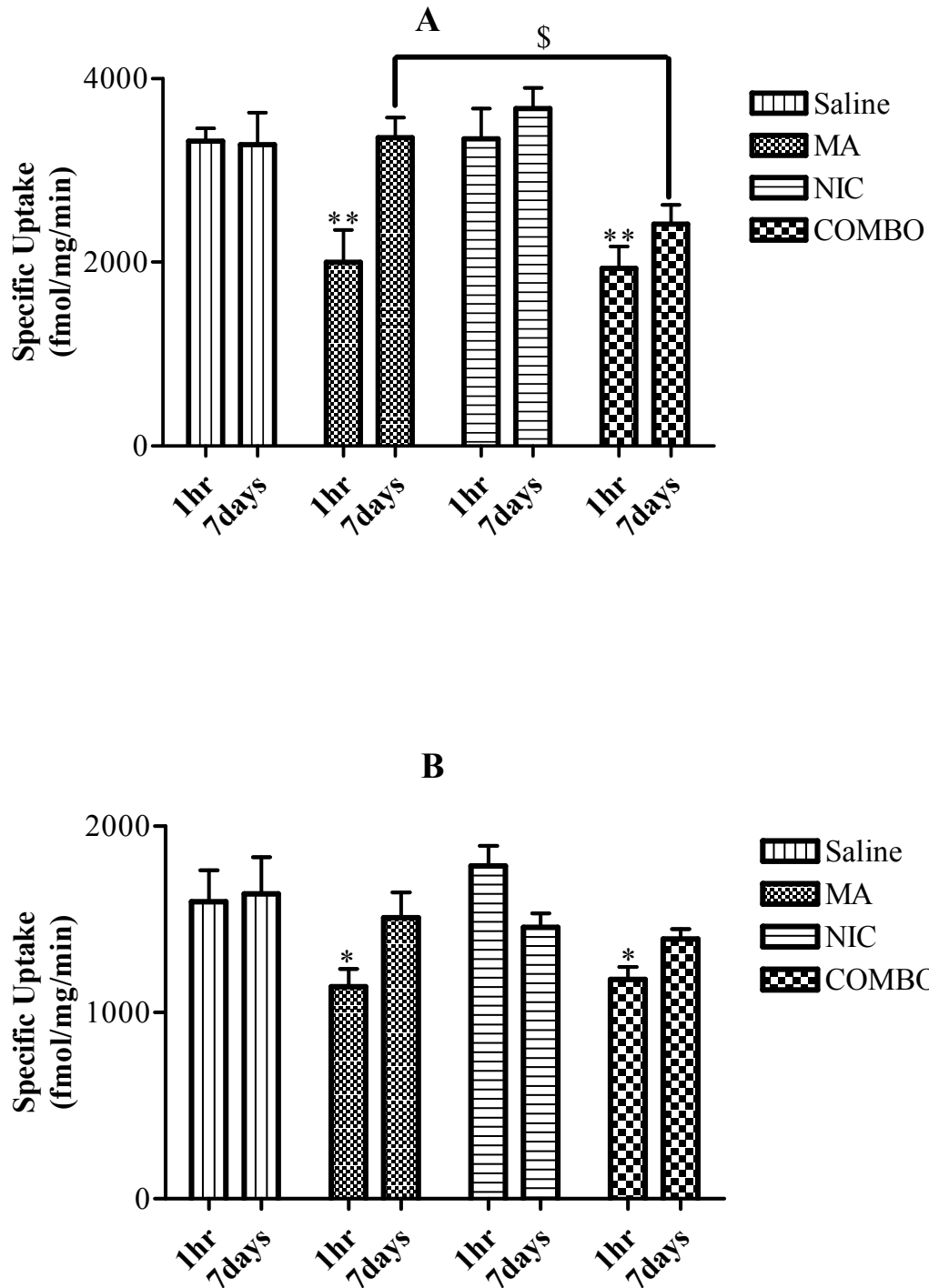


Figure 4-7. [³H]Dopamine uptake apparent V_{max} values expressed as specific uptake (fmol/mg of protein/min) at the 1hrPT and 7dayPT. (* p<0.05; ** p<0.01) \$ denotes difference between the MA and COMBO treated groups (p<0.05) at the 7dayPT STR time point. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

Table 4-1. Uptake Apparent Vmax Values (fmol/mg protein/min); Percent of Control and GBR Apparent Bmax Values (fmol/mg protein); Percent of Control Data represent the mean±SEM of four independent experiments, each conducted in duplicate. Astricks indicate difference between the identified group and the saline control group (*p<0.05; **p<0.01; ***p<0.001) \$ indicates difference between MA and COMBO treated groups at the 7dayPT time point (p<0.05).

[³H]DA Uptake Data

Treatment	Vmax-Specific uptake (fmol/mg protein/min)		Percent of control (%)	
	1hrPT	7dayPT	1hrPT	7dayPT
Striatum				
Saline	3318±139	3218±346	100±4.2	100±10.6
MA	1997±352**	3105±278 \$	60±10.6	94±8.4
NIC	3345±329	3674±223	99±7.1	112±6.8
COMBO	1934±237**	2663±157 \$	58±7.1	81±1.7
Nucleus Accumbens				
Saline	1594±168	1635±196	100±10.5	100±12.0
MA	1137±94*	1509±135	71±5.9	92±8.2
NIC	1786±108	1455±75	112±6.8	89±4.6
COMBO	1178±66*	1393±52	73±4.1	85±3.2

4.3.2 [³H]GBR12935 binding. The potent and selective dopamine uptake inhibitor [³H]GBR12935 was utilized to detect changes in the density of the DAT on rat STR and ACC membranes. Cold saturation curves (figures 4-8 and 4-9) from membranes of 1hrPT and 7dayPT animals were examined over a concentration range of 0.01nM to 5 μ M GBR12935. Cold saturation studies failed to demonstrate an alteration in the IC₅₀ values between any of the treatment groups or time points (figure 4-10). The 1hrPT regimen reduced MA and COMBO treated STR and ACC [³H]GBR12935 maximum specific binding to 54%, 51%, 61% and 67% of control values, respectfully (figure 4-11). The [³H]GBR12935 binding appeared to return to control values at the 7dayPT time point, as demonstrated by the values of control 94%, 102%, 100%, and 101%, respectfully. Our studies revealed apparent Bmax values, in accordance with previous reports from the literature (e.g. Janowsky et al., 1986; Page et al., 2000).

Results presented in figure 4-12 and table 4-2 represent the transformed data from figure 4-8 and figure 4-9 which demonstrate the changes in the DAT density expressed as % control (fmol/mg protein). A comparison of the [³H]DA uptake and [³H]GBR12935 binding studies is presented in table 4-3. The resulting cold saturation data was analyzed with a nonlinear computer-fitting program to estimate apparent Bmax (transporter density) and IC₅₀ values. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way analysis of variance (ANOVA) (Winer, 1971), followed by Bonferroni post hoc comparison, where appropriate, before data transformation to percent of control for clarity of presentation. Results were considered significant when P was < 0.05. All data analysis was performed using GraphPad Prism Version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

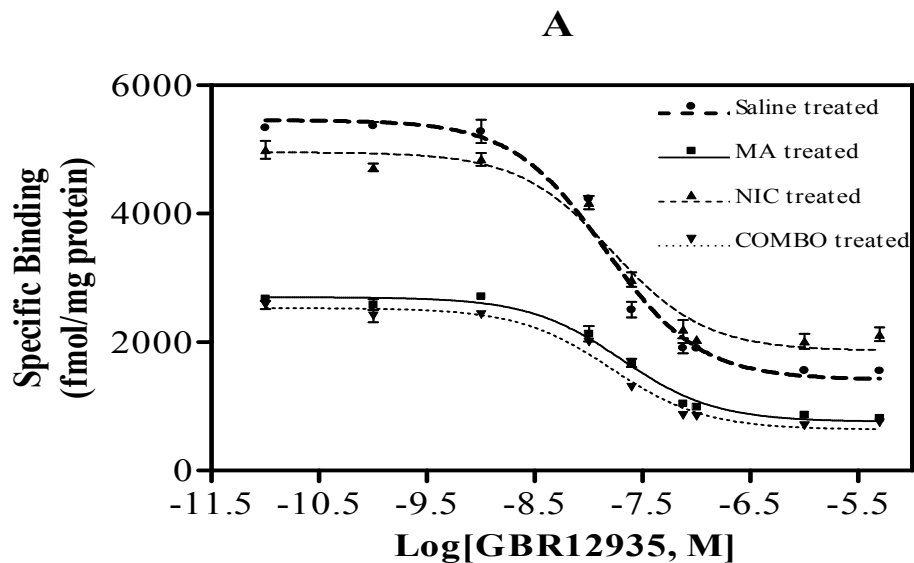


Figure 4-8. [³H]GBR12935 binding curves in the striatum. [³H]GBR12935 binding curves were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh STR synaptosomal preparations over a concentration range of 0.01nM to 5 μ M of unlabelled GBR12935. Data represents the mean (\pm SEM) of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

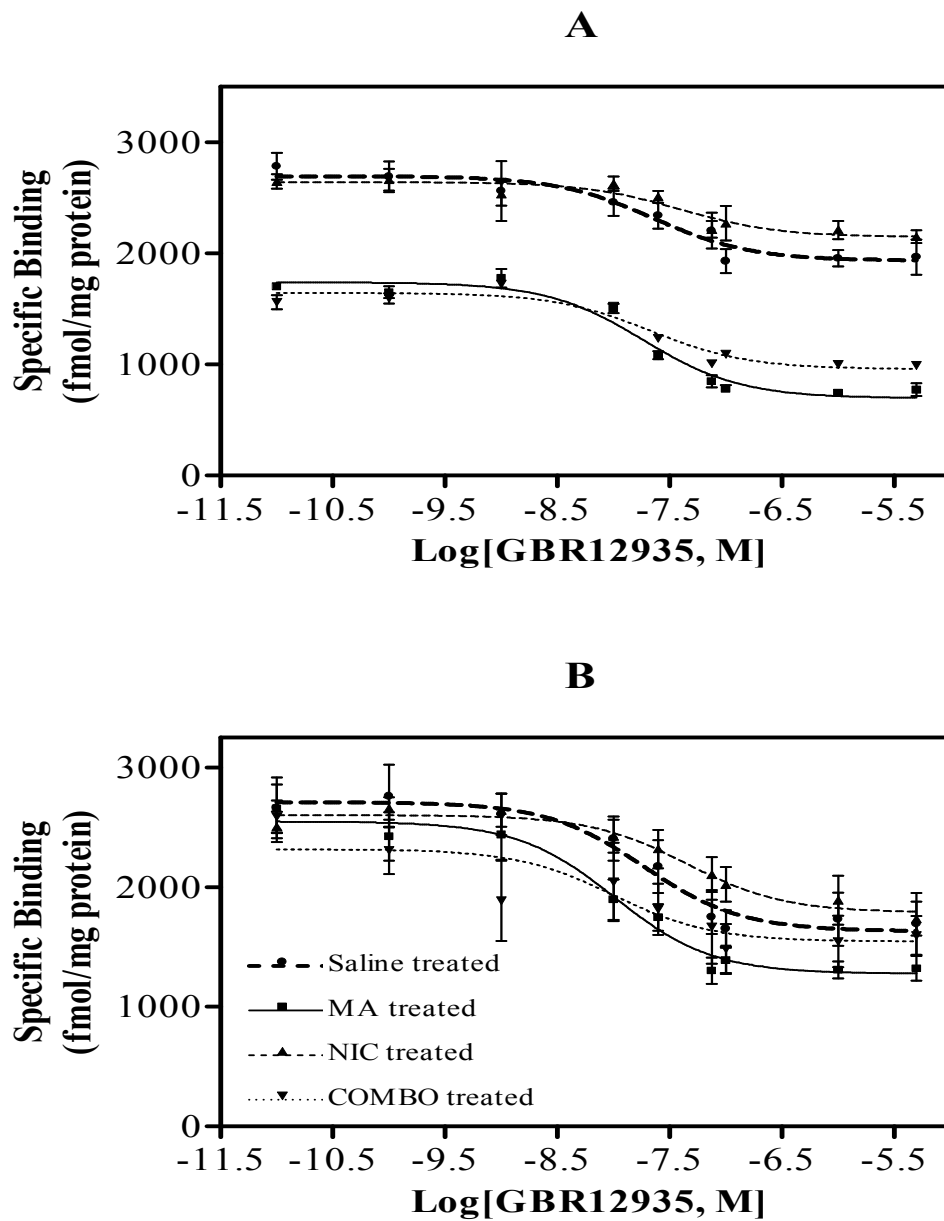


Figure 4-9. [³H]GBR12935 binding curves in the nucleus accumbens. [³H]GBR12935 binding curves were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh ACC synaptosomal preparations over a concentration range of 0.01nM to 5 μ M of unlabelled GBR12935. Data represents the mean (\pm SEM) of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

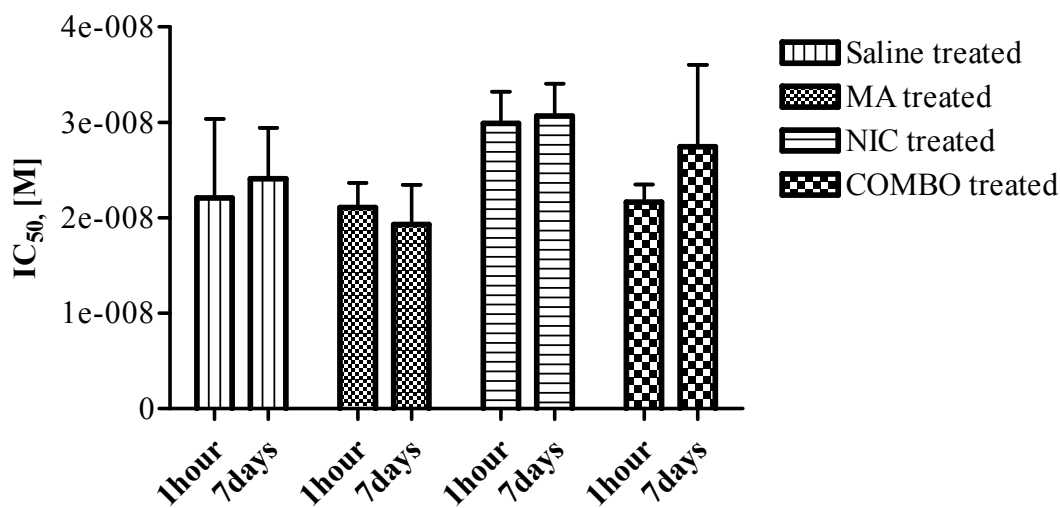
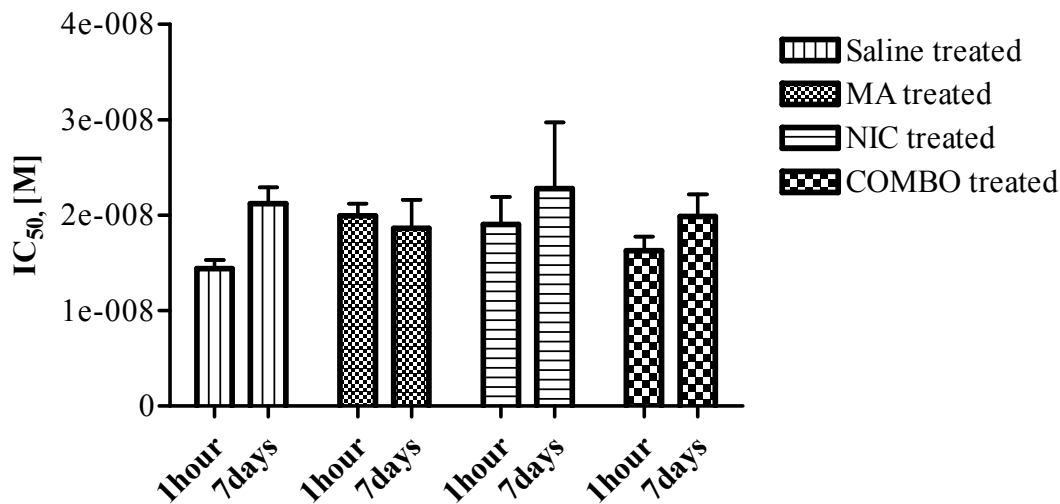


Figure 4-10. [³H]GBR12935 binding IC₅₀ values. [³H]GBR12935 binding IC₅₀ values as determined from nonlinear regression analysis expressed as molar equivalent at the 1hrPT and 7dayPT. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

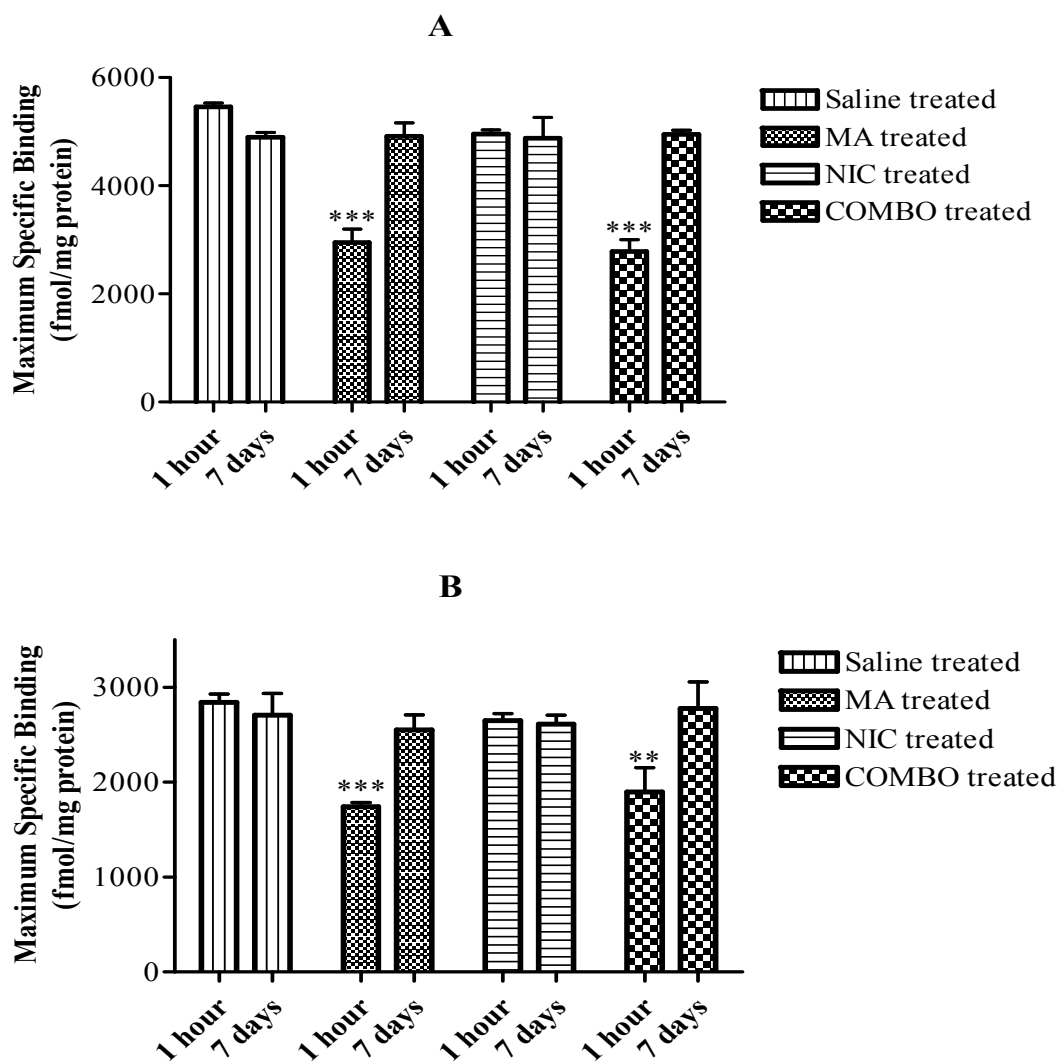


Figure 4-11. [³H]GBR12935 binding apparent Bmax values expressed as maximum specific binding. [³H]GBR12935 binding values (apparent Bmax) as determined from nonlinear regression analysis expressed as maximum specific binding (fmol/mg of protein) at the 1hrPT and 7dayPT. (** p < 0.01; ***p < 0.001) Each bar represents the mean(±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

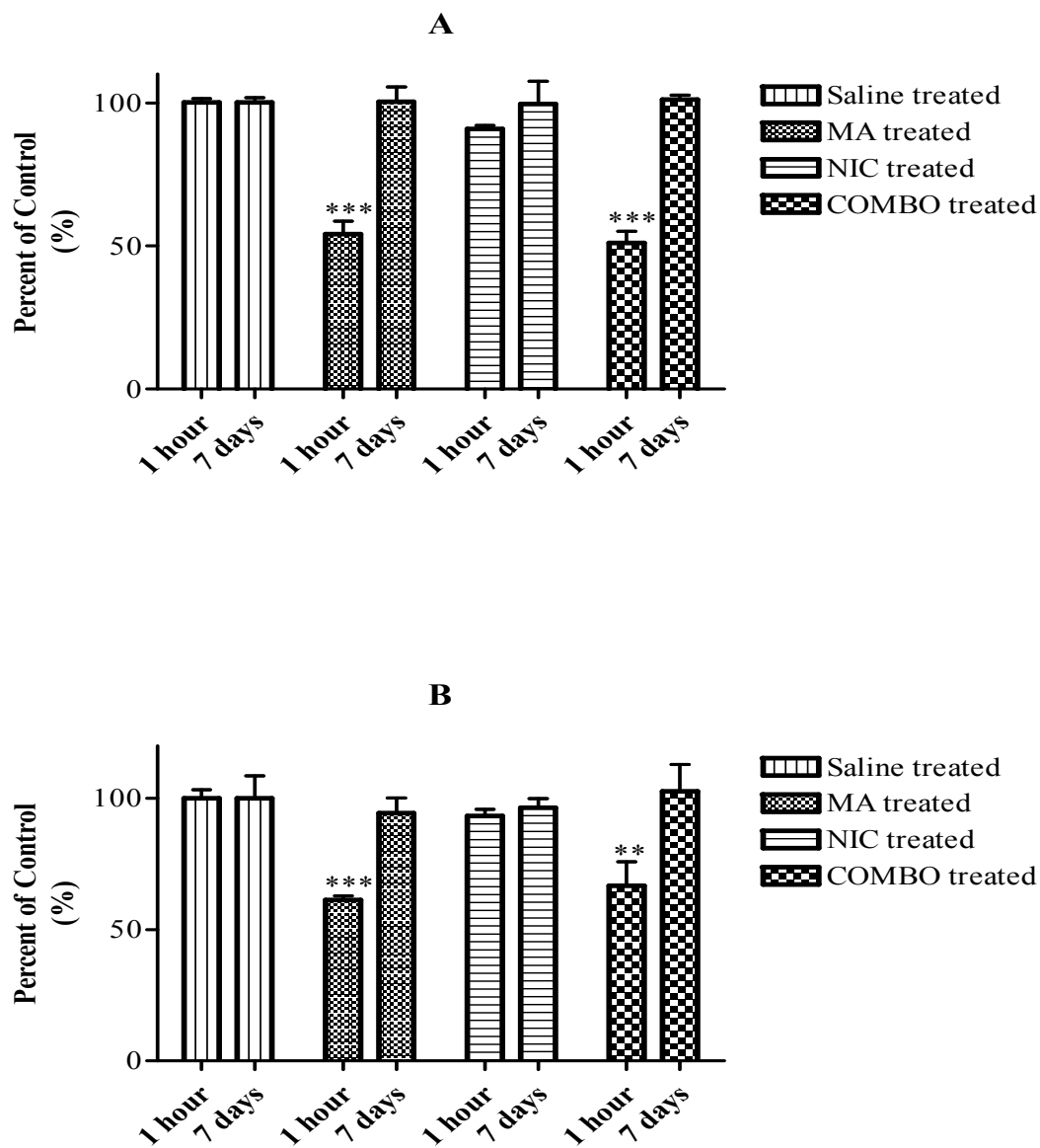


Figure 4-12. [³H]GBR12935 binding values (apparent B_{max}) as determined from nonlinear regression analysis expressed as percent of control (%) at the 1hrPT and 7dayPT. (** p < 0.01; ***p < 0.001) Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC.

Table 4-2. GBR binding data. Bmax-Specific binding (fmol/mg protein); Percent of control (%)

Treatment	Bmax-Specific binding (fmol/mg protein)		Percent of control	
	1hrPT	7dayPT	1hrPT	7dayPT
Striatum				
Saline	5456±77	4897±88	100±3.2	100±8.5
MA	2951±244***	4911±256	61±1.5	94±5.8
NIC	4955±74	4878±384	93±2.5	96±3.5
COMBO	2781±225***	4948±80	66±9.1	102±10.3
Nucleus Accumbens				
Saline	2837±90	2704±230	100±1.4	100±1.8
MA	1739±43***	2550±156	54±4.4	100±5.2
NIC	2647±72	2607±96	91±1.3	99±7.8
COMBO	1895±257**	2775±28	51±4.1	100±1.6

Table 4-3. Dopamine uptake and GBR binding comparison. The comparison of mean ± SEM values from four independent experiments, each conducted in duplicate for dopamine uptake and GBR12935 binding. The data has been expressed as percent of control after 1hrPT regimen. Dopamine uptake and binding was significantly different from controls (*p < 0.05; **p < 0.01; ***p < 0.001) as evaluated by ANOVA. (n = 4)

Treatment	STR Uptake 1hrPT	STR Binding 1hrPT	ACC Uptake 1hrPT	ACC Binding 1hrPT
MA	60±10**	54±4*	71±6***	61±1***
COMBO	58±7**	51±4*	74±4***	67±9**

4.3.3 [³H]Raclopride binding. The potent and selective antagonist of dopamine D₂ receptors, [³H]Raclopride, was used to detect changes in the density of the D₂ receptors on rat STR and ACC membranes. Cold saturation curves (figures 4-13 and 4-14) from membranes of 1hrPT and 7dayPT animals were examined over a concentration range of 0.01nM to 5μM unlabelled raclopride. Cold saturation studies failed to demonstrate an alteration in the IC₅₀ values or apparent B_{max} values between any of the treatment groups or time points (figure 4-15 and figure 4-16). Results presented in figure 4-17 and table 4-4 represent the transformed data from figures 4-13 and 4-14 and demonstrate the approximate density of DA D₂ receptors present on the membranes expressed as % control (fmol/mg protein).

Our findings were not consistent with Bennett et al., 1997, where they demonstrated approximately a 40% reduction in [³H]raclopride binding sites, which reportedly represented the DA D₂/D₃ receptors present in midbrain cell cultures, one week after five days of cell culture treatment with 10 μM MA. This value was noted to represent the entire D₂/D₃ receptor population in the midbrain cultures, including the autoreceptors on dopaminergic cells. However, our studies were in agreement with studies done by Dewar et al. (1989), where they observed a B_{max} value of 364±20.3 fmol/mg protein in rat caudate-putamen, compared to our results, which yielded 372±42 fmol/mg protein in the STR and 253±24 fmol/mg protein in the ACC. Additionally, the IC₅₀ values that were determined in our studies (16±6 nM) were in accordance with others reported in Kohler et al. [26 nM] (1985).

The presented data was analyzed with a nonlinear computer-fitting program to estimate apparent Bmax and IC₅₀ values. Best-fit models were determined by the F test (Munson and Rodbard, 1980). This data was analyzed by two-way analysis of variance (ANOVA) (Winer, 1971), followed by Bonferroni post hoc comparison, where appropriate, before data transformation to percent of control for clarity of presentation. Results were considered significant when P was < 0.05. All data analysis was performed using GraphPad Prism Version 4.00 for Windows, (GraphPad Software Inc., San Diego, CA).

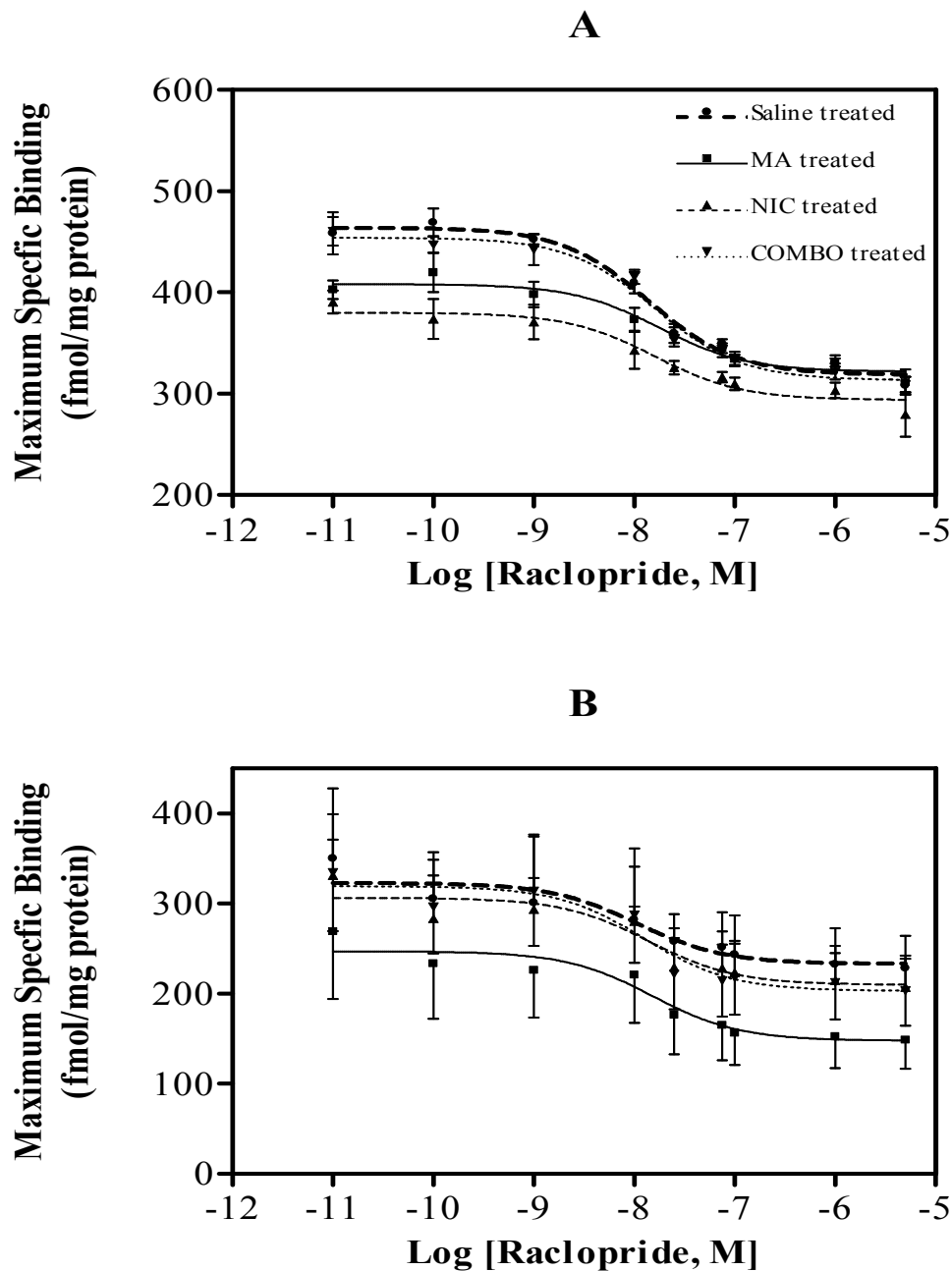


Figure 4-13. [³H]Raclopride binding in the striatum. [³H]Raclopride binding studies were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh STR synaptosomal preparations over a concentration range of 0.01nM to 5 μ M of unlabelled raclopride. Data represents the mean \pm SEM of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

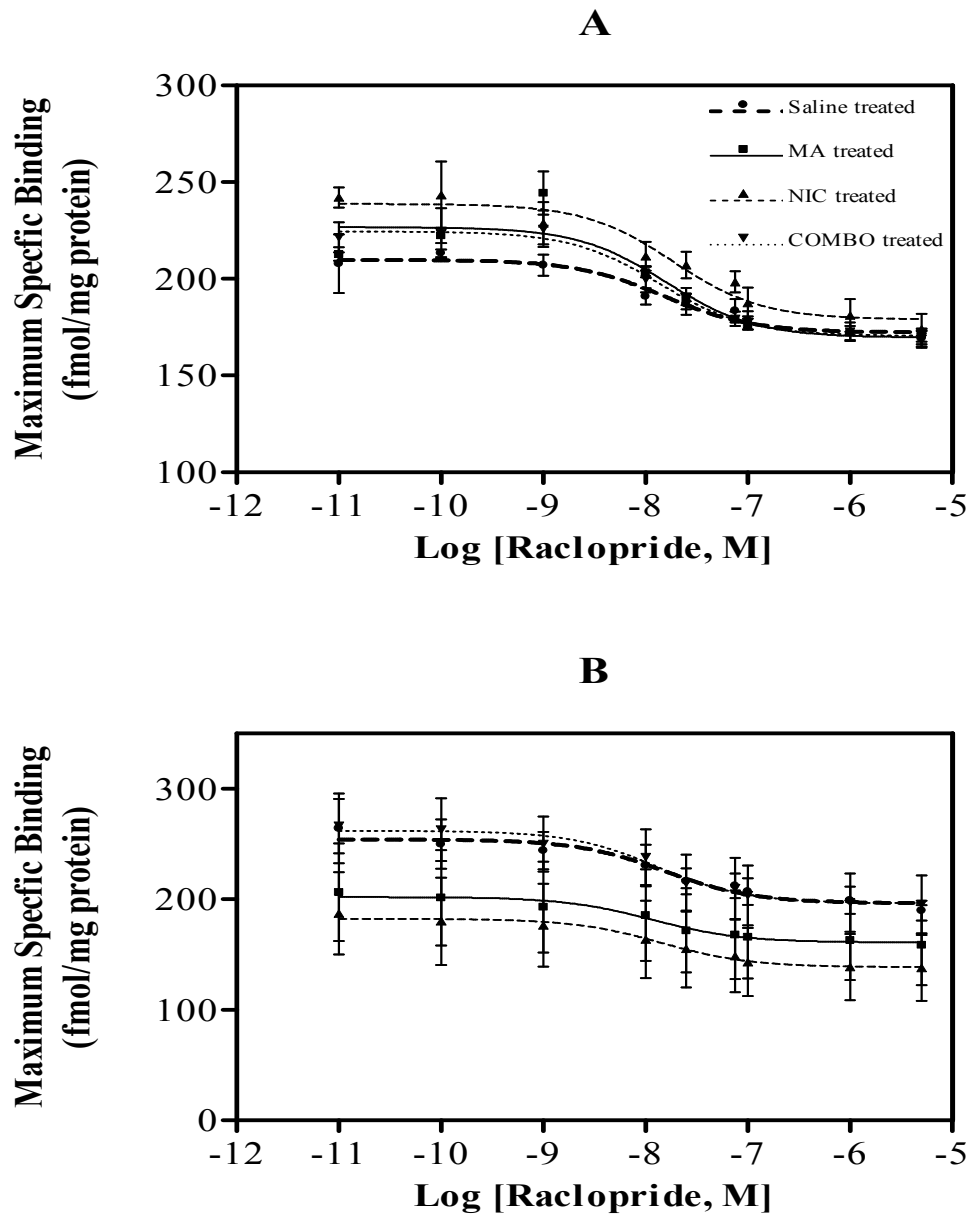


Figure 4-14. [^3H]Raclopride binding in the nucleus accumbens. [^3H]Raclopride binding studies were performed and competitive inhibition curves generated for the treatment groups listed. Results were obtained from fresh ACC synaptosomal preparations over a concentration range of 0.01nM to 5 μM of unlabelled raclopride. Data represents the mean \pm SEM of four independent experiments, each conducted in duplicate. (A) 1hrPT (B) 7dayPT

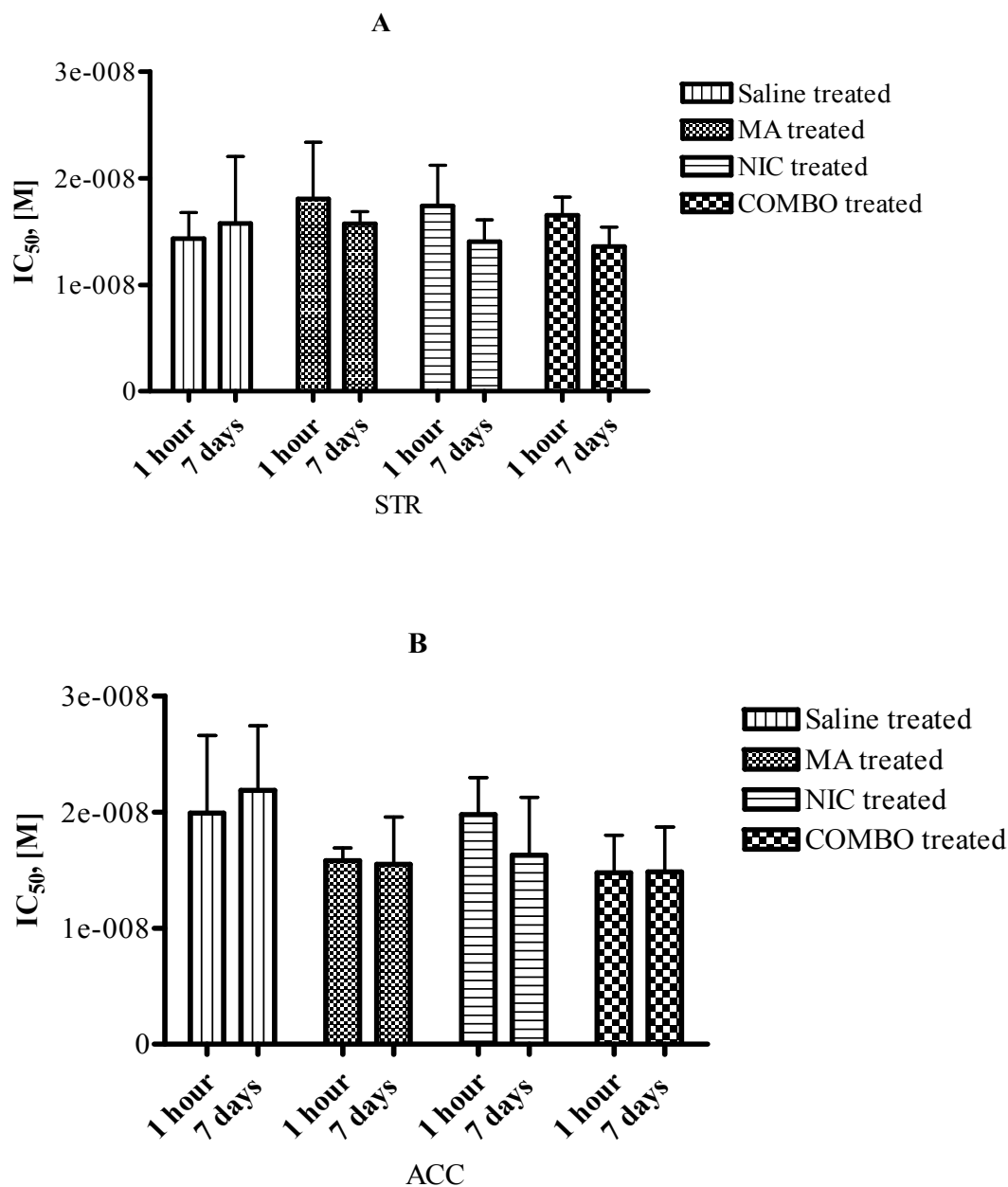


Figure 4-15. [³H]Raclopride binding IC₅₀ values. [³H]Raclopride binding IC₅₀ values as determined from nonlinear regression analysis expressed as molar equivalent at the 1hrPT and 7dayPT. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

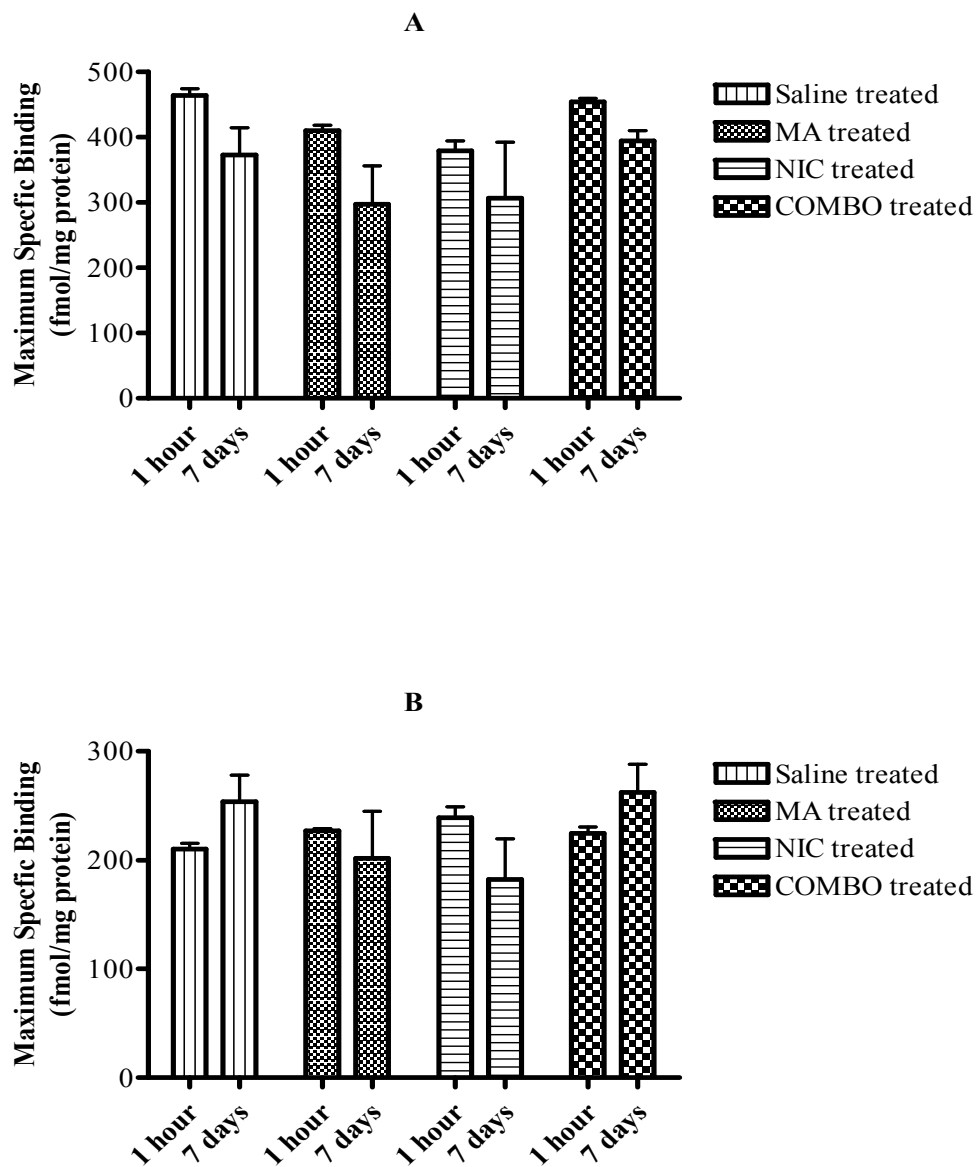


Figure 4-16. [³H]Raclopride apparent B_{max} values expressed as maximum specific binding. [³H]Raclopride apparent B_{max} values as determined from nonlinear regression analysis expressed as maximum specific binding (fmol/mg of protein) at the 1hrPT and 7dayPT. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

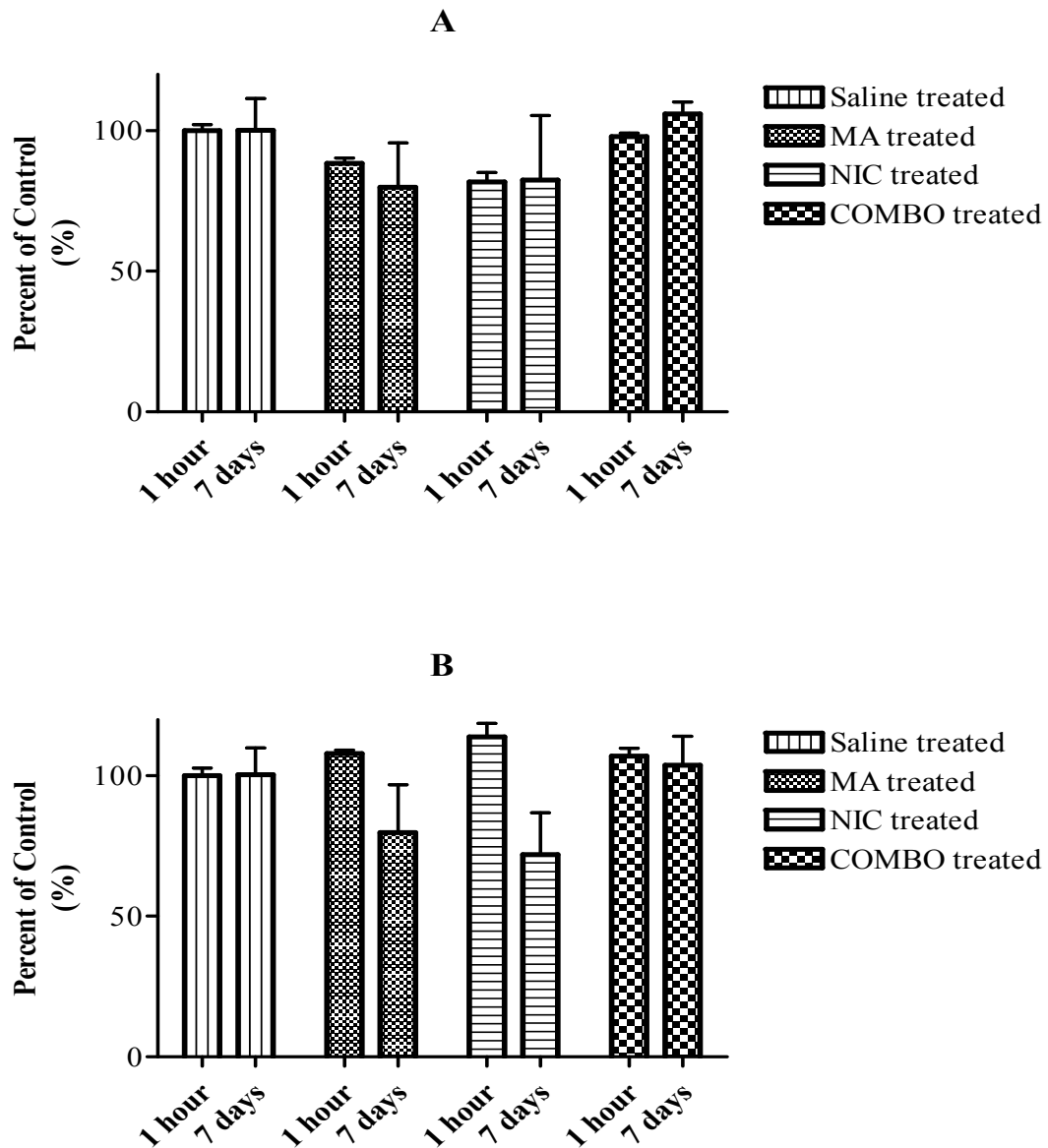


Figure 4-17. [³H]Raclopride apparent B_{max} values expressed as percent control. [³H]Raclopride binding values (apparent B_{max}) as determined from nonlinear regression analysis expressed as percent of control (%) at the 1hrPT and 7dayPT. Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

Table 4-4. Raclopride binding data.

Treatment	Bmax-Specific binding (fmol/mg protein)		Percent of control (%)	
	1hrPT	7dayPT	1hrPT	7dayPT
Striatum				
Saline	464±10	372±42	100±2	100±11
MA	409±8	297±56	89±2	80±15
NIC	379±15	306±80	92±4	83±17
COMBO	454±5	394±15	98±1	100±4
Nucleus Accumbens				
Saline	210±5	253±24	100±3	100±11
MA	226±4	201±42	107±2	80±16
NIC	238±10	182±37	103±5	80±15
COMBO	224±6	262±26	106±3	103±10

4.3.4 [³H]dopamine release. The final studies sought to determine the effects of the various drug treatments on the release profile of the STR and ACC tissue slices. The DA release potential was assessed in both brain regions at 1hrPT and 7dayPT time points by preloading each set of tissue slices with [³H]DA. The slices were then perfused for 30 minutes at a flow rate of 0.6 ml/min. in a wash out stage using release buffer.

A low, stable baseline of basal release (approximately 1%/min.) was established. After eight minutes (fraction 4), tissue was stimulated to release [³H]DA by a two-minute exposure to 30 mM KCl. The perfusion solution was returned to the non-stimulating releasing buffer for an additional 16 minutes, allowing the [³H]DA release to return to the basal level. Fractions were collected in two-minute increments. Release was reported as fractional release as previously described in Chapter 2.

Data were expressed as radioactivity released during the collection interval (fractional release, percent). The highest amount of fractional release was seen in fraction number 6. The peak release of [³H]DA (fraction 6) was used to compare treatment groups for the statistical analysis. Additional data analysis was done utilizing the sum of fractions 5-9, which would approximate an area under the curve (AUC) for the total amount of [³H]DA released in response to the stimulus. Finally, each of the treatment groups' AUC was compared to the average saline (control) treated group's AUC and reported as percent of control. Statistical significance was determined by utilizing a two-way ANOVA (Winer, 1971), followed by Bonferroni post-hoc comparison. Statistical significance was assumed at P values less than 0.05. All data analysis was performed using GraphPad Prism Version 4.00 for Windows, (GraphPad

Software Inc., San Diego, CA). All data were analyzed prior to transformation to percent control for graphical representation.

While the overall incorporation of [^3H]DA should be lower in MA and COMBO treated animals, because of the decrease in [^3H]DA uptake demonstrated in the DAT studies, this does not effect the fractional DA release, which is a ratio of the counts in the fraction to the total counts in the tissue. The reduction in [^3H]DA release observed after MA or COMBO treatments would not depend on the amount of incorporation of [^3H]DA into the cell, but the ratio of that released to that remaining in the cell.

[^3H]DA release stimulated by KCl (30mM) was 24% and 34% (STR and ACC, respectfully) of total radiolabeled DA in the saline treated brain slices, which will be used as the control value (figure 4-18 and table 4-5). In STR, the 1hrPT MA and COMBO treated tissue released 17% and 10% of the loaded [^3H]DA, a 30% and 60% reduction from control values, respectfully (figure 4-18A). Similar decreases were observed in ACC tissue with 1hrPT MA and COMBO treated ACC tissue values being 17% and 12%, a 50 and 65% reduction from control values, respectfully (figure 4-18B). The observed decreased [^3H]DA release appeared to be a temporary event in that both STR and ACC tissue release values returned to control values at the 7dayPT time point.

The peak fractional release values are presented in figure 4-18, figure 4-19, and table 4-5. In addition to the peak release values, figure 4-20, figure 4-21 and table 4-5 also represent the sum of fractions 5-9, which represents the total amount of [^3H]DA released in response to the stimuli. The sum of fractions 5-9 [^3H]DA release stimulated by KCl (30mM) in the saline treated tissue was 24% and 34% (STR and ACC, respectfully) of the total radiolabeled DA. A reduction of 31% and 32% was seen at the

1hrPT in STR and ACC in the MA treated rats, and a 52% and 48% reduction in the COMBO treated rats, respectfully. These values also returned to control values at the 7-day time point. The author utilized the total release (sum of fractions), instead of the peak release, to form an opinion about the outcome of the data, because it offers a more uniform measurement of the [³H]DA release.

The basal levels of [³H]DA release were reported in figure 4-22 as percent of fractional [³H]DA release. Figure 4-19 is the basal levels of [³H]DA release expressed as percent of control with the summary of the basal release data presented in table 5-5. Statistical differences were seen in both MA and COMBO treated groups in both STR and ACC at the 1hrPT time point. These differences were not seen in any of the groups at the 7dayPT time point as the basal release in both groups in both brain regions returned to the control value.

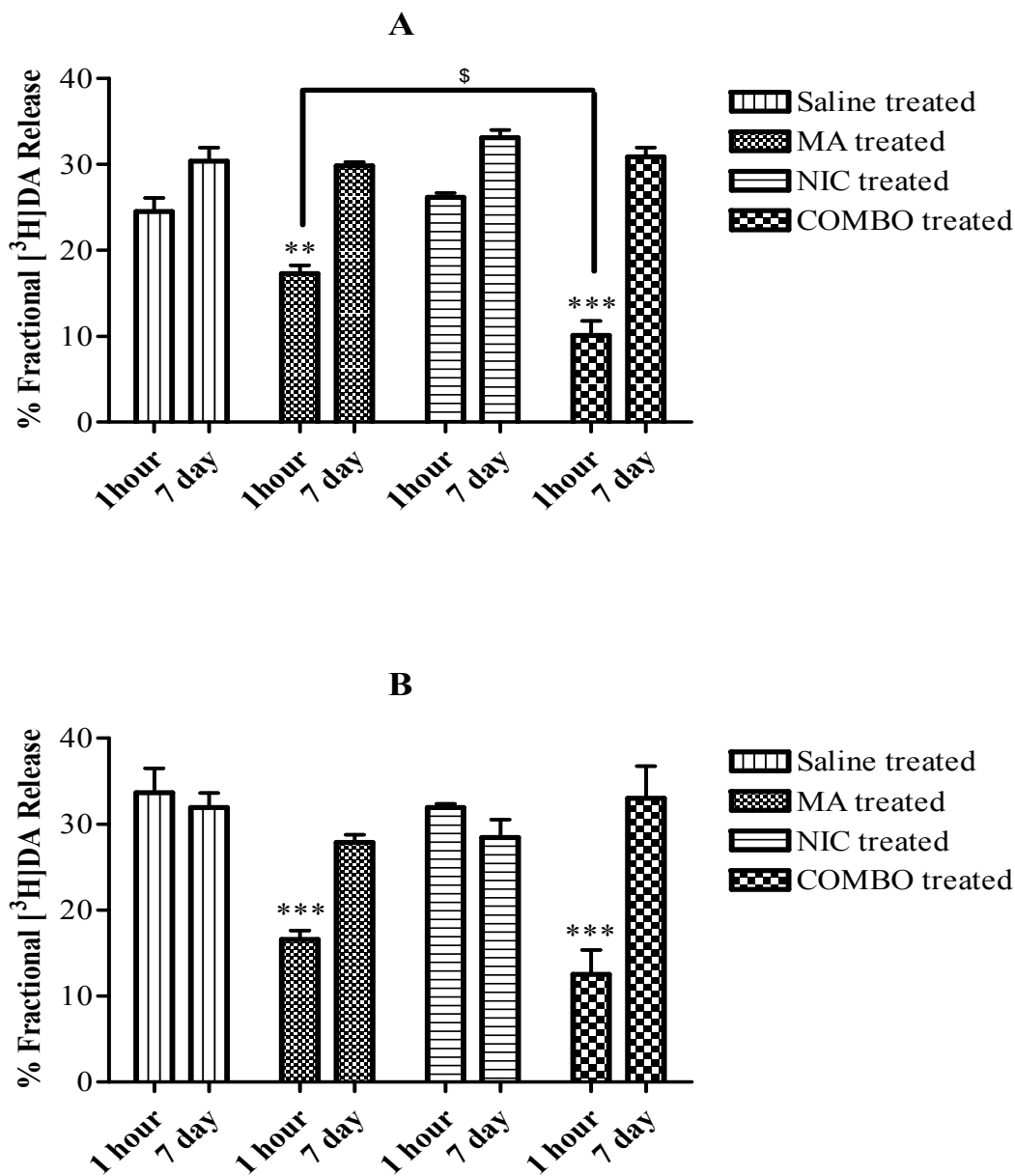


Figure 4-18. Evoked [³H]dopamine release expressed as percent of fractional [³H]dopamine release. Peak fractional [³H]DA release (expressed as percent of fractional [³H]DA release) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly decreased 1hrPT but not 7dayPT. (** p < 0.01; *** p < 0.001 = statistically different from the control group; § = statistically different from each other, p < 0.05) Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

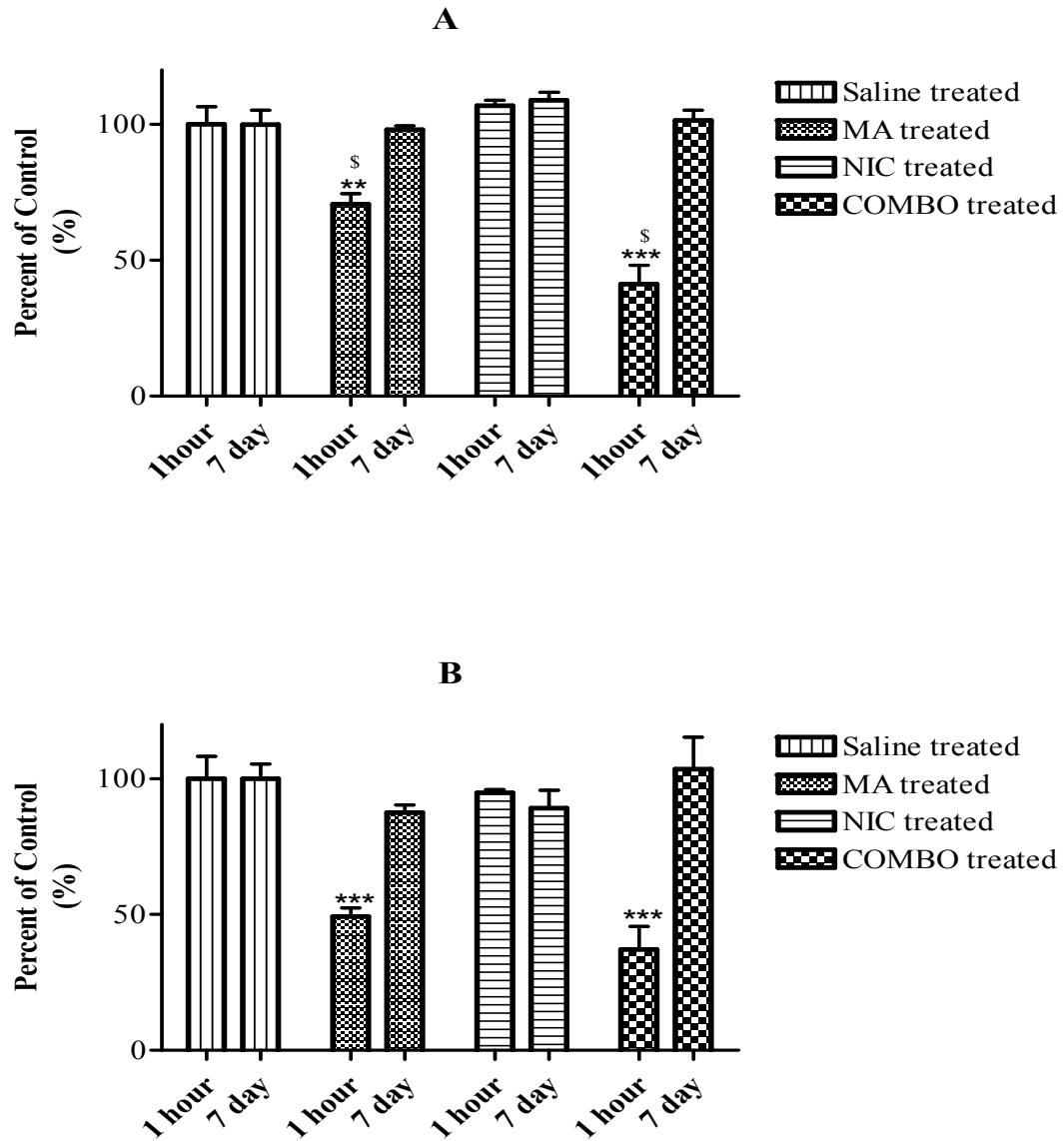


Figure 4-19. Evoked peak fractional [³H]dopamine release expressed as percent of control. Peak fractional [³H]DA release (expressed as percent of control) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly decreased 1hrPT but not 7dayPT. (** p < 0.01; *** p < 0.001 = statistically different from the control group; [§] = statistically different from each other, p < 0.05) Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

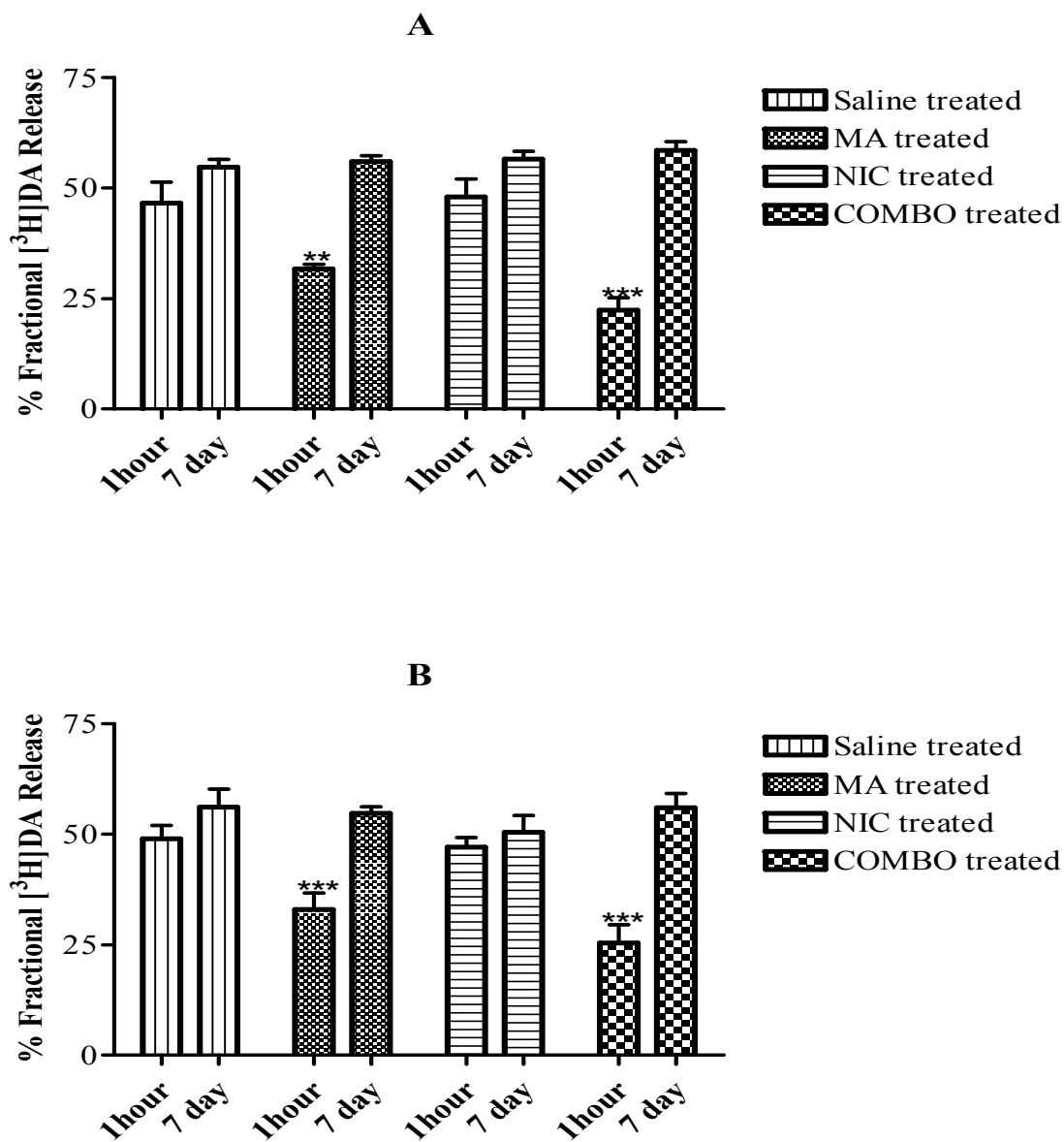


Figure 4-20. Sum of fractions 5-9 evoked [³H]dopamine release expressed as percent of fractional [³H]dopamine release. Sum of fractions 5-9 [³H]DA release (expressed as percent of fractional [³H]DA release) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly decreased 1hrPT but not 7dayPT. (***) p < 0.001) Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

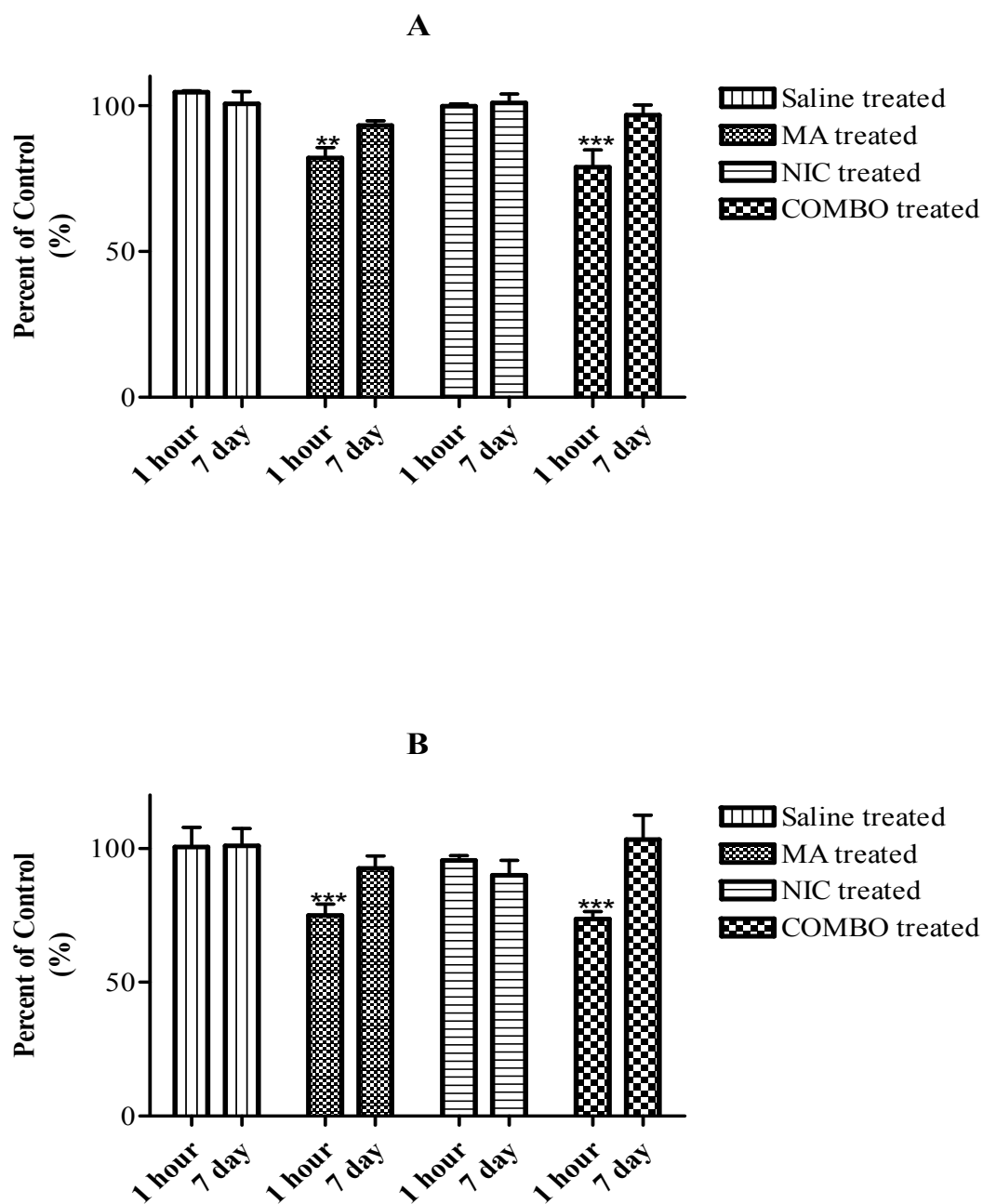


Figure 4-21. Sum of fractions 5-9 evoked [³H]dopamine release expressed as percent of control. Sum of fractions 5-9 [³H]DA release (expressed as percent of control) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly decreased 1hrPT but not 7dayPT. (***) p < 0.001) Each bar represents the mean (±SEM) of 4 samples run in duplicate. (A) STR (B) ACC

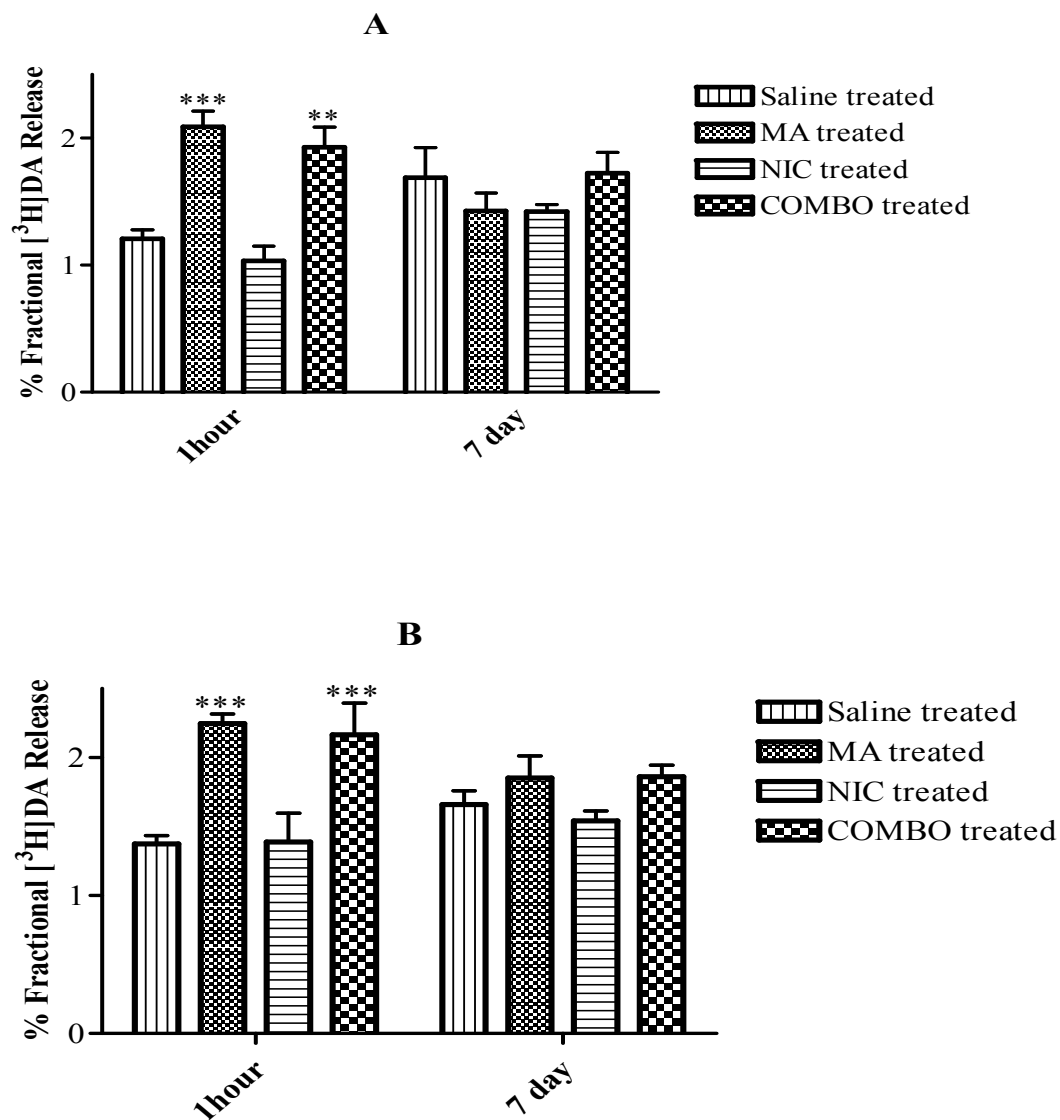


Figure 4-22. Basal [³H]dopamine release expressed as percent of fractional [³H]dopamine release. Basal [³H]DA release (expressed as percent of fractional [³H]DA release) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly increased at 1hrPT but not 7dayPT (***) p < 0.001). Each bar represents the mean (±SEM) of 4 samples run in duplicate.(A) STR (B) ACC

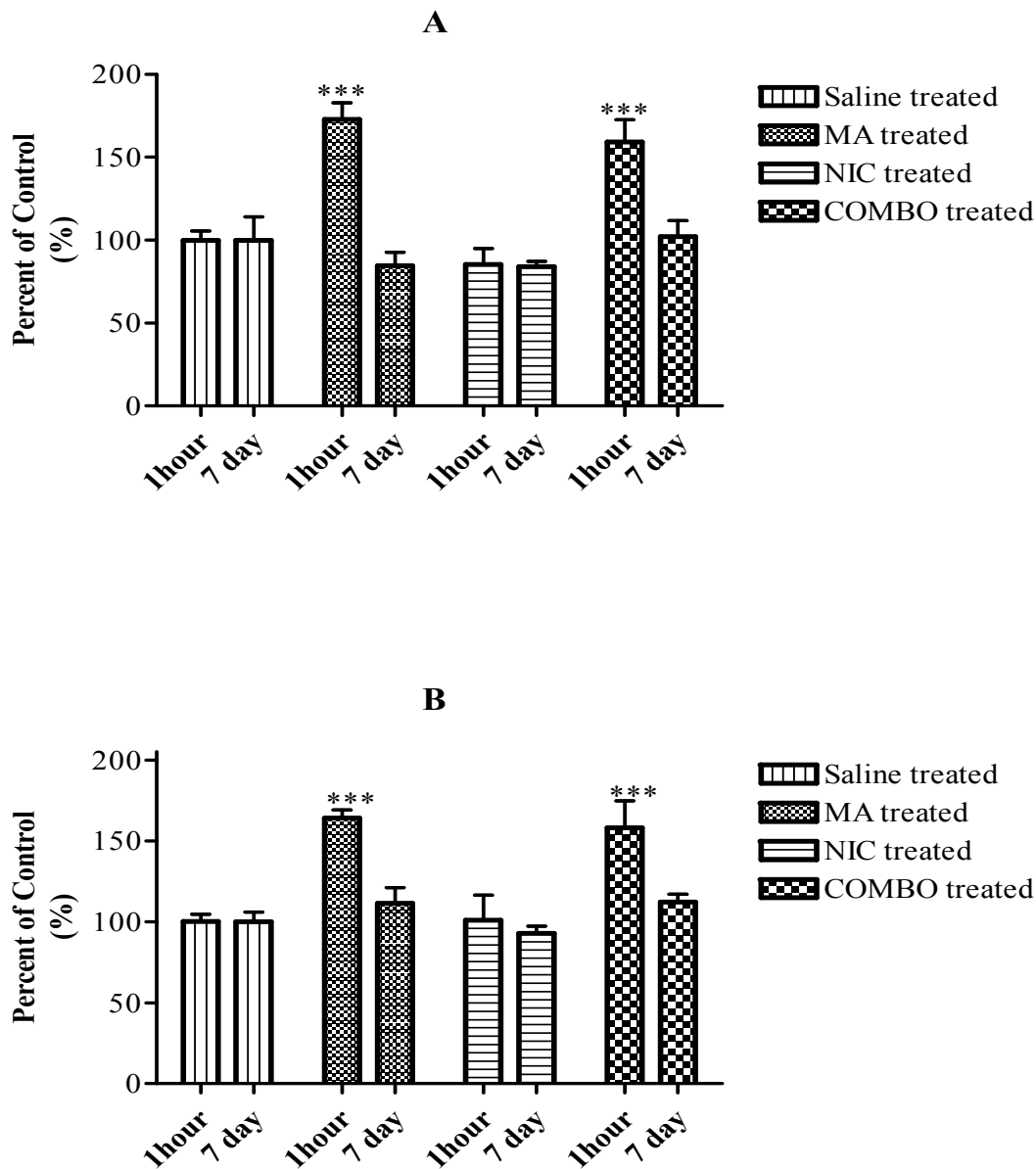


Figure 4-23. Basal [³H]dopamine release expressed as percent of control. Basal [³H]DA release (expressed as percent of control) either at 1hrPT or 7dayPT. Releasing agent was 30 mM KCl. [³H]DA release was significantly increased at 1hrPT but not 7dayPT (***) $p < 0.001$). Each bar represents the mean (\pm SEM) of 4 samples run in duplicate. (A) STR (B) ACC

Table 4-5. Comparison of evoked dopamine release and basal dopamine release.

Fractional Release Values

Treatment	Fraction 6 (%)		Sum of Fractions 5-9 (%)		Percent of Control (%)	
	1 hour	7 days	1 hour	7 days	1 hour	7 days
Striatum						
Saline	24±1.6	30±1.6	46±4.8	54±1.8	100±6.5	99±5.2
MA	17±0.9**,\$	30±0.4	32±1.0**	56±1.2	70±3.8	98±1.4
NIC	26±0.5	33±0.9	48±4.1	57±1.7	106±2.0	108±2.8
COMBO	10±1.7***,\$	30±1.1	22±2.8***	58±2.0	41±6.9	101±3.6
Nucleus Accumbens						
Saline	34±2.8	32±1.7	48±3.0	56±4.0	100±8.2	100±5.3
MA	17±1.0***	28±0.8	33±3.7***	54±1.5	49±3.1	87±2.8
NIC	32±0.4	28±2.1	47±2.2	50±3.7	95±1.3	90±6.5
COMBO	12±2.8***	33±3.7	25±4.1***	55±3.2	37±8.5	103±11.6

Basal Release Values

Treatment	Mean Basal Release of Fractions 1-4 (%)		Percent of Control (%)	
	1 hour	7 days	1 hour	7 days
Striatum				
Saline	1.4±0.06	1.66±0.10	99±5.6	100±14
MA	2.2±0.07***	1.85±0.16	173±10.1	85±8.1
NIC	1.4±0.21	1.54±0.07	85±9.6	84±3.3
COMBO	2.2±0.23***	1.86±0.08	159±13.1	102±9.7
Nucleus Accumbens				
Saline	1.21±0.06	1.68±0.24	100±4.5	100±6.0
MA	2.09±0.12***	1.43±0.14	164±5.0	111±9.6
NIC	1.03±0.12	1.42±0.05	101±15.2	93±4.4
COMBO	1.93±0.16**	1.72±0.16	158±16.7	112±4.9

4.4 Discussion

The reinforcing or addictive effects of MA are closely associated with their ability to enhance DA transmission within the mesocorticalimbic DA system, which extends from the ventral tegmental area to terminal regions in the nucleus accumbens (Kuhar et al., 1991; Roberts et al., 1977). Transport into the presynaptic terminal is the primary mechanism for terminating the effects of released DA (Giros et al., 1996). The majority of biochemical and pharmacological studies characterizing DA transport in the rat brain have utilized synaptosomes obtained primarily from the dorsal STR with few from the nucleus accumbens (Williams and Steketee, 2004). Substantial animal data now implicate the nucleus accumbens as a critical target in the mechanism of action of all drugs of abuse, including MA (Robinson and Berridge, 1993; White and Kalivas, 1998; McLeman et al., 2000).

The current studies were designed to examine the ability of MA co-administered with NIC to alter the release of DA, modify DA D₂ receptors, and to identify any concomitant changes in DA transport or transporter number not only in the STR, but also the ACC. Results presented in Table 4-1 demonstrate that [³H]DA uptake into STR and ACC synaptosomes, prepared from rats decapitated one hour after MA and COMBO treatment (three 5mg/kg i.p. injections in two hour increments), was decreased by 40% in STR and 30% in ACC, without modification of the apparent affinity (data not shown). Additionally, studies utilizing STR and ACC synaptosomes, prepared from rats decapitated 7 days after MA administration (described above), appeared to return to normal and did not deviate from control values. However, in the STR, the rats receiving the COMBO treatment did not return to normal saline values at the 7dayPT. There was a

significant difference between the MA and COMBO treated animals which received the same amount of MA in each injection. Therefore, one can infer that the co-administration of the two drugs together prevented the uptake from returning to normal. The ACC region showed no significant difference between the MA and COMBO treated animals at the 7dayPT and all values essentially reached the level of the saline control group.

Similar studies by Fleckenstein et al. (1997) demonstrated that similar effects are unrelated to residual levels of MA introduced by the treatment, as evidenced by the findings that MA concentrations in the synaptosomal preparations were less than 1% of the concentration required to decrease synaptosomal [³H]DA accumulation by 50% (Fleckenstein et al, 1997a;b).

The functionality of the DAT in each of the tissues (STR, ACC) at two different time points (1hrPT and 7dayPT) was assessed by determining IC₅₀ values and the apparent (Vmax). The Vmax indirectly assesses the specific rates of [³H]DA uptake. There was no difference in receptor affinity, as demonstrated indirectly by the constant IC₅₀ values, which did not differ significantly from control values. A 40% and 42% reduction in specific [³H]DA uptake was observed one hour after the last MA administration in STR and ACC, respectfully. The alterations in the apparent Vmax values as seen in figure 5-5 and reported in table 5-1 indicated a decrease in the specific rate of transport. Similar findings were reported by Nakayama et al. (1993), which demonstrated a 36% reduction in specific [³H]DA uptake in the STR 7 days after the last MA administration. These findings were determined after an increasing dose paradigm of MA lasting over one week. This data lead us to believe that the initial decreases we

observed with multiple doses on a single day may be sustained for up to a week, with a more chronic administration schedule.

The number of transport sites was found to be decreased, in addition to the alteration of specific [³H]DA uptake velocity changes. This was evidenced utilizing the highly selective dopamine uptake inhibitor GBR12935. Results of the 1hourPT MA and COMBO treated animals [³H]GBR12935 binding studies revealed a 46% and 29% reduction of transporter density in STR and ACC, respectfully. These are similar proportions to reductions seen in the uptake studies (Table 4-3) and to previously reported values (29% reduction in STR GBR12935 binding 7 days post last MA administration) by Nakayama et al. (1993).

The decrease in [³H]DA transport along with the modification of DAT protein density would indicate that a modification of the expression of the DAT had taken place. MA has previously been shown to rapidly and reversibly decrease the dopamine transporter activity. This change has been attributed to high doses of MA and its ability to promote free radical formation and inactivate the DAT thru oxidative events (Fleckinstein et al., 1997b; 1999). Our studies do not further elucidate the mechanism of this change; they do show that MA-induced dopaminergic changes in [³H] DA uptake and DAT density as demonstrated by specific [³H] DA uptake and GBR12935 binding studies are not altered significantly with the co-administration of NIC. Furthermore, our studies provide data to substantiate similar reductions in [³H] DA uptake and DAT density within two separate areas of the rat brain, the STR and ACC.

Regulation of DA transport function involves many factors, including phosphorylation or glycosylation of transporter protein, alteration of the membrane

potential, the fluidity state of the membrane, or modification of sulfhydryl groups (Povlock et al., 1996); all of which would alter transporter affinity. Our findings, which demonstrate a decrease in transport velocity (V_{max}) and density of transporter sites (B_{max}) with no change in affinity (apparent IC_{50}), leads us to believe that the transporter modification seen in our studies are not likely in response to the above listed factors.

Bennett et al. (1997) reported transporter function studies that contradicted our findings. The Bennett group demonstrated alterations in DA uptake involving transporter affinity for the substrate DA, and not changes in the number or rate of transporter sites. Their findings observed after MA treatment were admittedly reported as results not typical of a neurotoxic response, which would have resulted in a reduction in V_{max} and B_{max} , which is what our findings suggested. It is important to note the Bennett et al. (1993) studies were based upon data utilizing cell culture and our data is from rat brain synaptosomes. The controversial nature of these studies indicates the need to further understand the mechanism of DAT alteration in response to MA administration.

$[^3H]$ Raclopride was used to examine the DA D_2 receptor subtypes within the STR and ACC. The substituted benzamine drug has been shown to be a potent and selective antagonist of the DA D_2 receptors. *In vitro* studies have shown that $[^3H]$ raclopride binds with high affinity and with a low proportion of non-specific binding to rat striatal homogenates (Kohler et al., 1985). Kohler et al. (1985) demonstrated that the regional distribution of DA D_2 receptors to be greater in STR than ACC. Our $[^3H]$ raclopride binding studies revealed no significant changes in either IC_{50} or apparent B_{max} values at 1hrPT or 7dayPT. These findings suggest no alteration to DA D_2 receptor number, which contradicts work published by Bennett et al. (1997). Bennett's group reported a reduced

response to quinpirole, the DA D₂ receptor agonist, after MA treatment indicating that there were modifications in the DA D₂ receptors. Their group subsequently found these changes to be due to down regulation (Bennett et al., 1997).

Persistently elevated DA levels are known to down regulate/desensitize both pre- and post-synaptic receptors and may be responsible for DAT modifications, as well. We speculate that the controversial results are due to the length of exposure to MA. Our studies utilized one day of multiple doses in rats followed by examination at 1hourPT and 7dayPT time points. The other studies collected their data from cell cultured mesencephalic cells and exposed the cells to MA for five days. Differential treatment regimen most likely explains the differences seen in our results. It should become obvious that the mechanism of MA to alter the dopaminergic system changes with length of exposure. The current DA D₂ receptor studies yielded no significant changes or findings and it is thought that the above-described dosing regimens do not alter the receptors at either time point observed. Additional time points may need to be addressed to complete our understanding of DA receptor regulation.

The majority of non-human primate studies support the view that MA induced neurotoxicity accounts for reduced DAT binding (Villemagne et al., 1998). More recently, human studies have found that detoxified MA users have reduced DAT (Volkow et al., 2001a; Sekine et al., 2001) and DA D₂ receptor (Volkow et al., 2001b) binding in the caudate, putamen, and ACC, all of which are areas associated with psychostimulants, addiction, and pleasurable response. DAT and DA D₂ receptors are two of the most important proteins controlling extracellular DA concentrations (Torres et al., 2003; Schmitz et al., 2002). Previous reports have demonstrated enhanced drug-

induced extracellular levels of DA are associated with reinforcement (Vezina, 2004). Examining changes in DA D₂ and DAT function associated with MA and COMBO dosing regimens would most likely identify differences that would affect MA's psychostimulant effects.

The final set of studies compared the MA, NIC, and COMBO treatment regimens, with respect to [³H]DA release in the STR and ACC at 1hrPT and 7dayPT. Under experimental conditions used in the present study, release of preloaded [³H]DA appears to be of neuronal origin and to have physiological relevance. Although the radioactivity measured in the collected fractions may consist of a mixture of neurotransmitters and metabolites, the amount of tritium released from rat brain slices has been previously shown to represent a close estimation of the release of labeled or endogenous DA release (Parker and Cubeddu, 1985; Herdon and Nahorski, 1987). Furthermore, the release of metabolites, during the superfusion, was inhibited by the presence of the monoamine oxidase inhibitor pargyline in the superfusion buffer (Zumstein et al., 1981).

The stimulated release of DA from nerve terminals can take place by a number of mechanisms. The primary mode of release is by depolarization-evoked exocytosis from the vesicular pool, in which depolarization of the terminal membrane activates voltage-dependent Ca⁺⁺ channels; the resulting influx of extracellular Ca⁺⁺ then mediates the release process (Raiteri et al., 1978; Prince et al., 1996; Dobrev and Andreas, 1997). Depolarization can be produced by increasing the concentration of extracellular K⁺, which alters the membrane potential by an amount determined by the Nernst equation. This method was used in our studies to evoke the release of the preloaded [³H]DA. Dopamine can also be released from the cytoplasmic pool (Kalivas and Duffy, 1991;

Heeringa and Abercrombie, 1995). Amphetamine and MA have also been shown to produce reversal of the dopamine transporter, which causes DA efflux from the nerve terminal (Piffl et al., 1995; Giros et al., 1996)

The results of our studies demonstrate the ability of MA and COMBO treatment to alter the release of [³H]DA from nerve terminals in the STR and ACC. Analysis of the amount of [³H] DA released at its peak (fraction 6), after release triggered by 30 mM KCl, demonstrates a statistically significant 30% and 59% decrease from control values at 1hrPT in STR and ACC, respectfully. Additionally, statistical difference was determined between the MA and COMBO treatment groups. Additional analysis of the total amount of [³H]released (fractions 5-9) in response to the stimulus fails to show a difference between the two treatment groups, yet, still demonstrates the difference between each of the treatment groups and the control values.

Data from the 1hrPT ACC studies is similar to the STR studies, in that the MA and COMBO treatment groups reduce [³H] DA release to 51% and 63%, respectfully. Both the STR and ACC studies show that [³H]DA release returns to control values at the 7dayPT time point (figure 4-18, figure 4-19, and table 4-5). The investigator believes that the difference found between the MA and COMBO treated groups in the 1hrPT STR fractional release studies most likely represents a more rapid rise in [3H] DA release, and that the sum of fractions 5-9 confirms that there is difference between control values but not between the two treatment groups (table 4-3).

Previous research has shown sub chronic exposure to amphetamine reduces basal concentrations of DA in the ACC (Rossetti et al., 1992; Weiss et al., 1992, 1997; Gerrits et al., 2002). Under the experimental conditions of the present study, basal release of

[³H]DA was noted to be increased in MA and COMBO treated animals at the 1hrPT time point. The MA and COMBO treatments in the STR and ACC were elevated 73% (MA-STR), 59% (COMBO-STR), 64% (MA-ACC), and 58% (COMBO-ACC) (table 5-3). The aforementioned studies are not extensive enough to elucidate the mechanism for the rise in basal [³H]DA release seen with MA and COMBO treated animals. The authors can conclude, based upon the 7dayPT data that, whatever the cause, it is reversible.

Admittedly, it is difficult to speculate on the cause of the increase in basal [³H]DA release. The overall data generated with the current studies represents a reduction in DAT function and density at 1hrPT with the return to normal at 7dayPT. This would lead most to postulate a reduction in basal [³H]DA release; that is not the case. The demonstrated changes at 1hrPT with return to normal at 7dayPT in the DAT and [³H]DA release studies leads one to believe there may be an association between the two. The observed decrease in DAT activity with an increase in basal [³H]DA release in the studies falls short of explanation. Mechanisms whereby MA decreases the DAT activity have not been elucidated fully, though MA-induced hyperthermia has been associated with similar changes (Metzger et al., 2000). Further studies will be needed not only to elucidate the mechanism of DAT alteration, but also to determine the changes associated with basal DA release.

In conclusion, these studies examined DAT density and function, DA D₂ receptor binding, and [³H]DA release in two brain regions at two separate time points and were unable to substantiate our hypothesis that the co-administration of NIC would enhance the psychostimulant effects produced by MA. Future studies proposed will examine the

ability of MA to decrease the reactive oxygen species produced by MA administration, in hope of finding a neuroprotective effect associated with NIC and MA co-administration.

Chapter V

DISCUSSION

5.1 Introduction

The studies previously described utilized well-established enzyme inhibitor kits to identify alterations within two major CYP450 enzymes, CYP3A4 and CYP2D6. Additionally, two dopaminergic brain regions were investigated using [³H]DA uptake, [³H]GBR-12935 binding, and [³H]raclopride binding to assess DA uptake, DAT density, and DA D₂-like receptor density changes, respectfully. These studies were used to identify neurochemical and metabolic changes associated with the co-administration of NIC and MA.

The first set of studies helped to substantiate human CYP2D6 and rat CYP2D2 as the primary metabolic enzymes of MA. Additionally, these studies demonstrated that CYP3A4, one of the other major human metabolic enzymes, played little role, if any, in the metabolism of MA. Furthermore, metabolic changes associated with the co-administration of these two drugs resulted in the COMBO treated animals metabolism returning to control values significantly faster than when administered MA alone.

Neuropharmacological studies demonstrated alterations in DA uptake and the density of the DAT protein. Yet, no changes were seen in the D₂-like DA receptor density. Studies investigating the release of DA demonstrated alterations in the evoked DA release and basal DA release. The neuropharmacological and metabolic changes

associated with NIC when co-administered with MA vs. MA alone can be summarized as a prolonged decrease in functional [³H]DA uptake within the STR, a decrease in the evoked [³H]DA released, and a decrease in the inhibitory action on the CYP2D2 isozyme in rat liver as demonstrated by the COMBO treatment group displaying a more rapid return to saline control values than the MA treatment group. This set of studies has demonstrated pharmacological changes associated with NIC co-administration but has also lead the investigator to consider more indirect mechanisms of psychostimulant enhancement. Additionally, one may consider the possibility that NIC instead of psychostimulant enhancement may prevent adverse effects from occurring.

5.2 Methodological considerations and results

5.2.1 Drug composition. The entire premise of this project is that illicit MA is not a pure substance. Adulteration of street drugs to increase profit is not a new concept to law enforcement. Essentially, a person can either purchase, or make a certain amount of illicit drug, and then dilute it with a cheaper non-controlled substance and tremendously increase their profit margin. Over the course of one month, 54 drug samples provided by the Tulsa Police Department forensic laboratory that had previously been identified as compounds containing MA were examined. These street-grade drugs were analyzed for the presence of various adulterants or secondary substances.

Only one adulterant was identified with a frequency of occurrence that exceeded 95%. NIC was the most common secondary product found in the MA samples that were tested. Other substances found included a variety of phthalate compounds, which are used as plasticizers and have been observed in many of the illicit drug samples previously

examined. Trace amounts of these compounds are commonly found in drug samples that have been previously stored in plastic bags (unpublished observation). Phthalates act as fixatives for perfume, slowing down evaporation and making the scent linger longer. Consumer and industrial applications range from making nail polish flexible and screwdriver handles less brittle to helping make the time-release coatings on numerous pharmaceutical products. In addition, they help make lubricants, adhesives, weather stripping, and safety glass (<http://www.phthalates.org>). It is our belief that these are not adulterants, per se, but more likely contaminants that have been leached out of the plastic bags, or other containers the drugs have been stored in.

MA samples seized from MA lab sites were not included in our analysis because this MA did not represent the average quality of illicit MA commonly distributed to the average user. It is our belief that MA from clandestine labs has yet to be diluted and would skew our data; therefore, it was not included in our sample analysis. Our studies indicate a bimodal distribution of MA sample composition with one peak at 30-60% MA and the other peak at 80-100% MA. The statistics indicate a mean of approximately 57% MA with a mode of approximately 55%. After looking at a plot of our data, we were confident that we could approximate illicit MA composition with a 50:50 mixture (MA:NIC). As previously discussed, the quality of MA that one acquires on the street depends on the illicit manufacturing process and the number of times and amounts it is adulterated.

5.2.2 Animal model. The Sprague-Dawley rat has been successfully used to investigate suspected metabolic and dopaminergic alterations for decades. Of the three main rat strains--Wister albino, Sprague-Dawley albino, and Long Evans (aka Lister

Hooded)--rats, the most common species used in the investigation of MA-induced metabolic and dopaminergic alterations is the Sprague-Dawley rat (Fischer and Cho, 1979; Krueger, 1990; Fleckenstein et al., 1999; Brown et al., 2000; Garcia-Sanz et al., 2001; Mazei et al., 2002; Davidson et al., 2005).

Additionally, Law and Moody (1994) observed differences in amphetamine metabolism in Dark Agouti rats previously found to be due to their absence of CYP2D enzymes (Al-Dabbagh et al., 1981). Several *in vitro* models studying the dopaminergic changes associated with MA administration have been published (Metzger et al., 2000; Sandoval et al., 2001; Davidson et al., 2005). Decades of supported research, along with the availability of the Sprague-Dawley rats through a breeding colony at Oklahoma State University-Center for Health Sciences, led investigators to utilize the Sprague-Dawley rats for all of the current studies, when an animal model was needed.

5.2.3 Dose and dosing paradigm. Over the last decade or so, many different dosing paradigms have been used to study the effect of MA on the dopaminergic system. Cho et al., (2001) has documented the relevance of pharmacokinetic parameters of MA in Sprague-Dawley rats, finding that the half-life ($t_{1/2}$) of MA in humans is approximately 12 hours and 70 minutes in rats, respectfully. With respect to half-lives, an interval of one day (about 20 70-minute half-lives) between injections in rats is equivalent to about 10 days (about 20 12-hour half-lives) between injections in humans (Cho et al., 2001).

Many studies have looked at the dopaminergic response to a single MA administration with doses ranging from 0.3 mg/kg to 15 mg/kg (Miller et al., 2005; Bustamante et al., 2002). Other studies have looked at dopaminergic changes associated with constant infusion using minipumps (Bustamante et al. 2002; Davidson et al., 2005).

The majority of studies looking at the dopaminergic systems and neurotoxicity utilized a dosing regimen where rats were given 10 mg/kg, four injections two-hour intervals (Cass, 1997; Kokoshka et al., 2000; Metzger et al., 2000; Riddle et al., 2002). Such a treatment regimen is very helpful in documenting toxicity, both neurochemical and behavioral.

Our initial studies utilized the standard 10 mg/kg, four injection two-hour interval dosing regimen. Our mortality rate (~50%) was fairly high in the MA and COMBO treated rats in the 1hrPT, and especially the 7dayPT groups; a recent literature search revealed similar findings in other studies. Cass (1997) reported that in the 10 mg/kg, four injection, two-hour interval studies some MA treated rats became lethargic and lost postural control during the treatment period, and that they had to be placed on cold packs for 15 to 20 minutes, to reduce their body temperature and to decrease mortality rates. Davidson et al. (2005) reported a similar mortality rate and indirectly criticized authors for not reporting the additional care required to prevent similar mortality rates. We decided to reduce the dose and number of injections, and lengthen the dosing interval early on, because of our high mortality rates. The final dosing regimen chosen was 5 mg of MA/kg (MA), 5 mg of NIC/kg (NIC), or a mixture that included 5 mg/kg of MA and 5 mg of NIC /kg (COMBO), or 0.1 ml of 0.9% saline solution. The injections were given three times at 2 hour intervals. According to Cho et al. (2001) as described above, this dosing regimen would approximate a human using MA every 2.5 days, or approximately three times per week.

After thorough consideration, we believe this dosing paradigm would more appropriately fit the average MA user, who normally does not ingest neurotoxic levels of MA, which, along with very recent research (Davidson et al., 2005), helped us

substantiate such. Davidson et al. (2005) is the first documented use of this dosing regimen. His group describes single day dosing regimens as good models of overdose in the naïve non-tolerant user (Davidson et al., 2005), a claim that would not go without criticism. Nevertheless, this dosing regimen proved to be adequate for our studies, as we observed many dopaminergic and metabolic changes we can attribute to the MA and COMBO administration.

5.2.4 High throughput kits. Cytochromes P450 are the principle enzymes for the oxidative metabolism of drugs and other xenobiotics. Among the CYP450 enzymes, five forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, appear to be the ones most responsible for drug metabolism (Spatzenegger and Jaeger, 1995). Inhibition of CYP450-mediated metabolism is often the cause of many drug interactions (Murray, 1992). In the past, the ability to assess the ability of a drug to alter CYP450 metabolism often required high performance liquid chromatography (HPLC) separation for metabolite quantitation, with a relatively time consuming and labor intensive assay.

The availability of HTS kits for CYP450 inhibition using cDNA-expressed enzymes or liver microsomes have tremendously increased the efficiency of the CYP450 inhibition studies (Parkinson, 1996). The good correlation of results obtained using recombinant CYP (rCYP) enzymes with fluorometric assays and the HPLC assays has been previously compared and it was determined that there is not much loss in data quality in the use of HTS assays, further validating the use of easier and less expensive rCYP HTS kits (Masimirembwa et al., 1999; Bapiro et al., 2001). Knowing this, and based on previous studies published on the CYP450 metabolism of MA (Lin et al., 1997; Cho et al., 1999), our investigators elected to purchase HTS inhibitor screening kits

containing insect cell microsomes (Supersomes®) prepared from human baculovirus insect cell expressing CYP2D6 and CYP3A4 individual cytochromes from GENTEST Corp. and BD Biosciences (<http://www.gentest.com>).

5.2.4.1 CYP3A4. The aim of this study was to evaluate the inhibitory effects of MA, NIC, and COMBO utilizing a novel and selective HTS assay that utilized recombinant human CYP3A4 Supersomes®. The human CYP3A4 Supersomes® findings demonstrated that the compounds under investigation did not significantly inhibit CYP3A4-mediated metabolism. Our studies are in line with a review published by Cho et al. (1999), who described the various CYP enzymes that have been shown to participate in the metabolism of MA, including various isoforms of CYP2B, CYP2C, CYP2D, and flavin-containing monooxygenase (FMO). We concluded that this isoform was not a major metabolizing enzyme of the compounds of interest.

5.2.4.2 CYP2D6. Inhibition of CYP2D6-mediated metabolism by MA, NIC, and COMBO drug treatments was determined using the novel nonfluorescent probe AMMC. Chauret et al. (2001) reported the use of AMMC as a specific CYP2D6 probe in human liver microsomes. Chauret's group demonstrated that CYP2D6 is the only observable P450 enzyme involved in the metabolism of AMMC. All other CYP450 enzymes tested yielded <1% AHMC fluorescent metabolite relative to CYP2D6. These observations imply minimal contribution of other enzymes to AMMC demethylation to AHMC under similar conditions.

Additionally, Stressor et al. (2002) tested the isoform selective probe, AMMC, in rat liver microsomes and discovered that Sprague-Dawley rat CYP2D2 also displayed high selectivity for AMMC demethylation. Based upon the previous findings that rat

CYP2D2 and human CYP2D6 displayed complete selectivity with low concentrations of AMMC, we concluded that our studies tested the inhibition of the specific CYP enzymes of interest, CYP2D2 (rat) and CYP2D6 (human). This probe proved to be a scientifically reliable and economically feasible way to compare metabolic alterations in the human rCYP and *in vivo* RLM.

Our *in vitro* studies yielded IC₅₀ values that suggest the possibility that MA has the ability to inhibit metabolism via the CYP2D6 isozymes within a physiologically relevant drug concentration range. Upon completion of the rCYP studies, rats were injected with one of four drug treatment regimens, as described above, and 1hrPT or 7dayPT rat livers were harvested and microsomes isolated for the present studies.

A two-way ANOVA (time by treatment group) of the rat liver microsomal studies demonstrated significant time and treatment affect, ($F_{(1,24)} = 6.14$; $p = 0.02$) and ($F_{(3,24)} = 16.99$; $p < 0.0001$) respectfully; however, they failed to show an overall interaction within the ANOVA, ($F_{(3,24)} = 2.43$; $p = 0.09$). Rat liver microsomal studies demonstrated a significant inhibition in the 1hrPT MA ($p < 0.001$) and 1hrPT COMBO ($p < 0.05$), as compared to the saline control group. The 7dayPT revealed that the MA treatment group was the only group that differed statistically from the saline control group ($p < 0.05$). Thus demonstrating a return to control value for the COMBO treated group. A significant difference between the MA and COMBO treated groups ($p < 0.05$) was seen using the two-way ANOVA with Bonferroni post testing. There was no statistical difference observed between the 1hrPT MA and COMBO treated RLM groups when using the two-way ANOVA with Bonferroni post hoc testing. However, a slightly more liberal statistical method utilizing a one-way ANOVA with Newman-Kuels (NK) post tests

resulted in a significant difference between the MA and COMBO treated rat groups at 1hrPT. The NK testing has been successfully used in several journal articles in similar situations to determine the difference between groups (Wan et al., 1999). This difference leads the investigator to believe that there may be a significant difference in the treatment groups, though the ultra-conservative testing parameters did not find it.

The observation of the COMBO treated group CYP2D liver enzyme functions returning to control level indicate that the CYP2D-mediated liver functions have returned to normal. Based upon these observations, one can infer that the NIC treatment may enhance the return of CYP2D-mediated metabolic function to normal. NIC has been shown to block lesions produced by the mitochondrial toxin malonate (Beal et al., 1994). NIC has also been shown to attenuate MA toxicity (Huang et al., 1997; Stephans et al., 1998). Because NIC is the precursor molecule for the electron carrier NAD, its administration would be expected to increase the number of reducing equivalents available for mitochondrial oxidative phosphorylation, improving mitochondrial energy production. If the inhibition of MA metabolism is a consequence of energy depletion, NIC should at least attenuate such deficits by improving mitochondrial energy production. This is clearly demonstrated at the 7dayPT time point where the COMBO group has returned to saline control values. Additionally, this can be speculated and supported statistically with the one-way ANOVA with NK post tests at the 1hrPT time point but not with the more conservative two-way ANOVA with Bonferroni post testing. Thus, the statistical vs. clinical significance may still be debated.

Though the present studies are not extensive enough to elucidate a specific reason for this effect, the author speculates that NIC may serve as a precursor improving

mitochondrial energy production and returning the metabolic rate to normal more rapidly. An additional theory to consider may address an increase in transcriptional activation of the CYP2D6 (human) and/or CYP2D2 (rat) enzymes leading to the increased synthesis of CYP proteins, which also would lead to a more rapid return to normal enzyme function, decreasing the intensity and shortening the duration of drug effects, as described by Dossing et al. (1983). Furthermore, either of these alterations could explain the return of CYP2D function seen at 7dayPT, as compared to the MA treatment group, which is still experiencing inhibition. The energy related theory would be more plausible if the difference seen at the 1hrPT time point is a true difference as the author expects.

Levy (1995) hypothesized that all drugs that are metabolized to a significant degree by the same enzyme are inhibited by inhibitors of that enzyme and display the same spectrum of interactions. According to Levy's hypothesis, if NIC is metabolized by the CYP2D6 enzyme system it could potentially interact with other CYP-catalyzed metabolism. The recombinant human CYP2D6 Supersomes studies did not identify any inhibitory interaction between NIC and CYP2D6. IC_{50} values of >10 mM (CYP3A4 Supersomes) and > 180 μ M (CYP2D6 Supersomes) indicate no inhibitory effect on CYP3A4 or CYP2D6 activity. These results clearly demonstrate that inhibition of CYP3A4 or CYP2D6 by NIC is not the source of an interaction.

MA and COMBO treatments both resulted in the inhibition of CYP2D2-mediated drug metabolic reactions in the rat groups at the 1hrPT time point and clear differences were seen in the 7dayPT between the MA and COMBO treatment groups. One can speculate that the same would be true with humans, because of the known selectivity of the AMMC probe used, along with previous research that documents the involvement of

CYP2D6 in MA metabolism (Lin et al., 1997). The observation that MA associated inhibition of CYP2D2 metabolism is still present at 7dayPT provides sufficient evidence that the inhibition is not due to residual MA being present in the RLM. This also concludes that the inhibition seen is not a direct competitive inhibition but some modification that is present up to seven days after the last administration. It appears from our data that the co-administration of NIC speeds the recovery of drug-induced inhibition to control levels, as evidenced by the COMBO treated group 7dayPT results.

Our experiments have identified the inhibitory potential of MA and COMBO treatment in RLM. In order to elucidate the mechanism of this inhibition, further investigation needs to be done. The exclusion of direct competitive inhibition as a mechanism for the inhibition, as evidenced by the reduced inhibition at 7dayPT in the MA treated groups, leads us to believe that the NIC serves to increase the NAD resulting in an increase in the reducing equivalents improving energy production and improving the CYP-mediated metabolism. Another theory to consider is that the CYP protein expression may be altered. Future studies may include NAD studies to determine if there is a correlation between NAD and CYP2D function when exposed to MA and COMBO treatment. Other studies may focus on a quantitative measure of CYP2D2 protein at 1hrPT and 7dayPT time points, in order to correlate the amount of inhibition with the amount of CYP2D protein present. This was not done in our studies, as we used the total amount of protein in the microsomes.

We are confident that NIC either evokes an increase in reducing potential or is responsible for a rapid increase in production of CYP enzyme protein, as we do not believe NIC is responsible for CYP enzyme metabolic rate induction. Future studies

studies will assist in providing a conclusive decision about the 7dayPT time point and its degree of inhibition. In addition to the hypothesis that NIC increases the rate of recovery, the other possibility is that it prevents the extent of inhibition in the first place. This is evidenced in the 1hrPT studies, where the MA treated groups IC_{50} was 0.8 μ M and the COMBO groups IC_{50} was 32 μ M. If the NIC in the COMBO treated group decreased the amount of inhibition from occurring in the beginning, then the recovery rate could be the same and the COMBO treated group would return to control values before the MA treated group. It is this uncertainty that will require the CYP2D2 protein studies to further our knowledge and assist us with identifying the mechanism responsible for these changes. Additionally, one may want to consider dose response studies with the MA and COMBO treated groups, to determine if the amount of inhibition is dose dependent.

5.2.5 Neuroadaptation involved in addiction. Methamphetamine has a molecular site of action at the monoamine transporters, in particular the DAT. Although MA binds to three monoamine transporters, DA, 5HT, and norepinephrine (NE), it is the action at the DATs that are most central to both the motor activating and reinforcing (rewarding) properties of the psychostimulants (Giros et al., 1996).

MA acts as a false substrate and is transported into the cytoplasm engaging a heteroexchange mechanism which results in the reverse transport of DA from the cytoplasm to the extracellular space (Kuczenski, 1983). Thus, in addition to increasing interstitial DA levels by inhibiting the reuptake of synaptic DA that has been released via normal Ca^{++} -dependent exocytosis, MA transport promotes additional release of DA via reversal of the DAT. Upon transport into synaptic vesicles, MA is thought to act as a weak base and degrade the proton gradient that supplies the co-transport ion for

sequestering DA in vesicles (Sulzer et al, 1995). Confronted with a collapsing proton gradient, the extravesicular cytosolic DA concentration rises dramatically and contributes to reverse transport into the extracellular space. This mechanism appears to be most relevant to the releasing properties of MA (Liang and Rutledge, 1982).

Psychostimulants primarily suppress the firing of the STR and ACC when tested using anesthetized animals [see White (1996) for review]. This effect is primarily mediated by enhanced stimulation of DA receptors. MA administration alters the excitability of neurons by a number of actions. Increased extracellular DA levels potentiate the ability of the endogenous transmitters to modulate specific ion channels. DA as described above can enhance or reduce inwardly rectifying K conductance depending on D₁ or D₂ class of receptor. Such effects can reduce the excitability of the STR and ACC neurons thus altering the reinforcing/addictive properties of the drug.

Acute MA administration elevates extracellular DA not only in DA terminal fields but also within somatodendritic regions in the midbrain (Kalivas et al, 1989). The subsequent stimulation of D₂ somatodendritic autoreceptors results in three interrelated cellular changes: (i) a decrease in DA release and synthesis (Wolf and Roth, 1987) (ii) hyperpolarization and inhibition of DA cell firing (White, 1996), and (iii) an increase in velocity of DA transport (Welch and Justice, 1996). Under normal conditions, all three effects of DA autoreceptor stimulation would decrease the availability of DA in the synaptic cleft. Given that MA has exerted direct effects on the DAT function and thereby increased synaptic DA levels, autoreceptors activation under these circumstances results from the enhanced extracellular DA levels. For MA in particular, the transporter-reversal mediated release of DA at the nerve terminals effectively shunts the neuron from impulse

control, thereby preventing any role by autoreceptor stimulation to decrease extracellular DA content. Moreover, the increase in transporter velocity would likely amplify the transport of MA and thereby facilitate release. This increase in extracellular somatodendritic DA concentration provides profound stimulation of autoreceptors, and rendering long-loop feedback inhibition essentially redundant (White et al., 1995).

Repeated administration of MA produces a variety of alterations in DA neurons. Most of the changes are relatively transient and are clearly not involved in the maintenance and expression of behavioral sensitization or other persistent behavioral changes. However, it has been suggested by other investigators that such transient neuroadaptations may be necessary to trigger other alterations that are responsible for the maintenance and expression of addictive behaviors (White, 1996). Transient neuroadaptations that have been identified include DA autoreceptor subsensitivity (Gao et al., 1998), reduction of G protein levels (Striplin and Kalivas, 1993), and enhanced basal levels of extracellular DA (Kalivas and Duffy, 1993). Each of these suggest enhanced basal activity of DA neurons, potentially altering mechanisms involved in DA release and post-synaptic DA receptor sensitivity (White and Wolf, 1996).

In addition to the alterations in pre-synaptic DA function described above, there are a number of neuroadaptations in the intrinsic function of neurons. Among the first attempts at identifying changes in the brain that accompany MA administration was the study of DA receptor changes. Despite years of study, the consensus has been reached is that, with rare exception, measures of DA receptor affinity and density are seldomly altered for extended periods of time in animals that have received repeated psychostimulants including MA (Self and Nestler, 1995). This does not imply that

transmission through DA receptors is unaltered by MA addiction. Such measures simply reflect an effective homeostatic process by which DA receptors adapt not only to levels and frequency of ligand occupancy but also to internal information regarding levels of signal processing. Previous reports of DA receptors both pre- and post-synaptically report enhanced responses (Wolf et al., 1994). However, these changes are not always accompanied by alterations in DA receptor density, but perhaps reflect enhanced DA receptor signaling. These could include but are not limited to adenylyl cyclase, PKA, G proteins, or cAMP formation. The remaining sections will discuss our findings and their relevance within the so called model for addictive neuroadaptation .

5.2.6 Dopamine uptake and [³H]GBR12935 binding. Synaptosomes are the simplest brain-tissue preparation that preserves the functional activity of the pre- and post-synapsis, thus, they have been proven to be very useful in studying different events, including uptake, storage, synthesis and release of neurotransmitters (Hebb and Whittaker, 1958; Whittaker, 1993). Analysis of [³H]DA uptake along with [³H]GBR12935 binding studies showed significant decrease in apparent V_{max} and B_{max} with no change in IC₅₀ values, respectfully, in MA and COMBO-treated rat STR and ACC at the 1hrPT time point. These values indicated that DA uptake was functionally reduced equally in the MA and COMBO treated rats at 1hrPT and that this reduction in DA may be due to a decreased number of uptake sites. The significant reduction of [³H]GBR12935 binding sites in the STR and ACC seen in MA treated animals returned to control levels at the 7dayPT time point. However, the apparent V_{max} of the COMBO treated animal rat group in the STR remained decreased significantly (p < 0.05) from the MA treated group in the STR which returned to control value. Current and previous

[³H]DA uptake and [³H]GBR12935 binding studies indicate that binding sites are highly associated with the DA uptake site (Janowsky et al., 1986; Andersen, 1987; Nakayama et al., 1993). This data indicates that the single day multi-administration treatment with the sole MA only administration regimen (3 X 5mg/kg i.p., 2hr apart) does not create a long-lasting decrease in DA uptake sites. However, the co-administration with NIC causes a reduced DAT velocity at the 7 day post last injection treatment time point in STR. The significant reduction of [³H]GBR12935 binding sites in the STR and ACC seen in MA treated animals returned to control levels at the 7dayPT time point. The decrease in DA uptake initially results in an increased amount of DA within the neuronal synapse. However, over time the DA will be degraded by metabolic enzymes within the cleft and not recycled resulting in an overall decrease in the amount of available DA within the dopaminergic neuron. Thus the DA within the dopaminergic neuron will be regulated by the synthesis of dopamine from its tyrosine precursor. This may result in a decrease in the amount of available DA needed for basal dopaminergic activity or perhaps result in less DA being released when the neuron is activated and thus drive the user to desire additional MA use. The initial DA increase within the synapse accompanied with the delayed uptake seen in COMBO treated animals may indicate a possible additional addictive property resulting from the co-administration of MA and NIC. The mechanism by which this alteration occurs is unable to be described at this point. However, one theory will be described in the following section on evoked and basal DA release.

To our knowledge, only one other group has published work utilizing a similar dosing regimen. Davidson et al. (2005) described this single day dosing paradigm as a good model for overdose in the naïve non-tolerant abuser. A recent postmortem study

reported that chronic MA users had significantly decreased levels of DAT in the caudate and putamen (Wilson et al., 1996). McCann et al. (1998) utilized the first PET study to examine MA abusers and showed that chronic MA abusers DAT ($[^{11}\text{C}]$ WIN-35,428) binding in the caudate and putamen was reduced even after three years of abstinence. These results provided a very good case to support MA-induced neurotoxicity.

There is a large body of evidence from non-human primates showing MA-induced neurotoxicity with various doses of MA and different dosing regimens. For instance, Villemagne et al. (1998) demonstrated reduction of DAT binding. More recently, human studies have found detoxified MA abusers to reduce DAT (Volkow et al., 2001b; Sekine et al., 2001) and DA D_2 receptor binding in the caudate, putamen, ACC, and prefrontal cortex (Volkow et al., 2001b).

Dopamine D_2 receptors and DAT protein are two of the most important mechanisms controlling extracellular DA concentration (Schmitz et al., 2001; Torres et al., 2003). Changes in these proteins were used as markers to quantitate MA psychostimulant effects. Our studies focused on two brain regions, the STR and ACC, because of previous research and their known link to addiction and pleasurable effects. Additionally, our choice of examining the DA terminal region was influenced by studies showing a loss of DA function in the dorsolateral STR, but with relative sparing in the ACC following chronic MA treatment (Paulson and Robinson, 1996; Harvey et al., 2000). This ACC sparing purportedly related to a lower density of DAT sites (Broening et al., 1997), a hypothesis supported by protective effects of DAT blockers (Rothman et al., 2000) and DAT knockout (Fumagalli et al., 1998). As seen by Robinson and Becker, our data supported previous research that showed relative sparing in the ACC. We found no

significant changes between MA and the COMBO drug treatment regimens within the ACC. We did find that the MA and COMBO treated groups responded essentially the same with in the ACC. Our [³H]DA uptake and [³H]GBR12935 binding studies demonstrated lower levels of DAT functionality and quantity; the changes we observed were proportionate to each region and returned to control values within one week.

Wagner et al. (1980) reported that MA treatment caused an irreversible decrease in the number of DA uptake sites in the rat STR, and proposed that these deficits were indicative of nerve terminal degeneration. This has been referred to as “amphetamine neurotoxicity” and has previously been shown to deplete STR DA (Robinson and Becker, 1986). Our studies did not demonstrate long-term depletion and, therefore, would not consider neurotoxic levels. This data helps substantiate our dosing paradigm, as we did not want to reproduce an extreme MA, only the typical user. On the other hand, almost all chronic MA abusers build up to high doses gradually, as they develop tolerance to the autonomic effects of the drug.

There is a certainly an association between the different treatment regimens and the various dopaminergic effects that have been observed. Intermittent treatment with low doses or gradual escalating treatment with AMP produced long-lasting changes in behavior (Robinson and Camp, 1987). Gradually escalating the doses of MA has been shown to produce enhancement of stereotypical behavior with subsequent challenge dose seven days later (Ichikawa, 1988). Simultaneously, an increase of extracellular DA in the STR was observed using *in vivo* microdialysis. Robinson et al. (1988) reported elevated extracellular DA in the nucleus accumbens, after treatment with an escalating dose of AMP. While continuous MA administration produces the MA neurotoxicity syndrome,

repeated intermittent, or gradually escalating doses, which still produce behavioral sensitization, do not result in the depletion of DA brought about by the degeneration of DA terminals, but rather enhance the extracellular concentration of dopamine (Ellison and Eison, 1983; Robinson and Becker, 1986).

Though our studies did not utilize microdialysis methods, nor did we specifically determine the amount of extracellular DA, one can postulate that the decrease in DAT function and density in addition to MA-induced DA release would indicate an increase concentration of extracellular DA, which would agree with previous studies utilizing the repeated intermittent or gradually escalating dose models (Ellison and Eison, 1983; Robinson and Becker, 1986). Our theory has previously been documented with an observed rise in extracellular DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) utilizing *in vivo* microdialysis with single and repeated MA administration by Bustamante et al. (2002).

It has been well documented that MA induces the release of DA from presynaptic dopaminergic neurons (Kuczenski, 1983). As the amount of DA rises within the synapse, several neurological changes occur simultaneously. These studies have demonstrated that after a series of MA or COMBO administrations the ability of the presynaptic neurons to take up the excess DA previously released is diminished. The alteration in function of the DAT subsequently results in a rise in the concentration of DA within the synapse. In both the MA and COMBO treated animals at the 1hrPT time point a reduction of both DAT function and density is seen. However, the significant finding is at the 7dayPT time point where the rat previously treated with MA alone returns to normal DA functional uptake and DAT density, and the COMBO treated rats functional DA uptake remains

suppressed. This finding suggests that in the COMBO treated rats the levels of DA within the neurons' synapse will be higher than the MA treated rats due to a decrease in the uptake of the excess DA. The increase in DA within the synapse may illicit a longer duration of dopaminergic action and result in the depletion of DA within the presynaptic terminal leading the MA user to desire additional drug to supplement the synaptic cleft.

5.2.7 Dopamine release. The *in vitro* dopamine release was analyzed using radioactive [^3H]DA. Although the radioactivity measured in the collected fractions may consist of a mixture of neurotransmitters and metabolites, the amount of tritium released from rat brain slices has been previously shown to represent a close estimation of the release of labeled or endogenous DA release (Parker and Cubeddu, 1985; Herdon and Nahorski, 1987). Furthermore, the release of metabolites during the superfusion was inhibited by the presence of the monoamine oxidase inhibitor pargyline in the superfusion buffer (Zumstein et al. 1981). In order to avoid error resulting from possible differences between the different sets of experiments, tissues from experimental animals and control rats were always analyzed simultaneously. The release was stimulated by 30 mM KCl. High K^+ -stimulated release of dopamine *in vitro* is thought to reflect the *in vivo* release evoked by depolarization (Ochi et al., 1995). It is believed that the potassium ions directly induce exocytosis, due to depolarization of nerve endings.

MA is believed to serve as a substrate for the DA transporter and, thereby, acts as a competitive inhibitor of DA uptake (Liang and Rutledge, 1982). Once inside the cell, MA interacts with synaptical vesicles and increases cytoplasmic DA levels, promoting reverse-transport of DA through the plasma membrane transporter (Sulzer and Rayport, 1990). Low concentrations of MA cause redistribution of DA from the synaptic vesicles

to the cytosol (Sulzer and Rayport, 1990; Sulzer et al., 1992) and promotes significant release of DA and can, but does not always, produce overt signs of neurotoxicity. Lower concentrations are capable of inducing neuronal adaptations that result in altered dopaminergic function; i.e., reduced DA uptake (Bennett et al., 1993), and signs of oxidative stress (Cubells et al., 1994).

The results of the present study indicate that the KCl stimulated release of dopamine in MA and COMBO treated rats was significantly decreased at the 1hrPT time point in both STR and ACC. Previous studies are in agreement with the decrease in release (Bennett et al., 1997). There was a significantly decreased amount of DA released from the COMBO treated rats at the 1hrPT time point in STR. The amount of decrease was essentially the same in ACC. This may be due to the reduced amount of DA uptake seen in the STR neurons when previously treated with the COMBO regimen as described earlier. A decrease in uptake causes a decrease in the amount in the neuron available for release. The decrease in DA release was not seen at the 7dayPT time point. As described above, MA is believed to induce the release of DA thru reverse transport in the DAT protein. The prior exposure to MA or COMBO drug treatment is believed to deplete the vesicular and cytoplasmic pools of DA; there is a decreased amount of DA available for release at the 1hrPT time point. The author believes that during the course of the week, DA levels are replenished and that is why no decrease is seen at the 7dayPT time point. This theory accounts for the reduction in release seen at the 1hrPT time point, with return to control values at one week.

The release data from the single fraction (fraction 6) revealed a significant difference between the MA and COMBO drug treatments. This would agree with the

significant difference in MA and COMBO observed in the uptake studies described in chapter 4. The release data was analyzed with the AUC method, allowing examination of the total amount of DA released, not just the maximum release point. The significant reduction of evoked DA released between the MA treated group and the COMBO treated group in the STR is believed to be related to the significant increase in basal DA efflux seen with increased levels of NADP as described originally by Pearl et al. (2000). The resulting decrease in the amount of evoked DA from the COMBO treated rats may simply be due to the fact that the available stores have been depleted by the increase in basal DA released. This theory was examined by looking at the basal DA release prior to KCL evoked stimulation and statistically analyzing the difference between the MA and COMBO treated groups. Contrary to the studies by Pearl et al. (2000), we did not find a difference between MA and COMBO treated animals. We did find a significant difference between MA and COMBO treated groups and the saline control in both STR and ACC.

We observed an increase in the basal release of DA within the MA and COMBO treated specimens. This may help explain the decrease in evoked release. An increase in basal DA release would deplete the cytoplasmic and vesicular DA storage pools. It is also possible that the DA transport function can be altered directly or indirectly by pretreatment of MA and COMBO drug treatment, and the changes we observed are a consequence of transporter modification by one of the previously listed factors, including phosphorylation, glycosylation, membrane potential, or sulfhydryl group alteration. Alternatively, MA and COMBO, by promoting enhanced cytoplasmic DA levels, could have forced the transporter to operate in a 'reverse' manner; i.e., to expel DA. If the

transporter were transporting DA out of the cell, this may have necessitated the higher substrate concentration to force transport in the 'inward' direction, resulting in an increased amount of basal DA release until the cytoplasmic DA levels returned to normal.

5.2.8 [³H]Raclopride binding experiments. The intermittent nature of drug administration appears to be necessary for sensitization to occur, although several studies have reported a lack of sensitization following subchronic intermittent administration (Segal and Kuczenski, 1992; Wolf et al., 1994). MA binging by humans does not necessarily correlate with the dosing schedule used to 'sensitize' rats to subsequent doses of MA, but rather that binging occurs over a several day (average of 5) period involving repeated administrations of MA, during which time users forgo food and sleep (Miller, 1991). Humans use as much as 10-20mg/kg of MA over a 24-hour period to maintain a constant drug level (Goodman and Gilman, 1985), which is relatively easy to achieve due to the long half-life (approximately 12 hours) of MA (Cook et al., 1991). Our studies were done to mimic a several day binge, as described above, based upon the comparison of MA half-lives in rats and human.

We observed a reduction in [³H]raclopride binding to the DA D₂ receptors indicating that there were modifications in the DA D₂ receptor density rather than affinity, as demonstrated by an altered K_d and constant IC₅₀. This is most likely due to an indirect effect of MA promoting endogenous DA release, which subsequently increases DA D₂ receptor binding to shut down release. Similar alterations in presynaptic DA D₂ receptors have been reported to occur following AMP and MA pretreatment, which resulted in autoreceptor downregulation/desensitization (Seutin et al., 1991; Yamamada et al., 1991). Persistently elevated DA levels, as demonstrated by the increase in basal

DA release, may also be responsible for downregulation of the DA D₂ receptors, which would result in alterations in dopaminergic activity and local intracellular cAMP/PKA levels, as reported by Bennett et al. (1997).

Our data most closely resembles previous studies that utilized PET imaging to measure DA D₂ sites (Volkow et al., 2001b). PET studies have been shown to mainly represent postsynaptic DA D₂ receptors (Hume et al., 1996). Davidson et al. (2005) specifically looked at pre-synaptic autoreceptors and found that their results did not correspond to previous PET studies. Davidson et al., (2005) reported the differences may correspond to the possibility that there are fewer DA D₂ autoreceptors in the rats but, functionally, there is no difference in autoreceptor control of DA release, at least under their experimental conditions. This can be explained by the large receptor reserve for DA autoreceptors, and this has been shown for inhibition of DA release (Yokoo et al., 1988), DA neuronal firing (Cox and Waszczak, 1990), and DA synthesis (Bohmker et al., 1992). They also made note of transient nature of autoreceptor subsensitivity in the rat, which may only be evident in the first week after amphetamine injection withdrawal, previously demonstrated (Ellinwood and Lee, 1983; Lee and Ellinwood, 1989; Wolf et al., 1993; Davidson et al., 2005). This leads us to speculate that our findings suggest that DA neurons respond to MA and COMBO treatment by altering DA D₂ receptor number (presumably both pre- and post-synaptic).

5.3 Conclusion

The historical abuse of MA has been substantially documented in the previous chapters with ample literature citations. The physical properties, various routes of administration, dosages, purity, pharmacology, neurochemistry, and metabolism have been discussed in detail. The typical pattern of abuse from MA's clandestine production to its sale on the street has been established, along with recent news that clearly demonstrates the violence and urgency to further understand MA abuse.

A review of MA's pharmacology and toxicology clearly demonstrates the drugs potent addictive and neurotoxic effects, as evidenced by long-lasting alterations in the dopaminergic system, the best-established and most widely agreed upon neurochemical link to addiction in literature today.

MA has been recognized as a potent psychostimulant drug for decades. Over the years it has been diluted with numerous chemicals, in order to generate a higher profit margin in illicit sales. Today, the adulterant NIC has become almost exclusive with the dilution of MA, as evidence with over 95% of the samples submitted to the Tulsa Police Department forensic laboratory containing between 3 and 97 % NIC (unpublished data). Knowing that using NIC is not the most economical way to dilute MA leads one to believe there are possibly other aspects influencing the choice of this cutting agent. If it is not availability or economics dictating the cutting agent, there must be some improvement in desired effects.

It is this line of thinking that initiated the hypothesis that was tested. NIC, co-administered with MA, provides an enhancement in MA's psychostimulant effects somehow providing a more desirable experience for the user. Previous researchers have

briefly looked at the interaction of these two agents but never compared their co-administration. A recent literature search only identified two papers that addressed the use of MA and NIC together. In these papers, pretreatment with high doses of NIC was shown to attenuate AMP and MA induced STR DA depletion in rats (Huang et al., 1997; Wan et al., 1999). Our studies were unable to demonstrate similar results. We observed MA and COMBO treated group induced alterations in DA uptake, DA release, and CYP2D-mediated metabolism. Additionally, significant differences were noted between MA and COMBO treated animals in the STR that leads the investigator to believe there is some psychostimulant enhancement when these drugs are co-administered. The difference between MA and COMBO in the STR uptake coupled with the decreased evoked DA release in the STR signifies a significant difference between the MA and COMBO dosing regimen. The co-administration of these compounds reduces the DA taken up into the dopaminergic neurons leaving the synapse DA concentration higher longer which leads to a significant reduction in the evoked DA release. The combination of these two effects can offer one theory as to the increase in psychostimulant effects based on our current knowledge of DA addiction. However, the increase in metabolic activity seen in the CYP2D enzymes does not match these findings. Therefore, in order to gain a better understanding as to how these factors work in concert more research must be done. Further research will better our understanding as to the how these finding fit together and as to how they affect the psychostimulant effects and addictive potential of MA when co-administered with NIC.

We must conclude that under our experimental conditions, the co-administration of NIC with MA does not produce overwhelming evidence to synergistic metabolic or

dopaminergic changes. However, the studies provide an introduction as to the understanding of changes associated with MA and NIC co-administration. Additional studies must be completed to identify possible behavioral changes associated co-administration or other possible mechanisms for our proposed enhancement.

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Dissertation: NICOTINAMIDE CO-ADMINISTRATION WITH
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Scope and Method of Study: The adulteration of illicit methamphetamine (MA) with nicotinamide (NIC) has become so prevalent in the United States that researchers must now consider the possibility that NIC may possess some ability to enhance the psychostimulant effects of MA. The purpose of this project was to evaluate the metabolic and dopaminergic sequelae of MA, NIC, and their co-administration (COMBO). The effects of MA, NIC, and their COMBO on human CYP3A4 and CYP2D6 Supersomes® and isolated rat liver microsomes (RLM) at 1hr and 7 days post last treatment (1hrPT, 7dayPT, respectfully) was investigated with commercially available kits. The dopaminergic changes were evaluated by measuring [³H] dopamine uptake, [³H] dopamine release, dopamine receptor binding ([³H] raclopride), and dopamine transporter binding ([³H] GBR12935) at 1hrPT and 7dayPT in two different brain regions, the striatum (STR) and nucleus accumbens (ACC).

Findings and Conclusions: We observed MA and COMBO treated group induced alterations in DA uptake, DA release, and CYP2D-mediated metabolism. Our RLM studies demonstrated significant inhibition with a more rapid return to normal with COMBO treatment. The dopaminergic studies revealed characteristic changes within both brain regions with the COMBO treated group producing a sustained reduction in DA uptake over the MA group in STR. The COMBO also resulted in a significant decrease in the amount of KCL evoked DA released in the STR while both MA and COMBO treatment resulted in an increased amount of basal DA release in both brain regions. Under our experimental conditions, the co-administration of NIC with MA does not produce overwhelming evidence to synergistic metabolic or dopaminergic changes. However, the studies provide an introduction as to the understanding of changes associated with MA and NIC co-administration.

Advisor's Approval: David R. Wallace, Ph.D.