Development and application of analytical chemical methods for the simultaneous analysis of the behavioral and pharmacokinetic response to amphetamine in rats

By

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ABSTRACT

Amphetamine is a psychostimulant that is used clinically for the treatment of attention-deficit hyperactivity disorder and narcolepsy. Using a force-plate actometer, amphetamine has been determined to produce a predictable behavioral phenomenon known as 'focused stereotypy' in Sprague-Dawley and Fischer 344 (F344) rats. Chronic administration of amphetamine can result in behavioral sensitization, a phenomenon by which later doses result in a more intense behavioral response than the same, initial dose. In rats, the behavioral response to a determined dose of amphetamine varies between strains and between individual rats within a strain. These variations result in uncertainty as to whether the resulting behavioral differences elicited by the drug are pharmacokinetic or pharmacodynamic in nature. Microdialysis provides a means to collect extracellular fluid from a particular brain region which can then be measured to determine the pharmacokinetic response to amphetamine in this region. A high performance liquid chromatography method using tandem mass spectrometry detection was developed for the analysis of amphetamine, the amphetamine metabolites (p-hydroxyamphetamine, norephedrine and p-hydroxynorephedrine), dopamine and clozapine to attempt to measure these compounds in dialysate obtained from the striatum of rat brains. This method was combined with high resolution behavioral measures obtained from a

force plate actometer to determine how closely associated drug pharmacokinetics are to the behaviors they elicit. Results indicate that amphetamine induced behaviors are not clearly linked to amphetamine pharmacokinetics and that the observed behavioral differences are likely pharmacodynamic in nature. The atypical antipsychotic drug clozapine has been shown to lengthen the duration of amphetamine induced focused stereotypy in rats. Amphetamine/clozapine interactions cannot be ruled out as a possible cause of this phenomenon. Because microdialysis was ineffective in determining clozapine levels in rat brain, a liquid/liquid extraction method was used to determine whether amphetamine had an effect on whole brain clozapine concentrations. Using this method, it was determined that clozapine levels were not affected by amphetamine at the time point analyzed.

To my children, Stephan and Anna

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CHAPTER 1

Introduction

In studies of behavioral pharmacology, drug concentrations are seldom constant. Likewise, drug induced behavioral processes may change throughout the course of a single administration or they may change with successive treatments. Such behavioral changes over the course of a session may be due to the natural time course of the behavior, or they may be due to changes in drug concentration, or both. Similarly, behavioral changes that occur with successive treatments may involve physiological adaptations that alter the metabolism, and thereby the elimination kinetics of the drug over time. These effects highlight the importance of understanding the direct effects of drug concentration when analyzing an animal's behavioral response to the drug. The far reaching goal of the following studies is to simultaneously measure the behavioral effect and concentration of a drug in a single experiment to determine how drug concentrations are directly related to the behaviors they induce.

Amphetamine is a psychostimulant used both clinically and as a commonly abused recreational drug. First synthesized in 1887 by Lazar Edeleanu, amphetamine remained a drug without a disease until 1910 when Gordon Alles independently synthesized it and reported its stimulating effects

while trying to find an effective decongestant (Fleckenstein et al., 2007). The first insight into the mechanism of amphetamine was published in 1958 by Burn and Rand in which they observed that amphetamine and other similar substances release either noradrenaline or adrenaline or both. It has since been established that amphetamine affects numerous neurotransmitter systems including norepinephrine, dopamine and serotonin (Fleckenstein et al., 2007). Amphetamine is currently used to treat narcolepsy and attention deficit hyperactivity disorder and has been used by the military to keep troops alert and reduce fatigue during extended flights as recently as Desert Storm (Emonson and Vanderbeek, 1995).

Chemistry, pharmacology and metabolism of amphetamine

Amphetamine Chemisty

Amphetamine is the parent compound of a subclass of compounds known as ring-substituted amphetamines (RSA). The popular street drug methamphetamine (METH) is included in this subclass, as are the designer drugs methylenedioxymethamphetamine (MDMA), and methylenedioxy-N-ethylamphetamine (MDEA). Although these molecules are slightly different from one another structurally, they are similar pharmacologically and, with the exception of methamphetamine, are often interchangeably referred to as ‘ecstasy’ (Freudenmann et al., 2004; Kraemer et al., 2002). The compounds

are generally comprised of a phenyl ring connected by a two-carbon side chain to an amino group with a methyl group attached to carbon 1 of the side chain. This structure has an asymmetric center making amphetamine a chiral compound. The two possible enantiomers are d-amphetamine (dextroamphetamine) and l-amphetamine (Jori et al., 1978). The experiments involved in these studies involve d-amphetamine. d-Amphetamine has been reported to have greater CNS potency than l-amphetamine (Arnold et al., 1976). Analysis of behavioral responses in rats shows that doses of 2.5 mg/kg d-amphetamine and 11.5 mg/kg l-amphetamine produce about the same amount of locomotor activity over a 4 hour period (Segal, 1975). Observation of stereotypy showed that onset occurred 30 minutes earlier for d-amphetamine and ended about 30 minutes earlier than stereotypies for l-amphetamine treated animals. Dopamine release cannot completely account for these behavioral findings. Recent studies comparing dopamine release in the striatum of rats treated locally with d- or l-amphetamine showed that d-amphetamine showed only a slightly higher amplitude of dopamine release than the l- isomer. However, l-amphetamine alone or as a racemic mixture of l- and d-amphetamine produced a significantly faster rise time to reach maximum dopamine release (Glaser and Thomas, 2004). It was hypothesized that l- and d-amphetamine have different effects on dopamine transporters although the nature of these differences has not been determined.

Amphetamine pharmacology

Literature shows that the stimulatory effects of amphetamine come primarily from the release of catecholamines (Sulzer et al., 2005; Kuczenski, 1995). Under normal circumstances, reuptake of monoamines by monoamine transporters located on the presynaptic neuronal membrane inactivates monoamine signaling in the brain (Rothman and Baumann, 2003). While stimulants such as cocaine produce their effects by inhibiting the reuptake of monoamines, amphetamine functions as a substrate-type releaser. That is, it is transported into the cytoplasm by acting as a substrate for monoamine transporters. This has two effects. First, it promotes efflux of transmitter by a process of transporter-mediated exchange. Transporter mediated exchange is the process by which the transport of amphetamine into the cell is followed by the movement of neurotransmitter out to the extracellular compartment (Khoshbouei et al., 2003). The second effect of amphetamine is to increase cytoplasmic levels of transmitter by disrupting storage of transmitters in vesicles (Rudnick, 1997; Rudnick and Clark, 1993). This increases the pool of neurotransmitter available for release by the transporter. In effect, amphetamine blocks reuptake of monoamines, prevents packaging into synaptic vesicles which leaves more in the cytoplasm, and promotes release of cytoplasmic neurotransmitter through transporters. While amphetamine has comparable activity at the dopamine

transporter (DAT), the serotonin transporter and the norepinephrine transporter, the DAT is most frequently implicated in the reinforcing and abuse properties of the drug (Fleckenstein et al., 2007). Given the direct effect of amphetamine on dopamine transporters and release, higher extracellular levels of amphetamine in the brain should directly correlate with higher levels of dopamine insofar as dopamine availability is constant. Using microdialysis, it was demonstrated by Kuczenski and Segal (1997) that following subcutaneous administration of d-amphetamine, the striatal extracellular dopamine response and extracellular d-amphetamine concentrations closely parallel each other and that both substances exhibit equivalent elimination rates. Further evidence to support the role of amphetamine as having a direct effect on dopamine release, and that the effects are not related to activity at dopamine receptors, apomorphine, a dopamine D1 and D2 receptor agonist, does not alter amphetamine-induced dopamine release in the striatum (Kuczenski et al., 1990).

The direct effect of amphetamine on dopamine release indicates that it is possible that different behavioral effects exhibited by different rat strains may be related to the concentration of amphetamine reaching the brain. The effect of amphetamine concentration on behavior is one of the empirical issues addressed in many of the experiments reported in these studies. Relevant to these studies is the finding that F344 rats have higher concentrations of dopamine transporter protein than Lewis rats (Gulley et al.,

2007). This could imply that amphetamine would have either a greater effect or a more long lasting effect on F344 rats. It would be important, therefore to directly compare amphetamine levels between strains to determine if behavioral differences result from a higher level of amphetamine in the brain or if the observed differences are unrelated to amphetamine and are due instead to differences in dopamine availability, dopamine receptor density or other pharmacodynamic factors.

Amphetamine metabolism

Amphetamine is metabolized by the cytochrome p450 (CYP) family of enzymes. In humans amphetamine is metabolized along two pathways, hydroxylation or deamination. The hydroxylation pathway involves either hydroxylation of the aromatic ring (p-hydroxylation) to produce p-hydroxyamphetamine (OHamp), or hydroxylation of carbon 2 of the carbon chain (β-hydroxylation) to produce norephedrine (NOR). Deamination involves hydroxylation of the terminal amine to form phenylacetone (PA) and benzoic acid (BA) (Green et al., 1986). Based on studies which measured relative levels of metabolites in several species, the deamination pathway appears to be the primary pathway found in rabbits and guinea pigs while humans, dogs and other primates utilize both pathways (Green et al., 1986). However, rats are unique in that the hydroxylation reaction is almost exclusively utilized while deamination is relatively minor. The primary

metabolite found in rat urine is p-hydroxyamphetamine (60% of dose) while p-hydroxnorephedrine and norephedrine were found at very low levels (0.3%) The deamination products benzoic acid and phenylacetone were not detected (Dring et al., 1970). In rats, the CYP2D subfamily of enzymes is selectively responsible for hydroxylation of amphetamine as well as the amphetamine analogs methamphetamine and MDMA (Law et al., 2000). It is important to point out that in humans CYP2D activity is the result of a single gene product, CYP2D6. In rats, the activity of CYP2D results from expression of four genes, 2D1, 2D2, 2D3, and 2D5 (Matsunaga et al., 1989). Most publications do not distinguish a specific rat gene product (Law et al., 2000); however, a study by Tomkins et al. (1997) showed that CYP2D1 inhibition prolonged amphetamine-induced hyperactivity and reduced amphetamine self-administration in rats.

Amphetamine psychosis and abuse

Metabolic interactions with antipsychotic drugs

Amphetamine-induced stereotypical behaviors are often used as a preclinical model of psychosis (Robinson and Becker, 1986). The use of amphetamine for this purpose is based on observations that humans subjected to chronic or high doses of amphetamine may experience many of the positive symptoms of schizophrenia such as auditory hallucinations and

paranoia (Ellinwood and Cohen 1971). Simultaneous administration of multiple drugs introduces the potential for these drugs to compete for metabolic enzymes. The metabolic pathways of amphetamine in the rat are relevant in the context of amphetamine-induced stereotypy as a method of analysis of the effectiveness of antipsychotic drugs. It cannot be ruled out that metabolic interactions of antipsychotic drugs with amphetamine are a factor in observed behavioral effects of these drugs.

A broad range of enzymes are responsible for metabolism of antipsychotic drugs (Spina and de Leon, 2007). Clozapine is an atypical antipsychotic drug that has been reported to be effective in 30-60% of otherwise treatment-resistant schizophrenia patients (Meltzer et al., 1993). It has been suggested that individual differences in clozapine's efficacy may result from interindividual differences in enzymatic activity (Fang, 2000). In humans clozapine is primarily metabolized by CYP3A4 and CYP1A2 with additional activity by flavin-containing monooxygenase 3 (FMO3). A lesser amount of activity was observed for CYP2D6 and CYP2C19 (Fang et al., 1998).

Results of studies done in this lab indicate that clozapine may extend the stereotypy inducing effects of amphetamine in a dose dependent manner. Amphetamine sensitized rats that were trained to press a lever to receive a reward temporarily stopped responding following amphetamine treatment. When clozapine was administered 30 minutes following amphetamine

administration, the period of time before operant responding returned was significantly increased (Osterhaus et al., 2004). While the exact nature of this paradoxical finding is uncertain, we hypothesize that clozapine-amphetamine interactions may be a contributing factor. Risperidone is another atypical antipsychotic drug demonstrating clinical efficacy. Risperidone is primarily metabolized by CYP2D6 and to a lesser extent CYP3A4. Metabolic profiling would indicate that Risperidone has a greater likelihood of interacting with amphetamine metabolism due to the common reliance on the CYP2D enzyme.

Amphetamine use and abuse

The use of amphetamines as commonly abused recreational drugs is well documented. Although classified by the US Drug Enforcement Administration as a Schedule IIa drug due to its abuse potential, amphetamine continues to be used clinically in either the d-form or the racemic l- and d- form to treat attention deficit hyperactivity disorder (ADHD) and narcolepsy. Dexedrine and Adderall are among the brand names under which amphetamine is marketed. Amphetamine suppresses appetite and was used for weight loss as well as a nasal decongestant under the brand name of Benzedrine starting in 1937 and continuing through the 1970's. A small number of users responded to amphetamine by experiencing an increase in energy, productivity, and euphoria caused by the drug and its

abuse liability became apparent. Use of amphetamine for weight management has been discontinued in the United States although it is still available for this purpose in some countries (Toda and Abi-Dargham, 2007). The use of amphetamine in the d-enantiomer Dexedrine is still an alternative treatment for narcolepsy although it is largely being replaced by Modafinil. Modafinil is not considered a psychostimulant and does not cause the motor agitation and sleep loss associated with amphetamine (Szabadi, 2006). Along with methylphenidate, the most common clinical use of amphetamine is as a primary treatment of ADHD under the brand name Adderall (Fone and Nutt, 2005). ADHD is thought to result from disruptions in executive functions largely regulated by the prefrontal cortex (Doyle, 2006). It is hypothesized that disruption in the dopaminergic circuitry of the prefrontal cortex leads to a diminished ability to plan and maintain appropriate thoughts and actions in different contexts over time (Casey et al., 2007). Amphetamine may be effective as a treatment because it leads to an increase in extracellular dopamine levels of pathways associated with the prefrontal cortex.

Of the three primary metabolites of amphetamine in rats, p-hydroxyamphetamine (POHA), p-hydroxynorephedrine (POHN), and ephedrine, two have CNS activity and are also used clinically. POHN is used in the diagnosis of Horner's syndrome due to its monoamine releasing potential (Walton and Buono, 2003). Norephedrine (phenylpropanolamine) is a preferential alpha-adrenergic receptor agonist (Flavahan, 2004). Before

being recalled due to incidents of stroke, norephedrine was used clinically as an over the counter nasal decongestant and appetite suppressant (Ioannides-Demos et al., 2006).

Although very similar, methamphetamine differs from amphetamine both structurally and pharmacologically. Structurally, methamphetamine has a methyl group attached to the amine. While it is often stated that methamphetamine is more potent than amphetamine, data does not support this in rats. Evidence shows that d- amphetamine was more potent than d-methamphetamine at increasing extracellular levels of dopamine in the prefrontal cortex (Shoblock et al., 2003). Kuczenski (1995) showed that amphetamine is more effective at releasing norepinephrine than methamphetamine suggesting that the observed differences in potency may not be dopiminergic in nature. Other suggestions about the nature of higher potency reported by drug abusers may be that the additional methyl group on methamphetamine creates a more hydrophobic molecule that more readily crosses the blood brain barrier and penetrates cell membranes (Derlet and Heischober, 1990; Milesi-Halle et al., 2007). The methyl group may also interfere with metabolism leading to a longer drug effect (Melega et al., 1995). A plausible explanation may be that amphetamine and methamphetamine exhibit different potencies at key neurochemical pathways in the central nervous system due to different effects on dopamine, norepinephrine, and serotonin transmission (Fischer and Cho, 1979; Kuczenski et al., 1995). The

reinforcing effects of amphetamine, as with all addictive drugs appear to be mediated through a reward pathway in the mesolimbic dopamine system (Foy et al., 2007).

Deaths from amphetamine abuse usually occur in the occasional user rather than in the tolerant, chronic, high dose abuser (Ellinwood et al., 2000). However, non-lethal pathologies are common in chronic abusers. A phenomenon of particular interest is amphetamine, or drug-induced psychosis. The hallmarks of this disorder are delusions, hallucinations (usually auditory) and paranoia (Curran, 2004, Czerwinski, 1998). Another behavior associated with amphetamine psychosis is punding (Schiorring, 1981). Punding is a compulsive fascination with and performance of repetitive, mechanical tasks. It has been estimated that up to 1.2% of emergency room visits in inner-city hospitals may be related to amphetamine abuse (Gray et al., 2007). Treatment is generally carried out by the administration of typical antipsychotic drugs such as chlorpromazine, haloperidol, or thioridazine (Srisurapanont et al., 2001).

Striatum, amphetamine and stereotypy

Paradoxically, some drugs that increase monoaminergic levels do not cause psychosis and are not abused (Rothman and Bauman, 2003). Rats treated with chronic or high doses of amphetamine exhibit stereotypical behavior analogous to the human case of drug-induced psychosis. The

animal shows little or no locomotor activity but moves its head at what has been shown to be a 10Hz rhythm on the force plate actometer (Fowler et al., 2002). Stereotypies are thought to result from excess dopaminergic activity in basal ganglia-based neuronal circuits controlling integration and switching of motor responses (Haracz et al., 1998). The basal ganglia collects input from cortical motor regions and redistributes these signals to particular regions of the frontal lobes and brainstem involved in aspects of motor planning and execution (Mehler-Wex et al., 2006). The level of dopamine activity in the basal ganglia affects the baseline level of behavioral activation. Excess activation leads to execution of the animal's response to the environment to the exclusion of other possibilities (Ridley, 1994; Saint-Cyr et al., 1995). The striatum serves as the input center for the basal ganglia. It has been demonstrated that motor stereotypies in rodents can be induced by dopaminergic stimulation of the striatum and can be abolished by blockade of dopamine striatal neurotransmission (Creese and Iversen, 1972; Fibiger et al., 1973 (referenced from Canales and Graybiel, 2000)). It is the dopaminergic influence on striatal function that is the focus of expressed behaviors analyzed in the experiments presented in these studies.

Sensitization

Amphetamine-induced behavioral sensitization is the phenomenon by which repetitive administration of the drug leads to a progressive and long-lasting augmentation of certain behaviors (Segal and Mandell, 1974). Measurements of stereotyped behaviors associated with amphetamine confirms that repeated, intermittent administration of the drug causes these behaviors to occur more intensely, with shorter latency, and at a lower dose than before sensitization (Segal et al., 1980). Behavioral sensitization has been shown to last for months or years (Sato, 1992).

Behavioral sensitization can be separated into two components, initiation and expression. Lesion studies have shown that these components are separate anatomically. In general, initiation of behavioral sensitization occurs in the ventral tegmental area (VTA) while neuronal events associated with expression occur in the motive circuit which is a collection of interconnected limbic nuclei (Pierce and Kalivas, 1997). The mesolimbic system involving the medial prefrontal cortex (mPFC) is believed to be associated with behavioral sensitization (Banks and Gratton, 1995). One hypothesis asserts that dopamine neurons projecting to the mPFC from the VTA play an inhibitory role by activating inhibitory GABA interneurons. A decrease in dopaminergic activation of these inhibitory neurons would decrease the inhibition of excitatory neurons projecting to the nucleus accumbens and VTA (Richtand, 2006).

Individual differences in behaviors induced by amphetamine must be considered in the experiments presented here since a major theme of this study involves differences in the behavioral response to drugs. Rats can be categorized as high or low responders based on their locomotor activity in a novel environment (Piazza et al., 1990). Individual differences in reactivity to novel environments seem to at least partially predict sensitivity to amphetamine and other stimulatory drugs (Piazza et al., 1990). High responders show an increased propensity for amphetamine self-administration compared to low responders (Klebaur et al., 2001). In the human case, high novelty seekers have been shown to use drugs more frequently than low novelty seekers (Donohew et al., 1991) suggesting a potential role in drug abuse. Environmental reactivity and dose effect functions change with amphetamine experience such that sensitized animals show less variability in their behavioral response than naïve animals (Fowler et al., 1999).

Schizophrenia

Animal models and schizophrenia

Out of necessity for productive drug research, there has been a growing trend to measure drug effects on animal behavior in an effort to predict behavioral effects in humans. Schizophrenia is a neuropsychiatric disorder most commonly involving auditory hallucinations, disorganized

speech and thinking, and significant social dysfunction. Because many symptoms of schizophrenia involve primarily human characteristics related to language and complex social interactions, it cannot easily be reproduced in an animal model. However, models that demonstrate behaviors analogous to those seen in schizophrenic patients are useful in advancing our understanding of this complex disorder. Two common categories of models currently used in schizophrenia research are behavioral and pharmacological. Prepulse inhibition (PPI) is a behavioral model in which a weaker stimulus precedes a stronger stimulus causing a diminished reaction to the second, stronger stimulus. Schizophrenic patients generally have a lower PPI than a normal control. That is, they would have a high response to the strong stimulus even when preceded by a weaker stimulus. The dopamine theory of schizophrenia was supported by studies showing that amphetamine and the dopamine agonist apomorphine disrupt PPI when administered systemically to rats (Mansbach et al., 1988). PPI has been used as a screening tool for antipsychotic drugs. Atypical antipsychotics tend to increase PPI in animals treated with dopamine receptor agonists (Geyer et al., 2001).

Examples of the pharmacological approach involve drugs such as LSD and PCP which are known to produce hallucinations in humans. The hallucinogenic properties of LSD were first compared to schizophrenia by Wooley and Shaw in 1956. Although still relevant, schizophrenic patients do not report a significant worsening of symptoms when given LSD (Krus et al.,

1963; Stone, 1973). The current study involves characterization of the behavioral and neurochemical effects of amphetamine in rats. The use of amphetamine to elicit psychotic-like effects in rats has become a popular pharmacological approach to schizophrenia research based on two primary features. First, as previously stated, amphetamine leads to dopamine release. A wealth of literature suggests that schizophrenia is associated with increased brain dopamine (DiForti, Lappin, and Murray, 2007). Secondly, patients with amphetamine-induced psychosis exhibit many of the positive symptoms of schizophrenia such as auditory hallucinations, paranoia, and extrapyramidal locomotor effects. A finding that further links amphetamine-induced psychosis to schizophrenia is a study by Laruelle et al. (1996) which shows that amphetamine-elicted psychotic symptoms in schizophrenic patients with no prior drug exposure were associated with exaggerated dopamine release in the striatum compared to normal controls. It has become common in both the industrial setting as well as academia to attempt to predict the efficacy of potential antipsychotic drugs by measuring effects on rats engaged in amphetamine-induced stereotypy.

Current Studies

The specific goal of this study is to develop a method to measure the level of various compounds in rat brain while concurrently measuring amphetamine-induced behavior. The following experiments are part of an ongoing process

to characterize drug induced behaviors in rats. A limitation of behavioral studies involving pharmacological effects is how pharmacokinetics affects behavior on an individual basis. Characterizing an animal's behavior following administration of a drug can best be understood if the dynamics of the drug in that particular animal are known. A drug's effect on behavior can only be validated insofar as the drug's behavior within the organism is known. It becomes necessary with behavioral studies to assume that all animals involved are receiving and metabolizing the drug equally throughout the entire test population. Microdialysis offers one way of directly monitoring the pharmacokinetics of a drug to test this assumption. The purpose of the experiments in this study is to develop and test a method to measure drug pharmacokinetics while at the same time measuring behavior with the force-plate actometer. In this way, differences between strains as well as differences between subjects can be better characterized. While the experiments presented here are not exhaustive, the results will show the successes and limitations of such an endeavor which will lay the groundwork for improvement and development in attempts to correlate behavior with pharmacokinetic parameters.

Hypothesis #1: Brain levels of amphetamine, amphetamine metabolites, dopamine and the atypical antipsychotic drug clozapine can be collected and quantified from an ambulatory animal over a 4-hour period.

In experiments involving the administration of drugs to an animal, individual variation in the pharmacokinetic and neurochemical response of the animal is paramount to the observed behavior. The source of individual variations in behavior may be due to drug pharmacokinetics, or they may be due to individual variations in the animal's neurochemical response to the drug. Hypothesis one introduces a method to simultaneously measure the catecholamine neurotransmitter dopamine, the antipsychotic drug clozapine, the CNS stimulant amphetamine (Amph), and the amphetamine metabolites p-hydroxyamphetamine (OHamp), norephedrine (Nor), and p-hydroxynorephedrine (OHnor) collected from brain extracellular fluid via microdialysis. Dialysate was collected from awake, ambulatory animals in a modified force plate actometer chamber. An HPLC-tandem mass spectrometry method was developed for quantitative analysis due to the sensitivity, flexibility and reliability of measuring molecules directly. While other methods have been published that measure each of the compounds of interest, they rely on chemical attributes that are not shared by each of these molecules. Clozapine can be detected using ultraviolet (UV) detection (Llerena et al., 2001). Amphetamine and the amphetamine metabolites p-

hydroxyamphetamine, p-hydroxynorephedrine, and ephedrine can also be measured using UV detection but only after completing a derivitization step such as acetylation to make them visible in the UV spectrum (Veress, 2000; Soares et al., 2004). Dopamine is typically measured using electrochemical (EC) detection (Hjemdahl, 1984). It would be desirable to have a method in place that could measure a broad range of molecules with minimal sample preparation. Sample preparation is relevant because sample loss could occur through physical manipulations or incomplete chemical reactions as would likely be the case with a derivitization step. Loss of sample would require more starting material which could decrease resolution by requiring more time between microdialysis samples. Tandem mass spectrometry allows the measurement of dialysate directly, minimizing sample handling, maximizing sample volume and thereby maximizing temporal resolution.

Hypothesis #2:

Behavioral data obtained from rats with a microdialysis probe surgically implanted in the striatum and tethered to a microdialysis system is comparable to behavioral data previously obtained from untethered animals that did not undergo surgical probe implantation.

Hypothesis two addresses how the microdialysis apparatus will affect behavior. Because the force-plate actometer is sensitive to vertical force changes, a tethering mechanism, which is required for microdialysis, may

change or even mask the behavioral characteristics previously measured. This could limit the efficacy of the force plate method of behavioral measurement while used in the context of dialysate sampling.

Behavioral data obtained in these experiments will be compared to behavioral data previously obtained from untethered animals of the same strain to confirm the validity of this method. As previously stated, there are observed differences in the topography of expressed stereotypy in different rat strains. This is not only relevant from a behavioral and neurochemical standpoint, but it might also affect the integrity of the microdialysis apparatus. Considering that F344 rats tend to have a more horizontal stereotypy while Sprague Dawley rats exhibit a largely vertical stereotypy (Fowler, unpublished observations), it was determined that we would use representative rats from each of these strains to confirm the neurochemical and behavioral results. It is plausible that behavioral analysis can be performed on surgically modified animals without corrupting behavioral data as measured on the force-plate actometer.

Hypothesis #3: The heightened behavioral response of Fischer 344 rats to amphetamine compared to Sprague Dawley rats is due to higher levels of amphetamine in the striatum.

Behavioral differences in sensitized and unsensitized F344 and SD rats have previously been reported (Thisayakorn, 2006). Results showed that

F344 rats appear to be more sensitive to the stereotypy-inducing effects of amphetamine than Sprague Dawley rats based on scores of spatial confinement, locomotion and focused stereotypy. Strain has been reported to be a factor in plasma concentrations of amphetamine in F344 and Lewis rats (Clausing et al., 1996). A logical question resulting from this finding is whether the differences in the behavioral response to amphetamine between Sprague Dawley and F344 rats result from a disparity in the amount of amphetamine reaching the brain. Brain amphetamine levels and behavior in groups of animals from each strain were measured to determine if individual differences and strain differences in the behavioral response to amphetamine correspond to amphetamine concentrations in the striatum.

Hypothesis #4: Measured behavioral changes that occur throughout the sensitization process are due to changes in amphetamine pharmacokinetics.

Amphetamine-induced behavioral sensitization is associated with a heightened behavioral response following chronic administration of drug. Several lines of evidence show that permeability of the blood brain barrier may be altered by chronic amphetamine (Sharma and Ali, 2006) and acute methamphetamine (Bowyer et al., 2006) treatment in rodents. To test the hypothesis that changes in the permeability of the blood brain barrier over the course of behavioral sensitization result in higher levels of brain amphetamine

and the associated behavioral response, we simultaneously measured behavior and amphetamine levels during the first and last injection of a sensitizing series of six injections. Behavioral data collected on the force plate actometer was compared to amphetamine levels obtained from striatal extracellular fluid that was collected and analyzed via HPLC-MS/MS to determine if behavioral changes correlate with amphetamine concentrations in Sprague Dawley and Fischer 344 rats.

Hypothesis #5: There is no relation between the intensity and duration of the behavioral response to amphetamine and amphetamine concentration in the striatum.

The behavioral response to amphetamine has been shown to increase or remain constant for 1-2 hours following amphetamine administration (Fowler et al., 2003). The dopaminergic response to amphetamine in the caudate putamen shows that dopamine rapidly reaches a peak level within 15 to 30 minutes of amphetamine administration and that amphetamine follows a similar time course (Ferguson et al., 2003; Kuczinski et al., 1997). These findings indicate that the behavioral time course does not closely mimic the behavioral response. The experiments presented here simultaneously measured amphetamine levels in the striatum and the behavioral response in sensitized and unsensitized Fischer 344 and Sprague Dawley rats to help confirm whether observed amphetamine-induced behavioral responses

correspond to altered levels of amphetamine or if behavioral responses are largely independent of absolute amphetamine levels in this brain region.

Hypothesis #6: Clozapine metabolism is altered by concurrent administration of clozapine and amphetamine in rats.

Clozapine has been shown to extend amphetamine-induced stereotypy in rats as measured on the force-plate actometer (Fowler et al., 2007). Due to potentially common metabolic pathways of amphetamine and clozapine, it is plausible that clozapine extends the effects of amphetamine through metabolic interactions which lead to an extended presence of both clozapine and amphetamine in the brain. Difficulties in measuring clozapine in brain microdialysate required the development and use of an alternate method of determination of clozapine in brain homogenate. Behavioral measures were taken following each injection throughout the sensitization process. On the final injection day, amphetamine-sensitized rats were either administered amphetamine or saline followed 30 minutes later by clozapine. At 130 minutes following the initial amphetamine injections, rats were removed from the actometer, decapitated and whole brain clozapine concentrations were determined.

The preceding hypotheses are addressed in the following chapters. Although the goal of these studies is to establish links between drug

concentrations and behavior, results for each chapter are loosely divided into a neurochemical component and a behavioral component. Chapter two addresses hypotheses numbers one and two by introducing the chemical and behavioral techniques required for development of a method to concurrently measure brain dialysate and behavior in amphetamine treated rats. Chapter three addresses hypotheses three, four, and five. Experiments in chapter three employ the method developed in chapter two to concurrently measure amphetamine kinetics and amphetamine-induced behaviors in Fischer 344 and Sprague Dawley rats. Chapter four introduces a liquid/liquid extraction method to determine whole brain clozapine levels in amphetamine-clozapine treated rats to address hypothesis number six.

CHAPTER 2.

Method for the simultaneous measurement of behavior and brain dialysate in rats.

Introduction

Results of this chapter address hypotheses one and two and are expected to demonstrate: 1) An HPLC/MS-MS method can be developed to measure amphetamine, amphetamine metabolites and clozapine. 2) A microdialysis system can be developed that will allow extraction of extracellular fluid from the striatum of rat brains without corrupting behavioral data as collected on the force-plate actometer.

Automated behavioral measurements have been successfully performed in this lab using the force plate actometer (Fowler et al., 2001). The precision and sensitivity of this instrument has allowed us to quantify subtle, but significant differences between the behaviors of Fischer 344 (F344) rats and Sprague Dawley rats following d-amphetamine administration (Thisayakorn, 2006) without using behavioral rating scales. These findings indicate that F344 rats are more sensitive to the behavior-modifying effects of amphetamine. The measured behavioral differences between these rat strains in response to amphetamine led to the question of the neurochemical nature behind these differences and how that relates to behavior. Continuing

a laboratory culture of advancement and ingenuity we have decided to expand the capabilities of the force plate actometer by combining neurochemical measurements with the high resolution behavioral measurements already in place to determine whether drug pharmacokinetics contribute to behavioral response of these rat strains. This chapter introduces a technique which combines force plate actometer technology with microdialysis to concurrently measure brain neurochemistry and behavior in freely moving rats.

The experiments presented in this chapter involve microdialysis in the striatum of rat brain. Microdialysis is a method that was first conceived by Bito et al. in 1966 and first applied successfully in vivo in 1974 by Ungerstedt and Pycock. Microdialysis is a technique that allows continuous monitoring of the extracellular environment in living tissue by passive diffusion of molecules through a probe consisting of a hollow fiber dialysis membrane (Hansen et al., 1999). In addition to brain, microdialysis may be performed in a wide range of tissues including blood, liver, skin, muscle, and others (de Lange et al., 2000).

The microdialysis system employs a tethering mechanism attached to a soft rubber harness that is worn by the rat. The tether is a flexible spring that supports the tubing that allows perfusion solution (in this case artificial cerebral spinal fluid) to flow from a syringe pump, through a probe implanted in the brain, and back to a collection vial. The apparatus is designed to allow

the rat maximum flexibility of movement and such rats are frequently referred to as “freely moving”. However, physical principles indicate that some restriction of movement is inherent in the tethering mechanism and the harness that is worn by the rat so the term “minimally restrained” is probably more descriptive.

In behavioral studies, even small disruptions can greatly affect the outcome of an experiment. A very important advantage of the force plate actometer over other commercial actometers is its ability to detect subtle changes in vertical forces brought about by rat behavior. Due to the highly sensitive nature of the force plate, the disruptions caused by microdialysis may affect measurements of rat behavior compared to results obtained from rats that are completely unrestrained. An important purpose of this chapter is to determine whether the restrictive nature of the microdialysis tethering mechanism will significantly affect amphetamine induced behaviors as compared to untethered rats. This will be accomplished by analyzing known behavioral patterns and comparing these results to previously obtained results from untethered rats.

Amphetamine elicits a wide range of behaviors in laboratory rats. At repeated moderately high doses (5.0 mg/kg), rats show sensitization in the form of enhanced stereotypy scores (Kuczenski and Segal, 1986). Focused stereotypy scores as measured on the force plate actometer, are a combination of locomotor activity and power spectral force changes in the

vertical plane. These power spectra of vertical forces are expressed as a rhythm typically occurring at about 10Hz. The rhythm is due to the vertical head movements that are observed in rats engaged in focused stereotypy. When power spectral measurements indicate a high amount of activity but locomotor activity is low, resulting stereotypy scores are high (Fowler et al., 2001). Graphs containing raw power spectral and locomotor activity measurements will be generated from amphetamine treated rats subjected to microdialysis monitoring. Confirmation of successful behavioral measures will require the presence of the 10 Hz peak and suppressed locomotor activity during the first part of a four hour session. For confirmation of behavioral results obtained from rats exposed to the atypical antipsychotic drug clozapine, focused stereotypy scores will be generated and compared to previously seen patterns expressed by rats exposed to amphetamine followed 30 minutes later by clozapine.

The molecules analyzed to test this method include amphetamine, amphetamine metabolites (p-hydroxyamphetamine, p-hydroxynorephedrine and norephedrine), dopamine, and clozapine. In rats, amphetamine metabolism takes place primarily via hydroxylation by the cytochrome P450 2D subfamily of enzymes (Law et al., 2000). The major metabolites of amphetamine (figure 2-1) are p-hydroxyamphetamine, norephedrine, and p-hydroxynorephedrine (Ring et al., 1970). These metabolites have been shown to have neurochemical effects on norepinephrine (Brodie et al., 1970),

serotonin, and dopamine (Matsuda et al., 1989). It was suggested by Anggard, et al (1970) that these metabolites may also play a role in amphetamine-induced psychotic behavior in humans. However, Kuczenski et al. (1982) suggested that activity of amphetamine metabolites do not fully explain the augmentation of behavior seen in amphetamine sensitized rats. The method presented in this chapter attempts to measure behavior while concurrently measuring amphetamine and amphetamine metabolites via microdialysis to determine if a relationship between levels of these molecules correlate with behaviors elicited from amphetamine treatment.

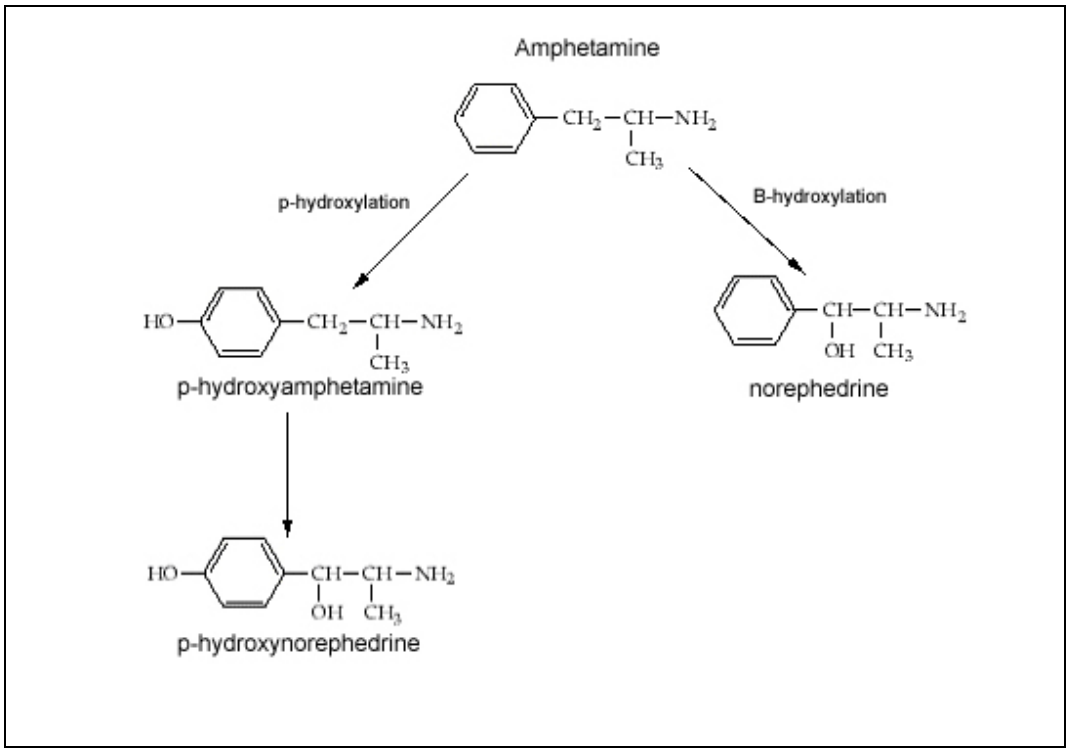
Clozapine was included in this study due to effects on stereotypy previously seen in this lab. Amphetamine-induced behaviors have been extensively used in simulation models of schizophrenia (Ellenbroek and Cools, 2000). Paradoxically, Clozapine, which is an effective atypical antipsychotic drug, has been shown to enhance amphetamine induced stereotypies (Robertson and MacDonald, 1984), and lengthen the focused stereotypy phase (Fowler et al., 2007). If clozapine and amphetamine compete for metabolic enzymes, this could partially explain these findings. Support for this comes from research showing that clozapine, like amphetamine, is metabolized by cytochrome p450 enzymes. The CYP3A and CYP1A subfamilies are believed to be primarily responsible, however the CYP2D subfamily may also play a role (Fang et al., 1998). As previously mentioned, the CYP2D subfamily is largely involved in amphetamine

metabolism. Concurrently monitoring clozapine and amphetamine levels in amphetamine-treated rats could offer insight into the role of drug interactions in amphetamine-induced behaviors.

Analyzing a range of molecules with different chemical properties presents a unique problem. HPLC-tandem mass spectrometry was chosen as the analytical method because it affords an opportunity to measure a wide range of molecules in a single microdialysis sample (described fully in methods). Mass spectrometry also allows the measurement of dialysate directly without a derivitation or solid phase extraction step that is necessary with other analytical methods (Soares et al., 2004). Using mass spectrometry we attempt to demonstrate the capabilities and limitations in quantifying these molecules in both standard in vitro solutions and in brain dialysate from behaving rats. Employing these chemical methods in dialysate obtained from rats undergoing behavioral analysis can result in a powerful technique with a broad range of applications.

Figure 2-1. Amphetamine metabolism in rats.

The two primary pathways involved in amphetamine metabolism in rats are para-hydroxylation of the ring structure and β-hydroxylation of the hydrocarbon chain. The primary metabolite is p-hydroxyamphetamine which undergoes further hydroxylation to form p-hydroxynorephedrine. Norephedrine is formed by meta hydroxylation of the hydrocarbon chain. Metabolism for both pathways occurs via the p450 2D subfamily of enzymes (Law et al., 1999).


Amphetamine
p-hydroxylation
B-hydroxylation
p-hydroxyamphetamine
norephedrine
p-hydroxynorephedrine

MATERIALS AND METHODS

Materials

Animals

Fischer (F344) rats were purchased from Charles River. Sprague Dawley rats were purchased from Harlan. Animals were received at the University of Kansas animal care facility at approximately 6-7 weeks of age and were observed in quarantine for two weeks prior to being accessed. Experiments were begun approximately 1 month after arrival. Animals were kept on a 12 hour light/dark cycle in standard plastic cages with non-aromatic wood chip bedding. Food and water were available *ad libitum* except during the four hour sessions in the actometer. Use of the animals was approved by the University of Kansas Institutional Animal Care and Use Committee, and procedures adhered to the National Institutes of Health 1996 edition of the Guide for the Care and Use of Laboratory Animals.

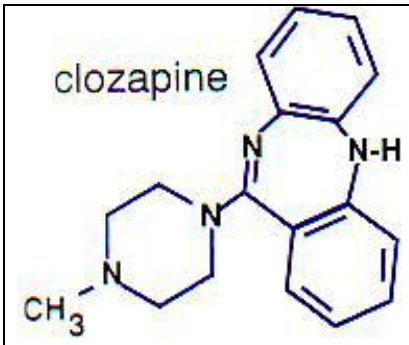
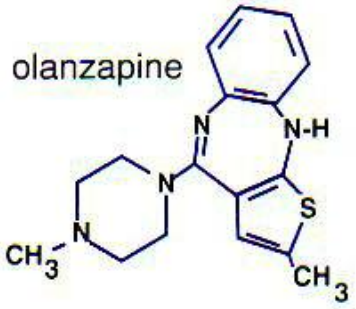
Solutions and Calibration Curves

Dopamine (3-hydroxytyramine), serotonin (5-hydroxytryptamine hydrochloride), d-amphetamine sulfate and the amphetamine metabolites DL-Norephedrine hydrochloride, p-hydroxynorephedrine, and p-

hydroxyamphetamine, were purchased from Sigma. Olanzapine was purchased from Lilly. For HPLC optimization and calibration curve determination, analytes were dissolved in an artificial cerebral spinal fluid solution containing 130mM NaCl, 24mM $NaHCO_3$, 10mM Glucose, 1.5mM $MgSO_4.7H_2O$, 3.5mM KCl, 1.25mM $NaH_2PO_4.H_2O$, 2mM $CaCl_2.2H_2O$ adjusted to pH 7.4. Olanzapine was used as the internal standard due to the structural similarity to Clozapine (figure 2-2). For preparation of the internal standard solution, olanzapine was dissolved in artificial cerebral spinal fluid with 100µM $HClO_4$ to a final concentration of 100nM. $HClO_4$ was included to slow oxidation of dopamine (Hows et al., 2004). Standards were prepared from a 1uM stock solution. To obtain calibration curve solutions, stock solutions were diluted to 256nM followed by serial dilution to final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 nM. Stock solutions were protected from light and stored at -20º C. Standards were prepared immediately prior to analysis. To obtain a calibration curve, the analyte solutions were added to an equal volume of internal standard solution resulting in final calibration curve concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 nM. For microdialysis analysis, 15µl of internal standard solution was added to vials prior to collecting 15µl samples for a final volume of 30µL per sample. The final concentration of internal standard for both calibration curve and rat dialysate sampling was 50nM.

Figure 2-2. Structure of the atypical antipsychotic Clozapine and the internal standard Olanzapine

Olanzapine is a multi-receptor analogue of clozapine and is derived from the structural modification of clozapine (Shen, 1999). As compared with the structure of clozapine, the chemical structure of olanzapine involves replacement of one of the side rings of the dibenzodiazepine molecule with a sulfur-containing 5-atom ring containing a methyl group at its 2-C position and deletion of chlorine in the remaining side ring.

clozapine  MW: 326.83	olanzapine  MW: 312.44

Behavioral measurement apparatus

The design of the force-plate actometer instrument is based on four force transducers located at each of 4 corners of a low weight, high stiffness and shear square plate. While the width of the plate can vary to some degree, all behavioral measurements performed in these studies were done on a plate that is 30 cm square. Force transducers are located at each of the four corners with the center of the transducers being 1 cm from the edge of the plate. This gives an effective distance of 28 cm between the centers of each transducer. A 5mm thick Plexiglas encasement is placed over the force plate and suspended approximately 2-5 mm over the plate so as not to make contact. The effective inside area of the force plate is 28cm X 28 cm. A 20 cm square opening was cut in the top of the Plexiglas enclosure to allow access for microdialysis sampling.

The force transducers serve two primary purposes. The first purpose is to determine the exact location on the plate of the center of mass of the object (rat or mouse) that is placed on it. The horizontal (or x,y) coordinates of the animal can be determined through an algorithm that calculates the relative force applied to each of the four transducers at a rate of 50 samples per second or more. Another important feature of the force transducers is to measure force changes in the vertical (or Z) coordinates. Measuring vertical force changes introduces a dimension that is not available in other popular

activity monitoring instruments (Fowler et al., 2001). By determining force changes, subtle aspects of behavior that cannot be seen through visual observation can be quantified. Force changes that are present during tremor and other stereotypical behaviors can be measured and monitored over a period of time. By combining measurements of rat locomotion in the x,y plane with measurements of force changes in the vertical axis, a determination of focused stereotypy has been achieved. Focused stereotypy scores are determined when small changes in the x,y plane, but large changes in the Z-force exist such as would occur when the animal is not locomoting but is moving its head with great force as is seen during amphetamine-induced focused stereotypy.

Microdialysis

The microdialysis setup included a CMA/100 microinjection pump with a 1000uL syringe (BAS). FEP tubing connected the syringe to an Instech swivel (375/D/22QM) weighing 17.6 grams. The swivel was mounted on a CMA/120 lever mount. A 12" flexible spring tether (1/4" wide, 10g) was attached to the swivel and tubing was passed through the center of the spring tether to the rat via a soft rubber harness (7g). The harness was purchased from BAS. Total mass of the swivel, spring tether, harness and tubing is 34.6 grams. A counterweight attached to the lever that held the swivel and tethering mechanism was positioned such that the swivel and tether were

exactly counterbalanced. The probe was a 3mm membrane length CMA/12 with a shaft length of 14mm and a membrane cutoff of 20 kiloDaltons. FEP tubing was attached to the inlet and outlet of the microdialysis probe from the rear of the animal's head.

Artificial Cerebral spinal fluid (130nM NaCl, 24nM $NaHCO_3$, 10mM Glucose, 1.5mM $MgSO_4.7H_2O$, 3.5mM KCl, 1.25mM $NaH_2PO_4.H_2O$, 2mM $CaCl_2.2H_2O$ – pH 7.4) was perfused at 1 µL/min and dialysate was collected in 12 X 32 mm total recovery vials (Waters Corp.) every 15 minutes (15 µL / sample).

Chromatographic equipment and conditions

A Waters Acquity Ultraperformance Liquid Chromatography (UPLC) instrument was used for separation of dialysate molecules. The Acquity system utilizes higher pressure so smaller diameter columns with smaller particle sizes may be used. This allows better separation and recovery for small injection volumes as are required in microdialysis sampling and analysis. The column was a Phenomenex Synergi Hydro RP (4 microm, 50 mm x 3.0 mm id). This is a C18 column with proprietary polar end-capping which provides hydrophobic and polar retention via polar interactions, hydrogen bonding, or electrostatic interactions. This allows for separation of a broad range of analytes (Kiridena et al., 2004). Flow rate was 0.3ml/min.

For the data presented in this section, a gradient method was used. Mobile phase 'A' consisted of 99:1 water:acetonitrile (v/v) with 0.6% formic acid. Mobile phase 'B' consisted of 99:1 acetonitrile:water (v/v) with 0.6% formic acid. HPLC injection volume was 20µL.

Mass Spectrometer

The HPLC instrument was attached to a tandem mass spectrometer for quantification of dialysate molecules following HPLC separation. The mass spectometer was a Micromass Ultima with a triple quadrupole analyzer. Detection was performed by positive electrospray ionization in the multiple reaction monitoring mode (MRM). The MRM transitions analyzed were clozapine (327→270), Olanzapine (313→256), p-hydroxyamphetamine (168.1→105.1), Dopamine (153.9→91), p-hydroxynorephedrine (152.1→117.1), Norephedrine (152.1→107.1), and amphetamine (135.9→91). Details of transitions will be covered in more detail in the results section.

Methods

Stereotaxic Surgery for implantation of guide cannula

Approximately one month after receiving animals, guide canullas were implanted into the striatum following IACUC approved rodent aseptic surgery policies and is described here.

Prior to surgery, rats were anesthetized by a subcutaneous injection of an acepromazine (0.67 mg/kg), ketamine (67.5 mg/kg), xylazine (3.4 mg/kg) mixture (diluted to 1.8mL with sterile injection saline). The area of the top of the head from the base of the skull forward to approximately even with the front of the eyes was shaved as closely as possible and ophthalmic solution was applied to the eyes to prevent drying.

The scalp was disinfected with three alternating applications of betadine and ethanol and an incision was made in the scalp along the mid-line. Small clips were then attached to hold the skin away from the mid-line. The underlying tissues were cleared to expose the skull. Using stereotaxic coordinates (AP = + 0.10 cm, Lat = -0.27 cm, Vert = -0.36 cm) the position for the probe was marked and three small holes were drilled nearby using a Dremel drill. A bone screw was inserted in each. These served to hold the epoxy and dental acrylic which keeps the guide cannula in position. The hole for the microdialysis probe was then drilled and the guide cannula lowered into this hole to a depth of 0.36 cm as determined by the stereotaxic coordinates. Dental acrylic was applied in several layers to form a cone shaped base that anchors the guide cannula in place. The animal was then given an injection of saline to prevent dehydration and placed on a heating pad until awake.

Microdialysis

Once the guide cannula was secured in place, the rats were allowed a 1 week recovery period prior to beginning microdialysis. On the morning of microdialysis, the rat was placed in the modified force-plate actometer chamber. A CMA microdialysis probe was connected to the FEP tubing and artificial cerebral spinal fluid was perfused for a few minutes until uninterrupted flow was observed in the outlet tubing. The implanted guide cannula was removed and replaced with the dialysis probe. This was done without anesthetic and was not outwardly traumatic to the animal. The animal was then left in the chamber for a minimum of 1 hour until flow reached equilibrium. Equilibrium was determined by weighing the dialysate produced over a 15 minute period. This period also allowed the animal to recover from the stress involved in being connected to the microdialysis tethering mechanism which may affect behavior. When dialysate reached a 15 minute weight of 15 µg, samples were collected. 15 µl of internal standard solution was placed in each of 18 total recovery vials and stored at -20 degrees C. For each sample collected, the respective vial was removed and placed in the sample collection rack. Following a 15 minute collection period the vial was removed and immediately frozen at -20 degrees. Samples were stored at -20 degrees until HPLC Mass Spec analysis could be performed. This was never more than one week following sample collection.

Mass Spectrometry

Tandem mass spectrometry provides unequivocal identification of molecules based on mass and fragmentation of the molecule into daughter ions specific to the parent molecule. Molecules are immediately introduced into the mass spectrometer following separation in the HPLC system. The tandem mass spectrometer method used for these studies employs positive electrospray ionization (Ashcroft, 1997; Yamashita and Fenn, 1984). Positive ion spray requires an ionization source (H^+), which was provided by the addition of formic acid to the HPLC mobile phase. From the HPLC system, sample emerges from the tip of a stainless steel capillary and high voltage is applied resulting in an aerosol of highly charged droplets. Nitrogen gas further evaporates the solvent and directs the spray through a sampling cone and into the mass spectrometer. The cone (or lens) voltage was adjusted individually for each sample to optimize each molecule's signal. Resulting ions then pass through the first quadrupole analyzer which, based on mass to charge ratio (m/z), selectively allows only the analyte of interest to pass. The resulting parent molecule then passes into the collision cell. The collision cell consists of a chamber containing an inert gas (argon). The selected sample ion collides with the molecules of argon and is fragmented resulting in daughter ions. Collision energy dictates the density of argon in the collision chamber and was adjusted for each ion of interest to optimize the production of a specific daughter ion. The fragments (or daughter) ions then pass into

the second quadrupole analyzer which allows only the selected daughter ion to pass through to the detector which monitors ion current, amplifies it, and transmits the information to a computer. The resulting signal is in arbitrary units based on the amount of signal from the detector. Due to the selective nature of this instrument, only one analyte at a time can be analyzed. Dwell time is the amount of time the mass spectrometer spends analyzing each molecule and was set at 0.05 seconds/sample.

A multiple reaction monitoring (MRM) method was developed for analyte analysis as follows. For determination of parent and daughter ions a 25 nM solution containing the molecules of interest (clozapine, olanzapine, p-hydroxyamphetamine, dopamine, p-hydroxynorephedrine, norephedrine, and amphetamine) was prepared in a 70% methanol solution and injected directly into the mass spectrometer. Samples were analyzed in MS1 mode to determine the optimal cone voltage and mass for the parent molecule. During MS1 mode the collision cell and second quadrupole are turned off. Operating the instrument in MS1 mode utilizes only the first quadrupole to optimize the signal and specificity of the parent molecule that will be allowed to reach the collision cell. After the parent molecule was optimized, the instrument was set to MS2 mode to determine fragmentation of the parent into daughter ions. MS2 mode allows the selected parent molecule to enter the collision cell where fragmentation takes place. The second quadropole was initially turned off so all resulting daughter ions could be viewed. A specific daughter ion

was selected based primarily on signal intensity. The collision energy was adjusted to result in optimal fragmentation of the parent ion into the selected daughter ion. The second quadrupole was then turned on to allow only the selected fragment to reach the detector. This process was repeated for each analyte of interest.

Experimental Method and Conditions

Sprague Dawley and Fischer 344 rats were received and the guide cannula implanted as described. Following a one week recovery period, individual animals were placed in the chamber. Although the actometer was not in a dedicated enclosure, lights in the room were turned off leaving only indirect light from an adjacent room. Some lighting was necessary for the experimenter to exchange microdialysis vials as needed. Precautions were taken to minimize disruptions including an insulated pad on the door accessing the room. After exchanging the guide canula for a probe as previously described, artificial cerebral spinal fluid was perfused at 1µl/min until equilibrium was achieved. No fewer than two 15uL samples were collected to achieve a baseline measurement prior to drug administration. The animal was given an intraperitoneal injection of amphetamine and behavioral recording was immediately started. For the first 5 injection days, an injection of saline was given 30 minutes into the session as a control for

the clozapine injection that was given on injection day 6. Samples were collected every 15 minutes with every 5th sample being weighed to confirm that flow and collection volume was consistent throughout the 4-hour session. This procedure was repeated every 4th day for 5 days for each rat to achieve sensitization. On the sixth injection day, animals were given an injection of Clozapine 30 minutes following the amphetamine injection.

RESULTS

For clarity, results are divided into three sections. Section one includes results from procedures involved in the development of analytical techniques and quantitative results from *in vitro* samples prepared in solution. The second section introduces results of chemical analyses obtained from brain dialysate. The third section includes behavioral data obtained from the force-plate actometer.

A. Results from analytical development

Chromatography

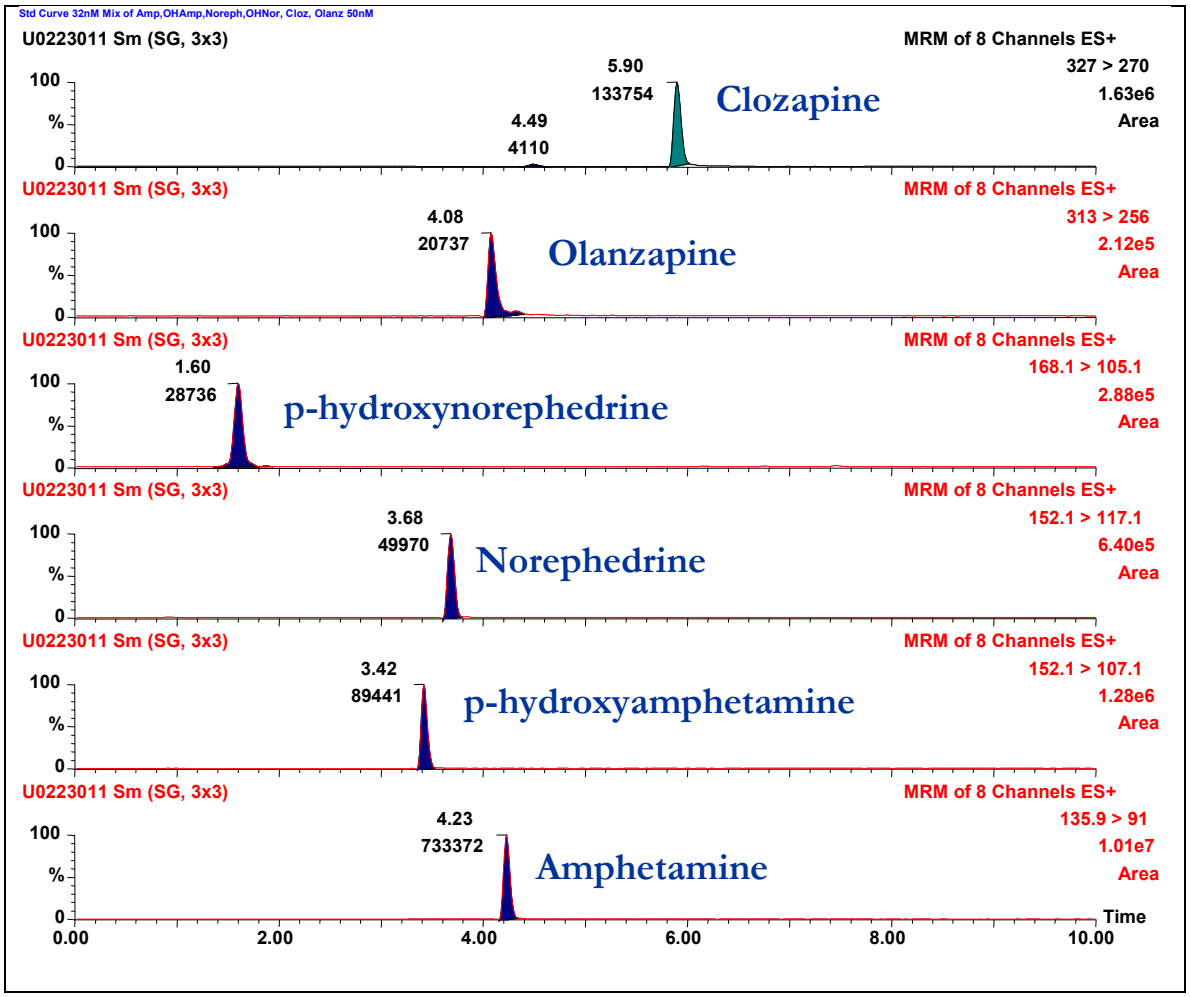
For HPLC elution the optimal gradient was found to be a 12 minute cycle beginning at 99% A (aqueous) and 1% B (organic) and sloping gradually to 70% A and 30% B over 5 minutes. The gradient was increased to 1% A and 99% B over one minute and held at this flow for 2 minutes before returning to 99% A. It was held at 99% A for 3 minutes to return to equilibrium prior to the next run.

HPLC analysis using the Phenomenex Hydro-RP column and the gradient described shows good separation (figure 2-3). Four of the six molecules elute between 3.5 and 4 minutes. At steeper gradients these molecules had poor separation (data not shown). p-hydroxynorephedrine,

being the most hydrophilic molecule eluted at around 1.6 minutes and Clozapine eluted last at about 5.5 minutes. All molecules eluted during the shallow phase of the gradient. Standard curves show linearity for each of the analytes. Limits of quantitation were determined as 10 times baseline.

Figure 2-3. HPLC-tandem mass spectrometry elution profile of Clozapine, Olanzapine (IS), p-hydroxyamphetamine, Norephedrine, p-hydroxyamphetamine, and Amphetamine.

Elution profile starting from top to bottom includes clozapine, olanzapine, p-hydroxynorephedrine, norephedrine, p-hydroxyamphetamine, and amphetamine. The x-axis represents the time line over which data was collected. The y-axis shows relative signal generated by the detector in percent of maximum. Number labels within the graph represent time of elution and peak integration for each peak. Results are from a 32nM standard prepared in artificial cerebral spinal fluid with 0.1M $HClO_4$ Molecules were separated by HPLC and detected by tandem mass spectrometry (see methods).


Std Curve 32nM Mix of Amp,OHAmp,Noreph,OHNor, Cloz, Olanz 50nM
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
5.90
133754
Clozapine
327 > 270
1.63e6
Area
4.49
4110
100
%
0
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
4.08
20737
Olanzapine
313 > 256
2.12e5
Area
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
1.60
28736
p-hydroxynorephedrine
168.1 > 105.1
2.88e5
Area
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
3.68
49970
Norephedrine
152.1 > 117.1
6.40e5
Area
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
3.42
89441
p-hydroxyamphetamine
152.1 > 107.1
1.28e6
Area
U0223011 Sm (SG, 3x3)
MRM of 8 Channels ES+
4.23
733372
Amphetamine
135.9 > 91
1.01e7
Area
Time
0.00
2.00
4.00
6.00
8.00
10.00

Mass Spectrometry

Molecules, fragmentation and transitions

Dopamine

MS2 analysis shows that dopamine produced daughter ions of m/z 119 and m/z 91 (figure 2-4a). The most abundant fragment was m/z 91. It was shown by Hao et al. (2001) that this transition in protonated dopamine likely occurs by the loss of an ammonia ion (NH_2^-) first, followed by the loss of H_2O and then the subsequent loss of a carboxyl group (CO). This results in an aromatic ring structure which was the most abundant fragment.

Clozapine

MS2 analysis of Clozapine (m/z 327) revealed relatively weak signals at m/z 296.1, 227, and 84.2 with a very strong signal at m/z 270.1 (figure 2-4b). Molecular weight analysis indicates that the most probable fragmentation resulting in a MW 270.1 daughter ion involves the loss of the carboxylated piperazine ring structure.

Olanzapine (Internal Standard)

Transition states of protonated olanzapine (*m/z* 313.2) were found using MS2 analysis to be m/z 256, and m/z 84, with smaller, insignificant fragments also detected (figure 2-4c). Fragmentation likely involves the

piperazine side chain (Smyth et al., 2004; Bogusz MJ, 1999) with the daughter ion composed of the remaining heterocyclic ring structure. The most abundant fragment of m/z 256 was used for the MS/MS analysis.

p-hydroxynorephedrine

MS2 transition states of the amphetamine metabolite p-hydroxynorephedrine (m/z 168.1) were 135, 133, 105, and 91 (figure 2-4d). The most abundant transition was 105.1. This corresponds to an aromatic ring with an attached CH_2 group.

Norephedrine

MS2 analysis showed that the major transition states of the amphetamine metabolite norephedrine (m/z 152.1) were 134 117, 115, 91 and 56 (figure 2-4e). The most abundant transition was 117.1 corresponding to an aromatic ring with a CHCH group attached, although this was not confirmed

p-Hydroxyamphetamine

p-Hydroxyamphetamine (m/z 152.1) has an identical parent mass to norephedrine and the two differ only in the placement of the hydroxyl ion. MS2 analysis of the transition states of p-hydroxyamphetamine revealed daughter ions of m/z 134 and 107 (figure 2-4f). The most abundant transition

was 107.1. The molecular weight of this fragment corresponds to loss of the $CH_2CHCH_3NH_2$ group leaving the phenol group. . Norephedrine also has a daughter at m/z 117. NJoerephedrine also has a daughter at m/z 117. Interference was anticipated but because HPLC elution times would be different, this was not a concern in terms of quantitation.

Amphetamine

Major transition states of protonated amphetamine (m/z 135.9) were m/z 137.2, 119, 91, 81.1, and 65.3 (figure 2-4g). As with dopamine, the most abundant transition resulted in a daughter ion of m/z 91. This is the molecular weight of the aromatic ring structure and is the likely site of fragmentation given that the structure of amphetamine is similar to that of dopamine.

Limits of quantitation

Lower limits of quantitation were determined using the criteria of 10 times baseline with a lowest concentration tested of 500 picomolar (0.5nM) as described in the methods section. The limit of quantitation for dopamine was determined to be 50 nM (figure 2-5). This value is higher than basal striatal dopamine levels which are between 7 and 20 nM in the rat brain striatum (Chen, 2004). This limits the use of tandem mass spectroscopy in dopamine determination in this paradigm. For norephedrine and p-hydroxynorephedrine the lower limit of quantitation was found to be 2 nM. The lower limit of

quantitation for p-hydroxyamphetamine was 1 nM. For amphetamine and clozapine the lower limit of quantitation was found to be below 0.5nM which was the cutoff for determination. Figures 2-6 through 2-10 show standard curves of amphetamine, p-hydroxyamphetamine, norephedrine, p-hydroxynorephedrine and clozapine for values within the quantifiable range.

Figure 2-4. Transition states of molecules following selective fragmentation of parent ions by MS2 analysis

MS2 analysis of dopamine, clozapine, olanzapine, p-hydroxynorephedrine, norephedrine, p-hydroxyamphetamine, and amphetamine. Graphs show relative signal of daughter ions following fragmentation of parent ions. X-axis values are the mass to charge ratio of all molecules exiting the collision cell. Peaks indicate intensity of signal of the fragment reaching the detector. In each case the daughter ion with most abundant levels were selected for the MRM method

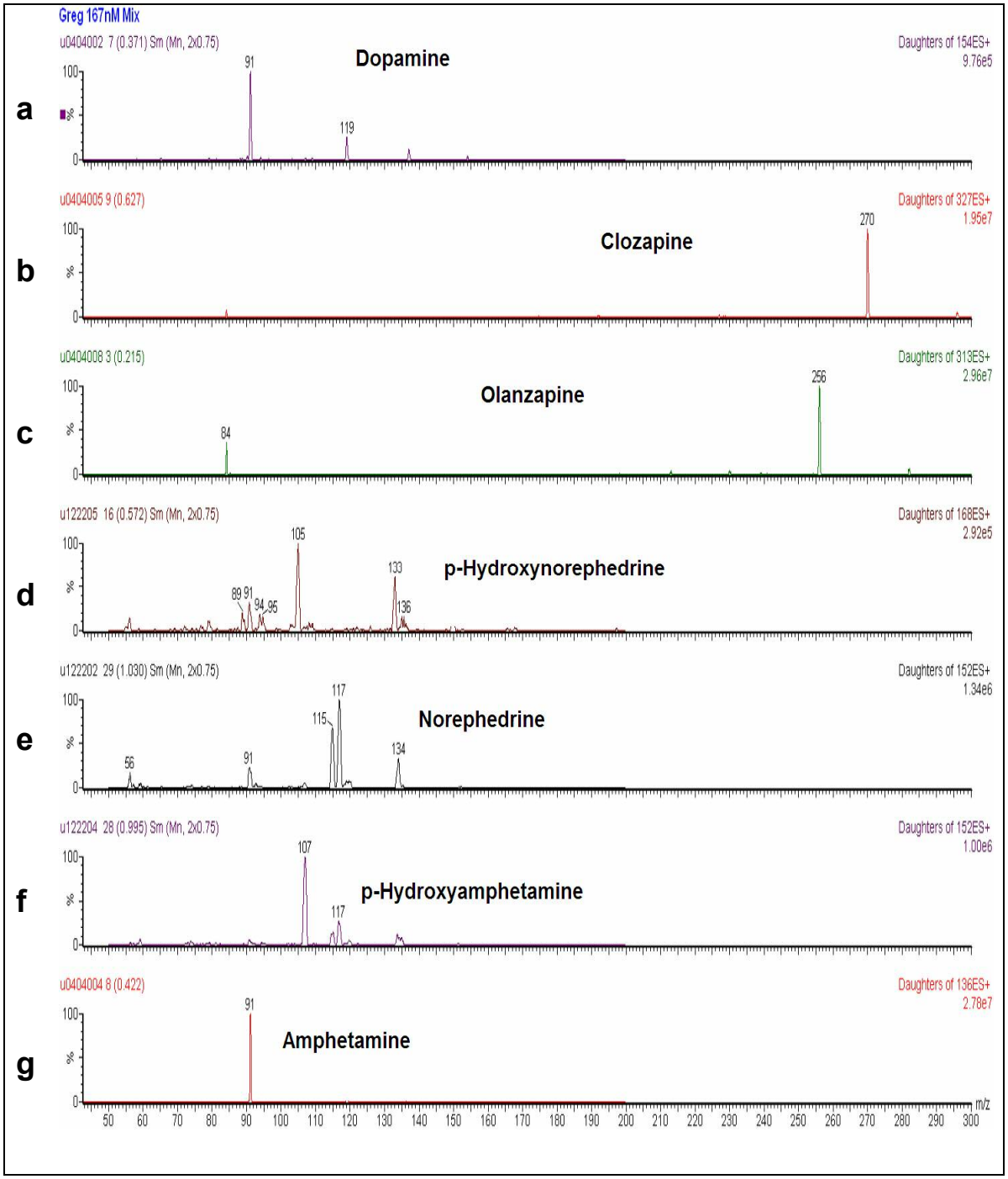

Greg 167nM Mix
a
u0404002 7 (0.371) Sm (Mn, 2x0.75)
Daughters of 154ES+
9.76e5
Dopamine
91
119
b
u0404005 9 (0.627)
Daughters of 327ES+
1.95e7
Clozapine
270
c
u0404008 3 (0.215)
Daughters of 313ES+
2.96e7
Olanzapine
256
84
d
u122205 16 (0.572) Sm (Mn, 2x0.75)
Daughters of 168ES+
2.92e5
p-Hydroxynorephedrine
105
133
89 91
94 95
136
e
u122202 29 (1.030) Sm (Mn, 2x0.75)
Daughters of 152ES+
1.34e6
Norephedrine
117
115
134
56
91
f
u122204 28 (0.995) Sm (Mn, 2x0.75)
Daughters of 152ES+
1.00e6
p-Hydroxyamphetamine
107
117
g
u0404004 8 (0.422)
Daughters of 136ES+
2.78e7
Amphetamine
91
%
m/z
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300

Figure 2-5. Mass spectrometry trace of dopamine at lower limit of quantitation. Tracing shows dopamine at 50 nM. The x-axis reflects time of elution. The y-axis reflects percent signal detected. Limits of quantitation are determined as having signal amplitude greater than or equal to 10 times baseline.

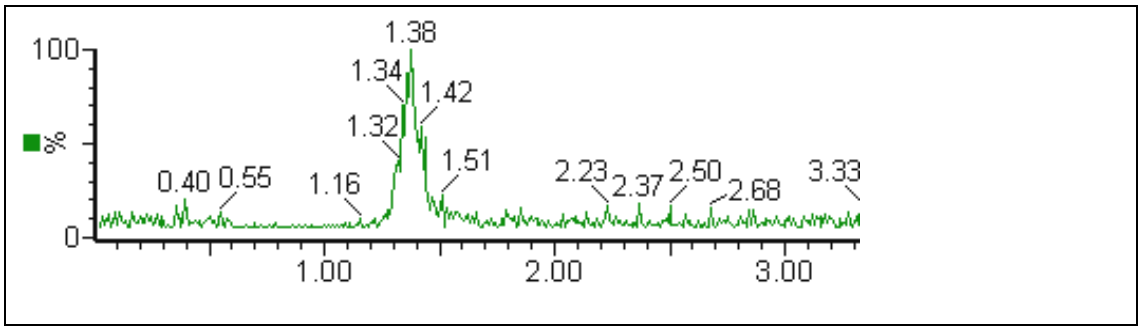
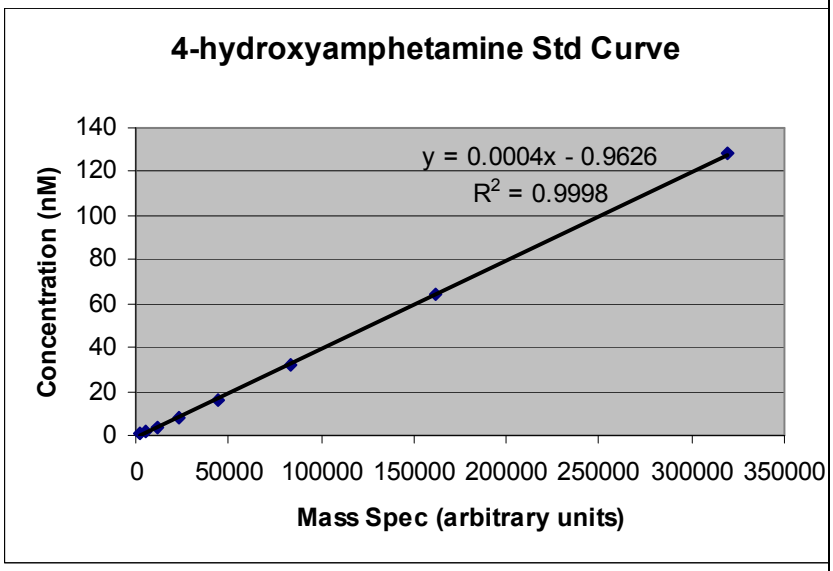

100
%
0
1.38
1.34
1.42
1.32
1.51
0.40
0.55
1.16
2.23
2.37
2.50
2.68
3.33
1.00
2.00
3.00

Figure 2-6 – Figure 2-10. Standard curve for p-hydroxyamphetamine, norephedrine, p-hydroxynorephedrine, clozapine and amphetamine.

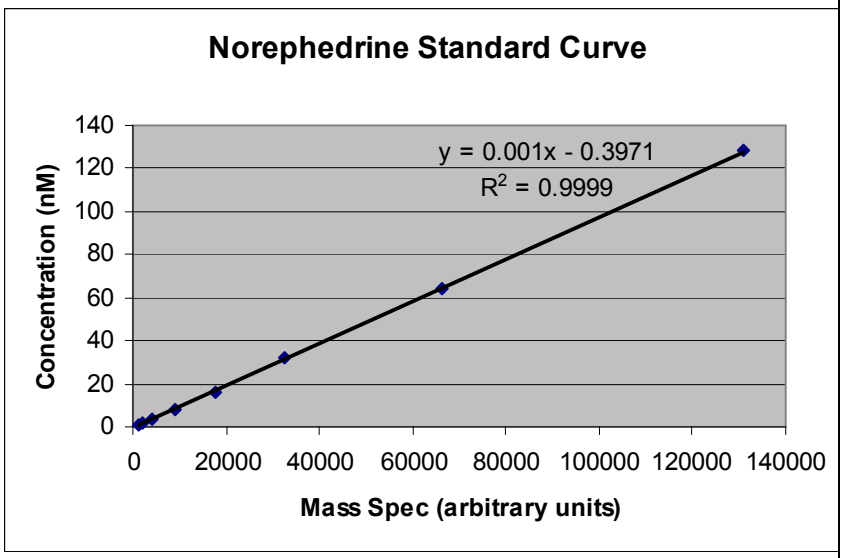
For all graphs standards were prepared from stock solutions and adjusted by serial dilution. Values for y-axes include concentration of prepared solutions. Values for x-axes are detection amplitude in arbitrary units as reported by mass spectrometry. Tables on the right are values used to plot graph. Values in the left column are mass spectrometry results in arbitrary units. Values in the right column are nanomolar analyte concentration. Ranges include lower limits of quantifiable concentrations.

Figure 2-6



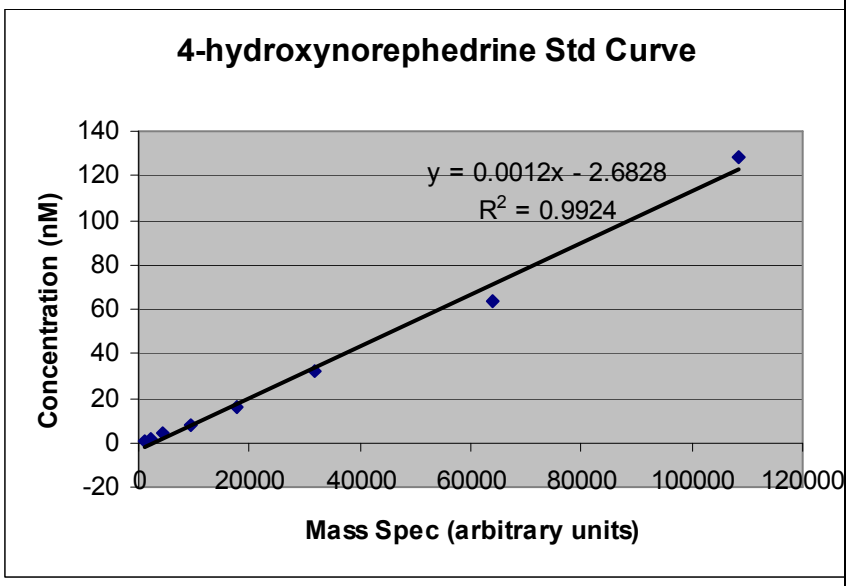
Mass Spec	nM
2586	1
5698	2
11501	4
23495	8
44318	16
83695	32
161717	64
319051	128

Figure 2-7



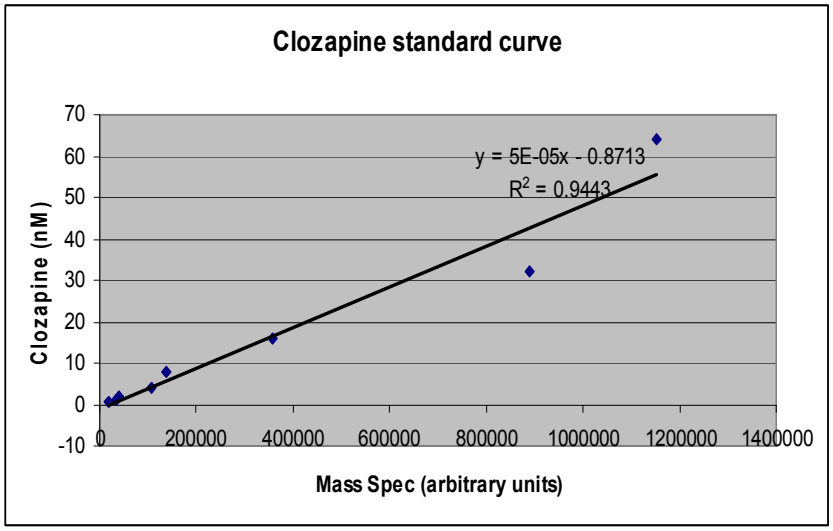
Mass Spec	nM
1102	2
2207	4
4221	8
8874	16
17584	32
32724	64
66177	128
131124	256

Figure 2-8



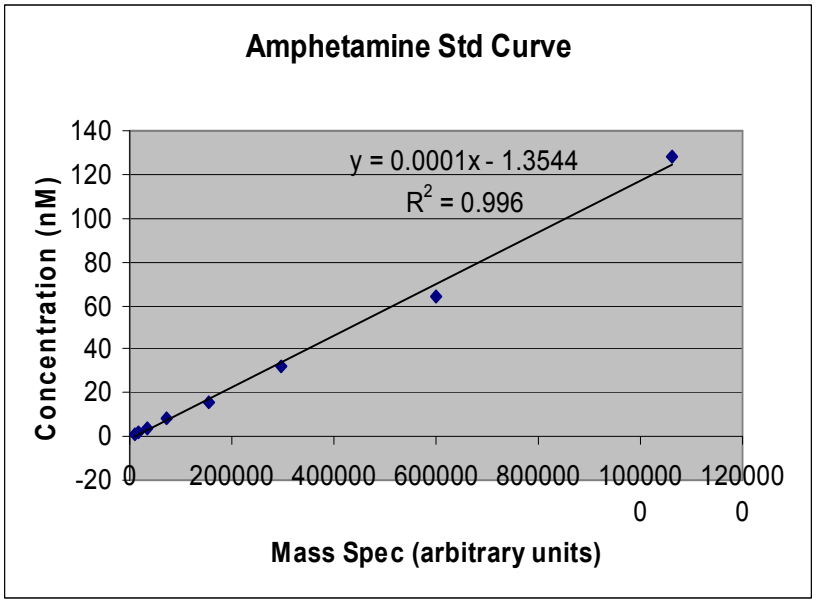
Mass Spec	nM
911	2
2304	4
4219	8
9414	16
17788	32
31874	64
64053	128
108541	256

Figure 2-9



Mass Spec	nM
16899	0.5
34323	1
40682	2
107992	4
138941	8
358784	16
889279	32
1152476	64

Figure 2-10



Mass Spec	nM
8888	0.5
16293	1
33611	2
73726	4
156082	8
296218	16
599810	32
1062049	64

B. Results of brain microdialysis quantification, *in vivo* sample

Dopamine, clozapine, norephedrine, p-hydroxyamphetamine, p-hydroxynorephedrine

For all animals in all cases p-hydroxynorephedrine, norephedrine or dopamine and clozapine could not be detected. Levels of p-hydroxyamphetamine were detected at low levels but often below the lower limits of quantitation. Available data for a single Sprague Dawley rat (figure 2-11) shows that p-hydroxyamphetamine levels appear to rise over the course of the sensitizing series of injections.

Olanzapine

Olanzapine data showed that analysis in standard solutions of artificial cerebral spinal fluid produced consistent results but there was variability in the striatal microdialysis samples. These results appear to be exclusive to dialysate samples since all brain samples showed fluctuating results while olanzapine used in standard curves that were prepared from artificial cerebral spinal fluid and analyzed during the same run produced consistent results. Figure 2-12 demonstrates the variability of olanzapine in brain dialysate as

compared to the stable isotope analogue of amphetamine (amphetamine-d_{11}) when held constant at 50nM throughout a 4 hour session.

Amphetamine

Results for amphetamine show that amphetamine was consistently quantifiable in both prepared solutions and in brain microdialysate. Amphetamine was well above lower limits of quantitation for all animals and in all time frames. Pharmacokinetic analysis of brain microdialysate confirms that amphetamine follows first order kinetics with a maximum concentration (Cmax) at 30 minutes (figure 2-13). Strain analysis of Cmax showed variability between Cmax values for each injection for both strains (figure 2-14). No significant differences were seen between groups or between injection days due to the small sample size. Half life values were between 45 and 90 minutes for both strains (figure 2-15). On the sixth injection day when clozapine was administered, no differences in amphetamine levels were seen between previous injection days suggesting that clozapine did not influence amphetamine concentrations. Results for half-life values showed no differences following clozapine administration on day 6. F344 rats showed an extended half life following clozapine administration but this value was not significant in this study. A larger sample size would be needed to show significance.

Figure 2-11. p-hydroxyamphetamine levels for each of 5 injection days.

Graph shows p-hydroxyamphetamine levels for each of 5 injections of a sensitizing series of 5.0 mg/kg d-amphetamine injections. The x-axis represents injection number. Injections were given every fourth day. The y-axis represents p-hydroxyamphetamine levels in nM. Values are average concentrations throughout a 4 hour session.

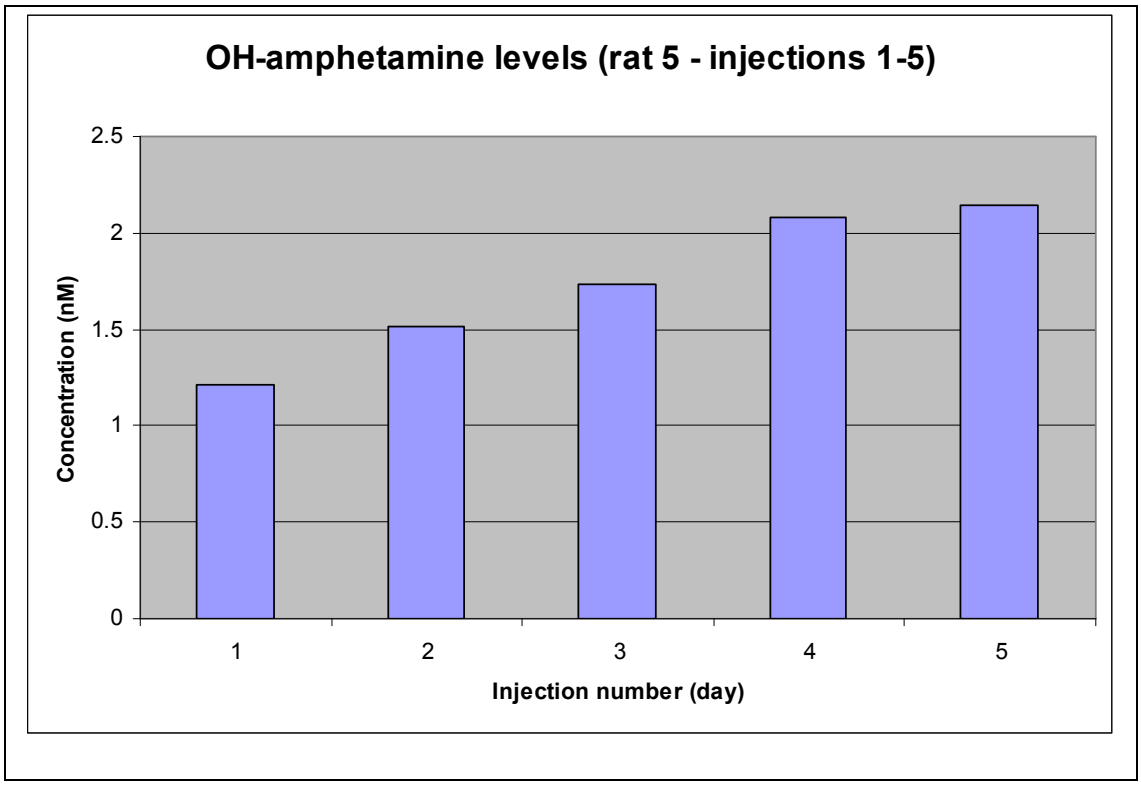

OH-amphetamine levels (rat 5 - injections 1-5)
Concentration (nM)
2.5
2
1.5
1
0.5
0
1
2
3
4
5
Injection number (day)

Figure 2-12. Tandem mass spectrometry results of olanzapine over a 4 hour session.

Graph shows variability of olanzapine in brain dialysate over a 4 hour session. Brain extracellular fluid was perfused into a collection vial containing artificial cerebral spinal fluid (0.1M $HClO_4$) with 100nM olanzapine. Samples were collected every 15 minutes. Olanzapine was held constant throughout the run. D11 amphetamine is shown under identical conditions. Variability for olanzapine was far greater than d-11 amphetamine under the same conditions indicating that olanzapine variability was not reflective of instrument variability. Because an internal standard is intended to correct instrument detection variability, it was determined that olanzapine was unacceptable as an internal standard.

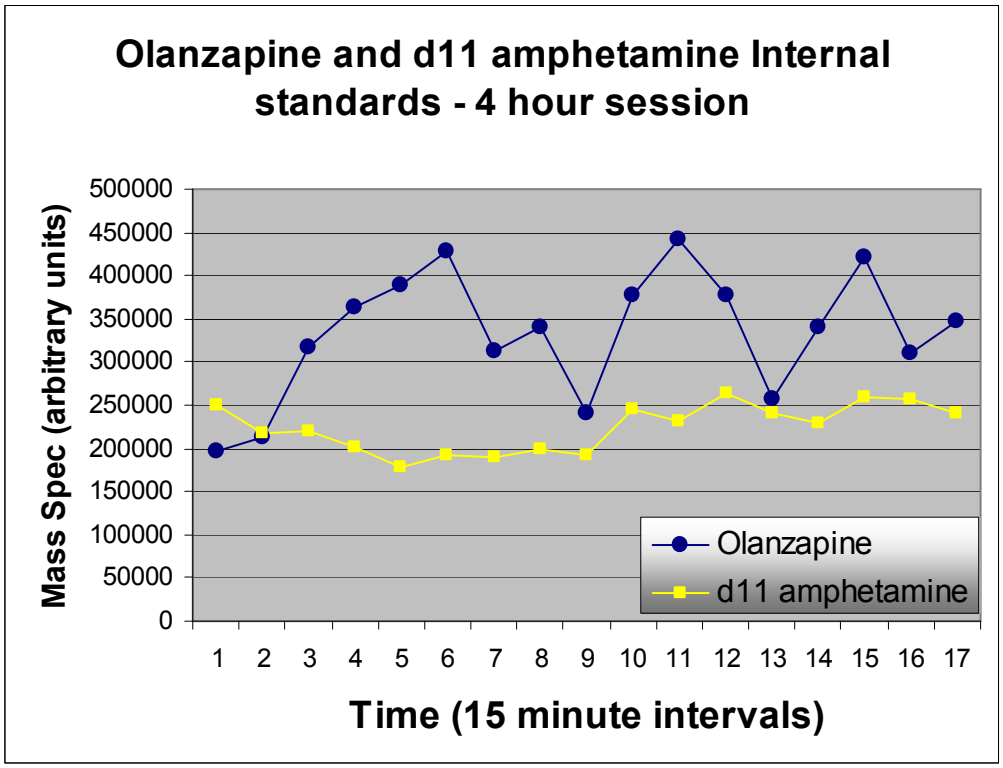

Olanzapine and d11 amphetamine Internal standards - 4 hour session
Mass Spec (arbitrary units)
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time (15 minute intervals)
Olanzapine
d11 amphetamine

Figure 2-13. Amphetamine concentration in striatum of Sprague Dawley and F344 rats

Graph shows average amphetamine levels as measured by microdialysis at 15 minute intervals throughout a 4 hour actometer session. Values are average amphetamine concentrations of injections 1-5 of a sensitizing series of 5.0mg/kg d-amphetamine (IP). Two Sprague Dawley and two Fischer 344 rats represent each strain. The x-axis represents time in 15 minute intervals. The y-axis represents amphetamine concentration in nM. Brackets are standard error of the mean. Both strains show first order kinetics with a rapid absorption phase followed by a slower, logarithmic elimination phase.

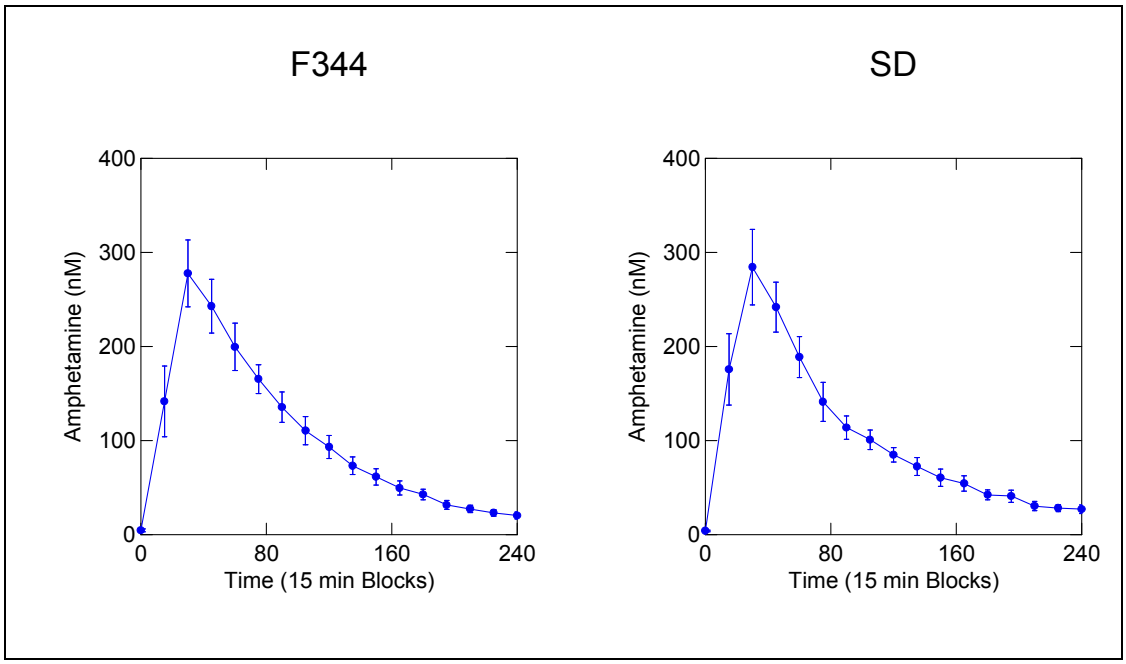

F344
SD
Amphetamine (nM)
400
300
200
100
0
0
80
160
240
Time (15 min Blocks)

Figure 2-14. Amphetamine Cmax by strain for each of six injections

Values reflect maximum amphetamine concentration of samples collected over 15 minute intervals during a 4 hour microdialysis/actometer session. Injections of 5.0 mg/kg d-amphetamine were administered every 4 days for a total of six injections. Injections 1-5 (x-axis) reflect the sensitization phase during which animals received an initial injection of amphetamine followed 30 minutes later by saline. On injection day 6 animals received amphetamine followed 30 minutes later by 10.0 mg/kg clozapine. No significant differences could be seen between injection days or between groups due to the small sample size.

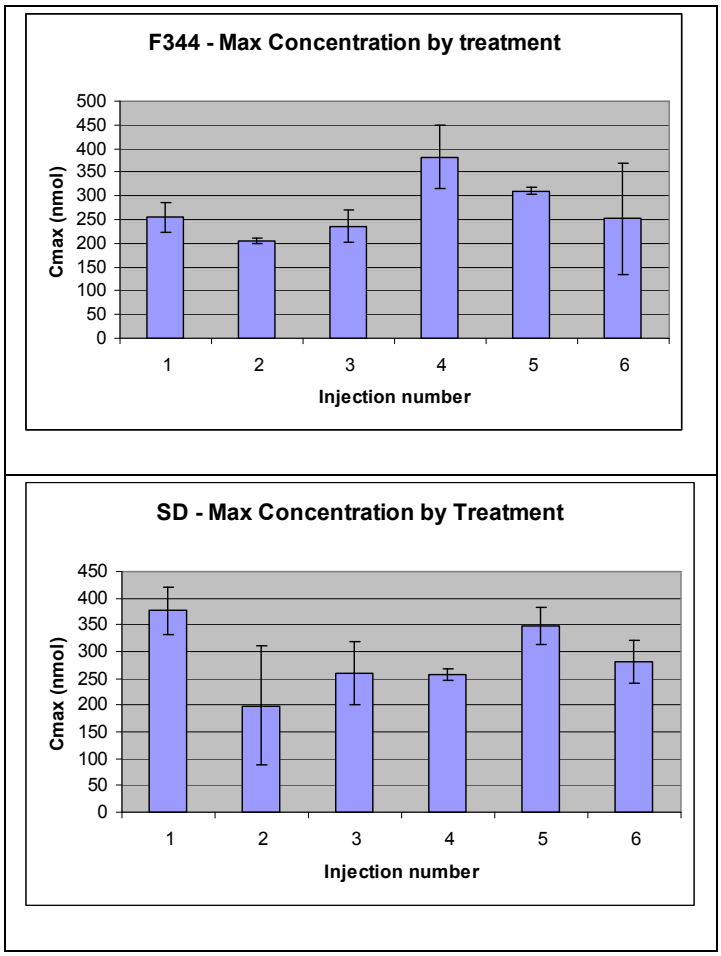
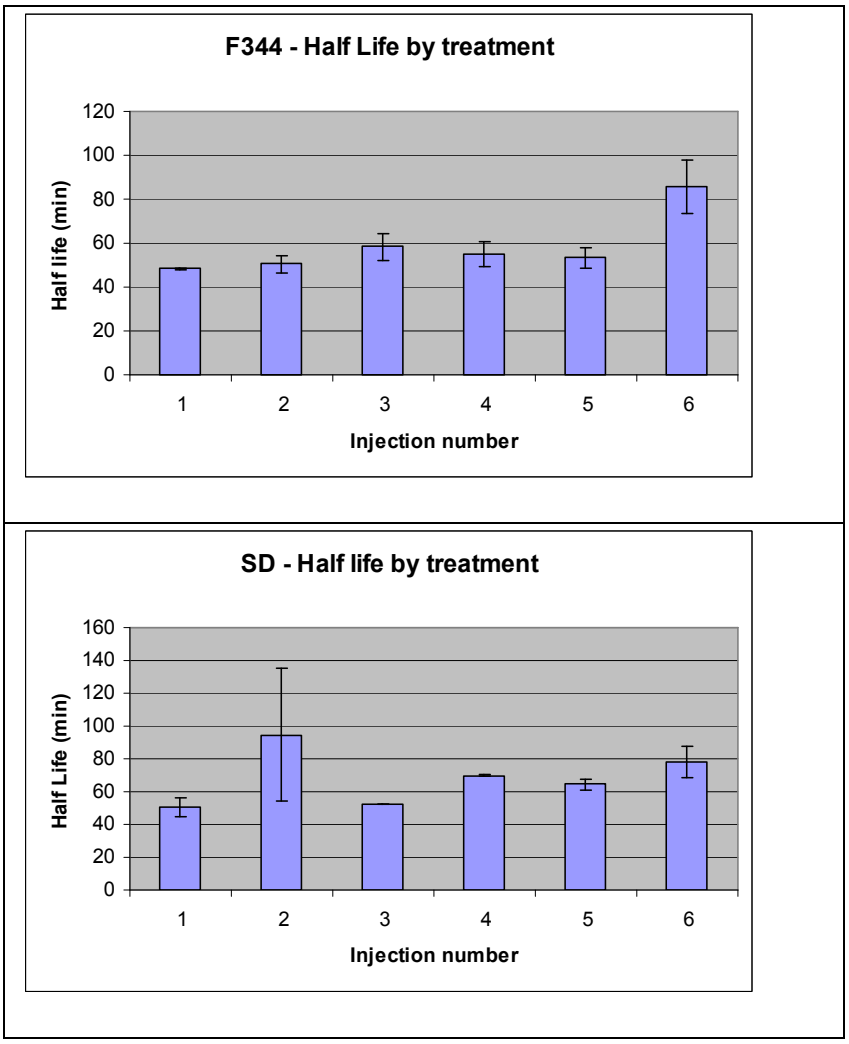

F344 - Max Concentration by treatment
Cmax (nmol)
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
Injection number
SD - Max Concentration by Treatment
Cmax (nmol)
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
Injection number

Figure 2-15. Amphetamine Cmax by strain for each of six injections

Values reflect amphetamine half life in brain microdialysate as calculated by Pharsight WinNonLin software. Half life values were based on the slope obtained from the terminal elimination rate constant and calculated as the value at half the distance from Cmax to the final data point. Samples were collected over 15 minute intervals during a 4 hour microdialysis/actometer session. Injections of 5.0 mg/kg d-amphetamine were administered every 4 days for a total of six injections. Injections 1-5 (x-axis) reflect the sensitization phase during which animals received an initial injection of amphetamine followed 30 minutes later by saline. On injection day 6 animals received amphetamine followed 30 minutes later by 10.0 mg/kg clozapine. No significant differences could be seen between injection days or between groups due to the small sample size however Fischer 344 rats showed an elevated half-life value for clozapine injection day 6.


F344 - Half Life by treatment
Half life (min)
120
100
80
60
40
20
0
1
2
3
4
5
6
Injection number
SD - Half life by treatment
Half Life (min)
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Injection number

C. Behavioral and molecular analysis

Figure 2-16 shows results of force plate actometer data obtained from a tethered Sprague Dawley rat included in this study from which microdialysis data was obtained (top) compared to results from an untethered, amphetamine treated Sprague Dawley rat (bottom). Data for both rats are from a 4 hour session on the fifth day of a sensitizing series of 5.0 mg/kg d-amphetamine at a time when both rats would be considered completely sensitized to the behavioral effects of amphetamine. Each column represents one hour. Individual frames include activity over a three minute period. Line graphs on the left of each column are power spectra of the vertical force variation showing peak rhythmic activity during each time frame. Square boxes to the right of the spectral data show the locomotor trajectory of each rat during that 3-minute frame. Power spectra in both graphs show a peak rhythm at about 10 Hz starting within 10 minutes of receiving amphetamine and lasting between 1 and 2 hours. Such a peak is characteristic of Sprague Dawley rats. Also evident in both rats was the decrease in locomotor activity during the stereotypy phase.

Figure 2-17 shows results of force plate actometer data obtained from a tethered Fischer 344 rat included in this study (top) compared to results from an untethered, amphetamine treated Fischer 344 rat (bottom). F344 rats

differ from Sprague Dawley rats primarily in the degree to which they express vertical head movements. This is evident from the broader, shorter duration of expression of peaks at 10 Hz seen in both tethered and untethered rats. Suppression of locomotor activity during the stereotypy phase is also apparent in both graphs. Figure 2-20 shows behavioral results from each rat during the sensitization phase (injections 1, 3, and 4 shown), plus data from clozapine treated animals on injection day 6.

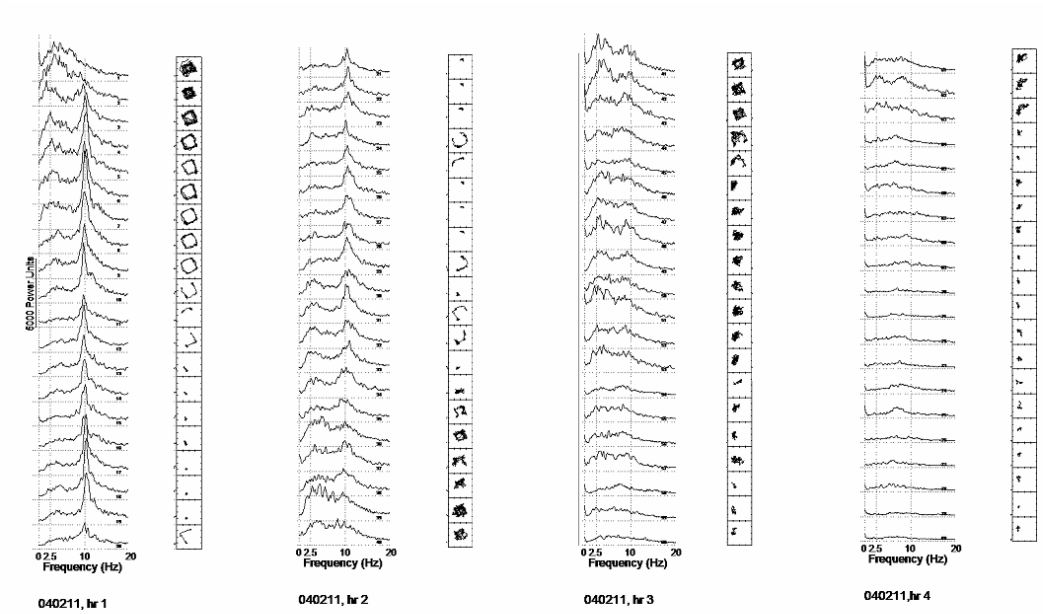
Stereotypy scores for Fischer 344 (left) and Sprague Dawley (right) rats are shown in figure 2-18. Results show that there was little expression of focused stereotypy during injection 1 (top row) and that behavioral sensitization took place between injections 1 and 5. Figure 2-19 shows that clozapine has an effect on stereotypy. Fischer 344 rats (top) show that stereotypy is prolonged in rats treated with clozapine 30 minutes following amphetamine treatment compared to controls receiving saline instead of clozapine. Sprague Dawley rats (bottom) show a temporary decrease in stereotypy score immediately following clozapine administration at 30 minutes. This was previously reported (Fowler et al., 2007) and is characteristic of amphetamine treated Sprague Dawley rats administered 10mg/kg doses of clozapine at 30 minutes.

Figure 2-16. Power spectra and locomotor trajectory data comparing a tethered Sprague Dawley rat undergoing microdialysis versus an untethered Sprague Dawley rat with no microdialysis.

Graphs for both animals reflect force-plate actometer data for injection day 5 of a sensitizing series of d-amphetamine. Behavioral sessions lasted 4 hours and were started seconds after receiving 5.0 mg/kg amphetamine. The top graph shows data obtained from a Sprague Dawley rat included in this study (Rat 4) that was undergoing simultaneous microdialysis sampling and behavioral analysis on the force plate actometer. The bottom graph shows data from an animal that was not undergoing microdialysis and was enclosed in a light and sound attenuating chamber. Raw data from the force plate actometer was analyzed using quantization software such as Pascal, MATLAB, and Systat. Graphs show 4 columns representing each hour of the 4 hour session. Each column has been separated into a power spectral component expressed as frequencies of oscillations of movements on the force-plate (rectangular boxes on the left), and locomotor trajectories showing the movement trajectories of the center of force of the rat on the force-plate (square boxes on the right). Each frame represents activity during a three minute period. Data shows that both rats express a power spectral peak at about 10 Hz reflecting vertical head movements associated with focused stereotypy beginning ten to fifteen minutes following amphetamine administration. Both animals also show decreased locomotion associated

with these peaks, although locomotion seemed to initially be higher in tethered rats included in this study. Points represent periods of spatial confinement during which the animal was awake (as seen by power spectral activity), but not locomoting. This period of focused stereotypy was followed by a period of elevated activity before returning to a low level of activity. Both graphs reflect the critical activities of peak force oscillations at 10Hz and decreased locomotor activity during the focused stereotypy phase.

Tethered, sensitized Sprague Dawley rat - 5.0mg/kg amph



Untethered, sensitized Sprague Dawley rat – 5.0 mg/kg amph

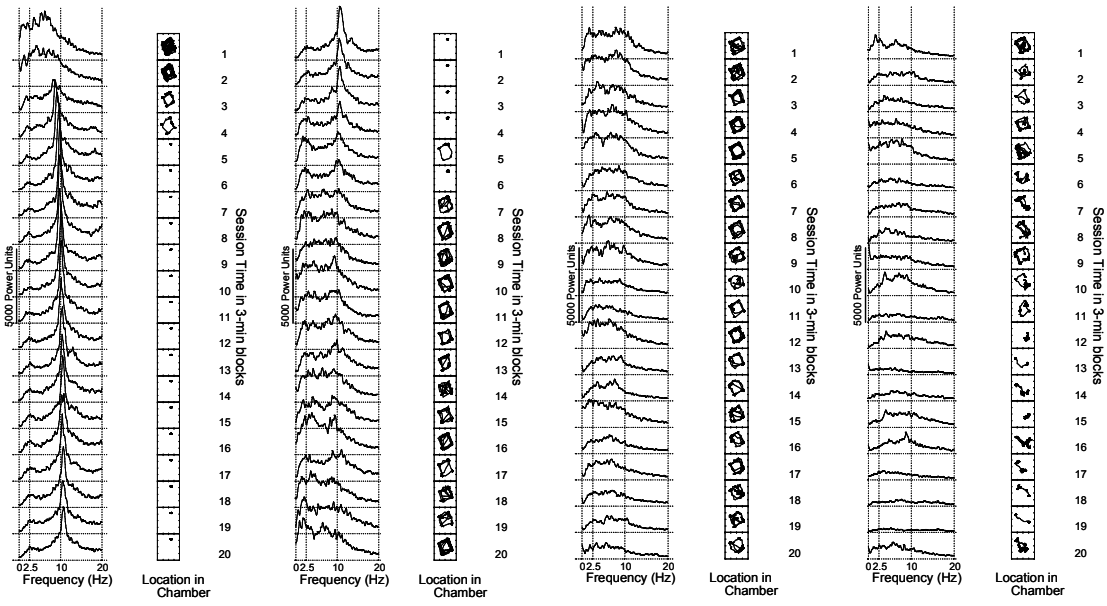
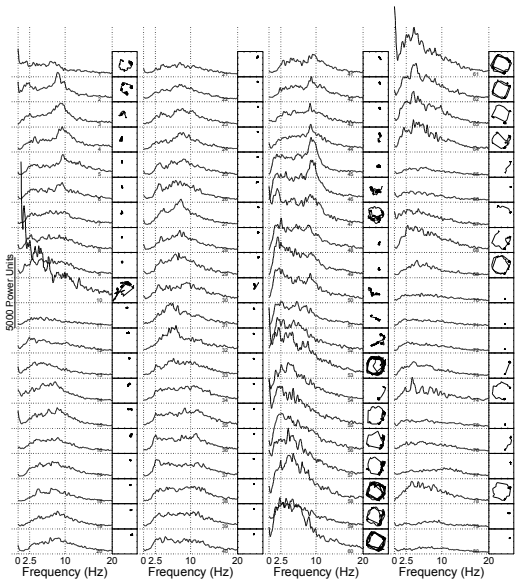


Figure 2-17. Power spectra and locomotor trajectory data comparing a tethered Fischer 344 rat undergoing microdialysis versus an untethered Fischer 344 rat with no microdialysis.

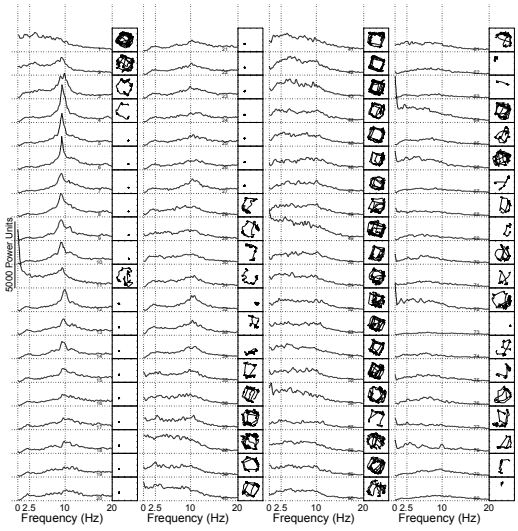
The top graph shows data obtained from a Fischer 344 rat included in this study (Rat 3) that was undergoing simultaneous microdialysis sampling and behavioral analysis on the force plate actometer. The bottom graph shows data from a Fischer 344 rat that was not undergoing microdialysis and was enclosed in a light and sound attenuating chamber. Graphs for both animals reflect force-plate actometer data for injection day 5 of a sensitizing series of d-amphetamine injections. Behavioral sessions lasted 4 hours and were started seconds after receiving 5.0 mg/kg amphetamine. Both animals received a saline injection at 30 minutes (corresponding to frame 10 of the graphs). Both animals show a 10Hz peak that is wider and with lower amplitude than that seen for Sprague Dawley rats. The peak was also only prominent during the first few frames before largely subsiding or disappearing. This transient peak at 10 Hz is characteristic of F344 rats. Data also show spatial confinement in terms of the locomotor trajectory tracing during the focused stereotypy phase.

Tethered, sensitized F344 rat – 5.0 mg/kg amph



030205, hr 1 030205, hr 2 030205, hr 3 030205,hr 4

Untethered, sensitized F344 rat – 5.0 mg/kg amph



011503, hr 1 011503, hr 2 011503, hr 3 011503,hr 4

Figure 2-18. Focused stereotypy for Fischer 344 and Sprague Dawley rats through a sensitizing series of amphetamine injections.

Graphs show mean focused stereotypy scores for F344 and Sprague Dawley rats following 5.0 mg/kg d-amphetamine. Scores of focused stereotypy are derived from measures of locomotor activity and power spectral data. A high focused stereotypy score would result from an animal expressing little or no locomotor activity but expressing a high amount vertical activity as would result from the animal staying in one place but rapidly and repetitively moving its head. Focused stereotypy scores increase throughout the sensitization process with the largest increase occurring between the first and second injection.

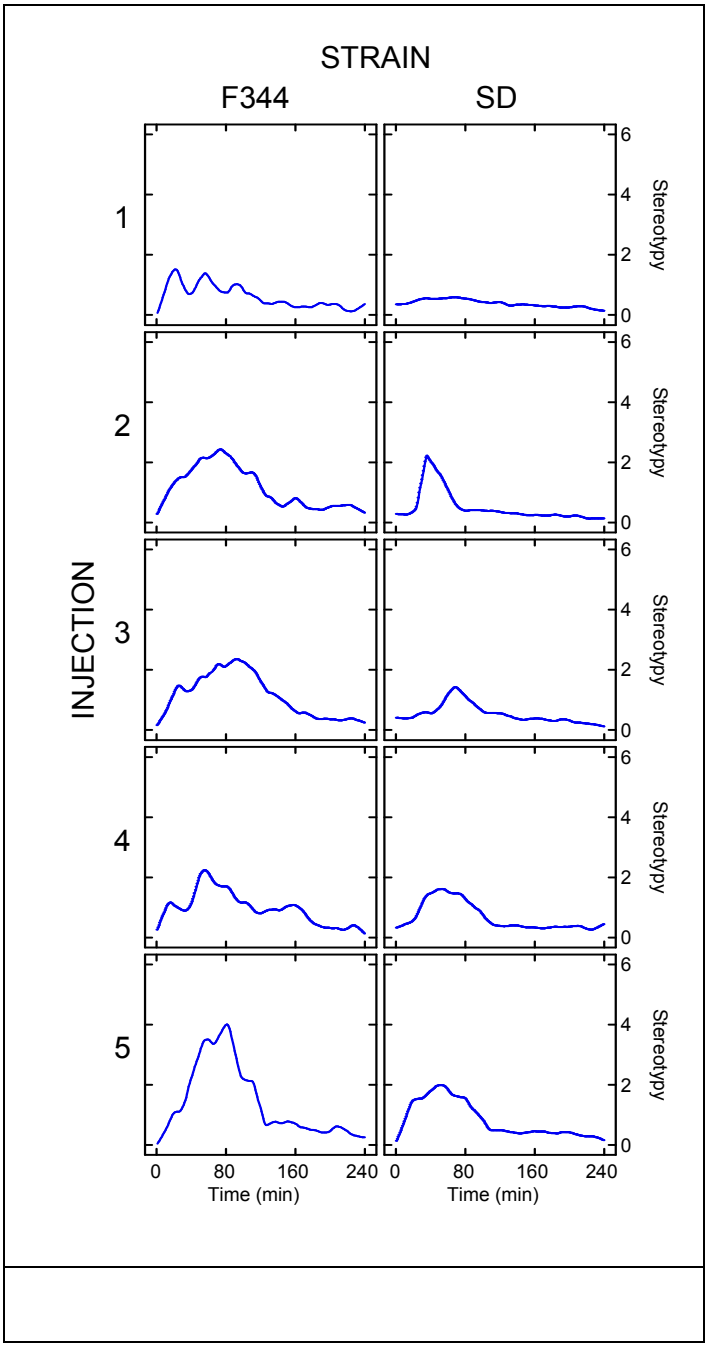
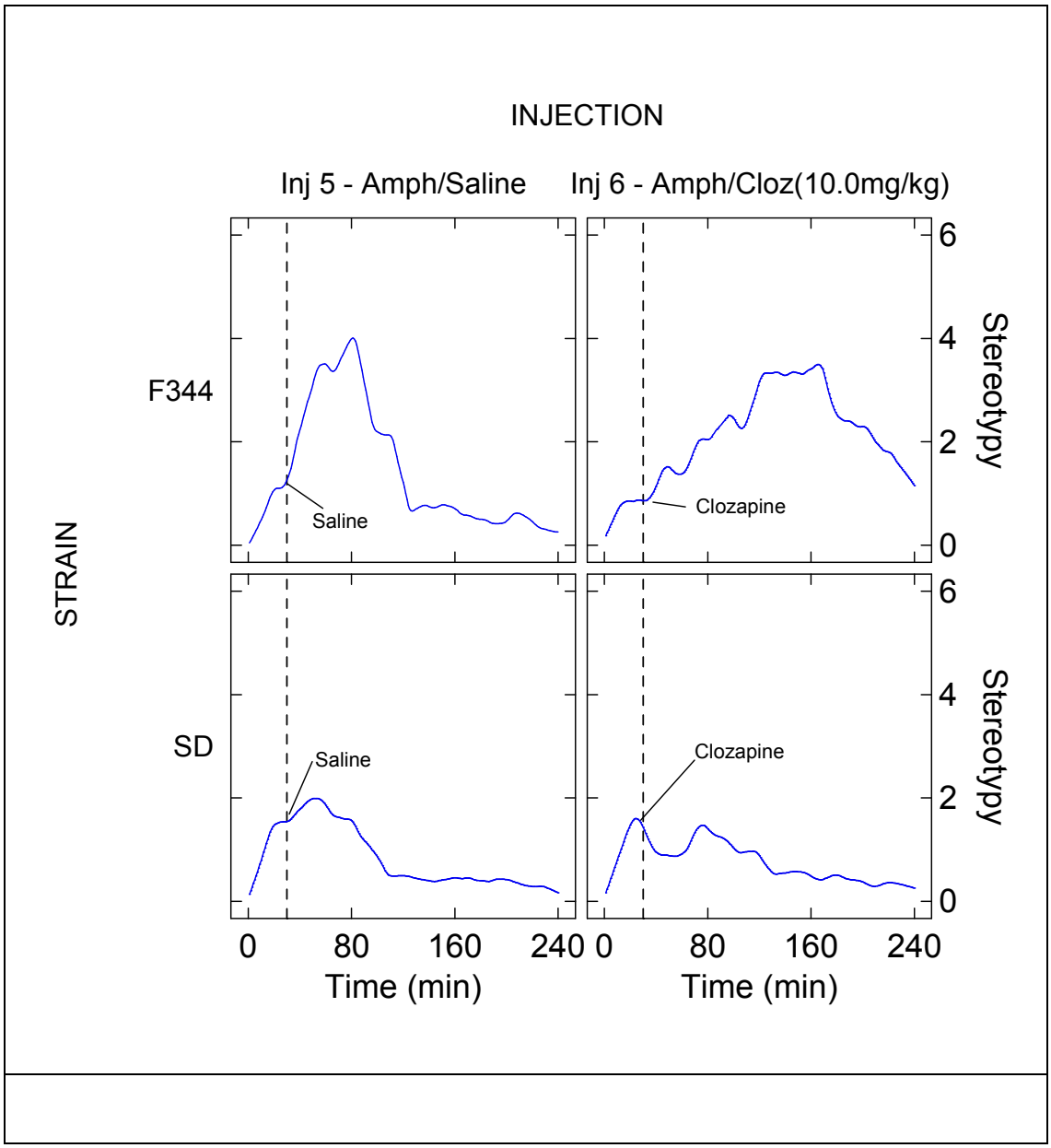

STRAIN
F344
SD
INJECTION
1
2
3
4
5
Stereotypy
0
2
4
6
0
80
160
240
Time (min)

Figure 2-19. Focused stereotypy for Fischer 344 and Sprague Dawley rats for animals receiving amphetamine plus saline, or amphetamine plus clozapine.

Graphs show scores of focused stereotypy for rats simultaneously undergoing microdialysis and behavioral analysis on the modified force plate actometer. The top two graphs are Fischer 344 rats and the bottom two graphs are Sprague Dawley rats. All animals received 5.0 mg/kg amphetamine immediately prior to beginning the session and either 1ml/kg saline or 10.0 mg/kg clozapine at 30 minutes (represented by the vertical, dashed line). The graphs on the left function as controls and include data from the fifth injection of the sensitizing series during which the animals received saline at 30 minutes. Graphs on the right represent data from the same animal 4 days later that received clozapine at 30 minutes. The charts show that F344 rats receiving clozapine show a longer focused stereotypy phase than saline treated controls. Sprague Dawley rats receiving clozapine do not show as much of an extension of focused stereotypy duration, but do show a transient decrease in focused stereotypy score following clozapine administration.


INJECTION
Inj 5 - Amph/Saline
Inj 6 - Amph/Cloz(10.0mg/kg)
STRAIN
F344
SD
Saline
Clozapine
Saline
Clozapine
Stereotypy
Stereotypy
0
2
4
6
0
80
160
240
Time (min)
Time (min)

DISCUSSION

Results from this chapter demonstrate that neurochemical data can be collected via microdialysis without extensively affecting key behavioral data as measured on the force plate actometer. Results of data from the chemical analysis portion of this study indicate that HPLC separation with tandem mass spectrometry detection is a viable option for most of the compounds tested. However, a discrepancy was found between results obtained in prepared *in vitro* solutions versus those obtained from perfused brain dialysate. All compounds prepared in solution were successfully separated on the HPLC instrument and detected by mass spectrometry. Amphetamine, amphetamine metabolites and clozapine all had limits of detection in the low nanomolar to picomolar range. Dopamine was shown to have a lower limit of quantitation of 50 nM which is well above basal dopamine concentrations of 4-20 nM previously found in the striatum (Chen, 2005). Limits of detection for amphetamine metabolites in solution were below previously reported whole brain concentrations (Kaddoumi et al., 2004; Honecker and Coper, 1975). Of the amphetamine metabolites, results show that only p-hydroxyamphetamine was detected in microdialysate, and then only transiently. Clausing et al. (1995) were unable to detect p-hydroxyamphetamine in Caudate/Putamen and Substantia Nigra dialysate using HPLC with fluorescence detection. The results of p-hydroxyamphetamine concentration that were obtained from this

study indicate that levels may increase over the course of sensitization. But the available data is only from one animal and not conclusive (figure 2-11). Of all compounds tested in this study, only amphetamine showed consistent results in brain dialysate for all animals throughout the entire four hour session.

The internal standard Olanzapine could not be consistently measured and thereby considered unacceptable as an internal standard for microdialysis sampling. While the reasons for the inconsistent results obtained from olanzapine are not clear, a possible reason is a matrix effect caused by the brain dialysate. The exact mechanism and origin of this matrix effect is not fully understood (King et al., 2000). The origin may be competition between the analyte, in this case olanzapine, and undetected ions formed in the HPLC-MS/MS interface (Matuszewski et al., 2003). Competition for ionization would allow the analyte of interest to enter the mass spectrometer quadrupole analyzer in a non-ionized form. A non-ionized molecule would not match the mass to charge (m/z) requirement specific to that molecule and would be excluded from passing through the first quadrupole and into the collision chamber for fragmentation and detection. Another likely possibility is precipitate formation. Olanzapine is a lipophilic molecule with poor solubility in aqueous solutions (Eli Lilly and Co., 2004). Olanzapine is reported by the manufacturer to be soluble in organic solvents and aqueous acids. A change in pH resulting from exposure to cerebral

spinal fluid (pH 7.3) with a high salt content could result in reduced solubility in dialysate samples.

Olanzapine was chosen as an internal standard due to the structural similarity to clozapine. For measurements of small molecules, a superior approach is use of the stable isotope analog of the molecule of interest (Bakhtiar and Majumdar, 2007). Amphetamine-d_{11} has an identical structure and chemical properties as amphetamine. Eleven hydrogen atoms comprising the ring structure of the amphetamine molecule have been replaced by the deuterated isotope of hydrogen resulting in a heavier molecule. The slight difference in mass results in selective detection in the tandem mass spectrometer. Because the chemical and structural properties are not significantly altered, amphetamine-d_{11} accurately represents amphetamine concentration.

Clozapine was not detected by brain microdialysis even though concentrations are likely higher than quantifiable limits. Like olanzapine, clozapine is a lipophilic molecule with poor solubility in aqueous solutions. It is likely that clozapine in brain is largely protein bound and thereby not available in brain dialysate (Zhou et al., 2005). Whole brain determination of clozapine levels have been successfully performed in this lab in mice using liquid/liquid extraction methods (Osterhaus et al., 2003), which may be an alternative option.

Behavioral data obtained from the force plate actometer on rats subjected to microdialysis testing was comparable to data previously obtained from rats tested in enclosed light and sound attenuating chambers. Inspection of graphs shown in figures 2-16 and 2-17 indicate that that both Sprague Dawley and Fischer 344 rats included in this study express comparable behavior both in power spectral output and graphs of locomotor trajectory to rats given the same amphetamine dose, but no microdialysis. The key behaviors as outlined in the introduction section include a peak at 10Hz along with a decrease in locomotor activity during the focused stereotypy phase (1-2 hours). Careful analysis of raw data from individual animals throughout the behavioral sensitization process indicates that there were several behavioral disruptions throughout the sessions. These disruptions can be seen in the individual charts of locomotor activity and power spectra shown in figure 2-20. Many of these disruptions can be attributed to three primary causes. First, the actometers used in this study were not enclosed in light and sound attenuating chambers as were the actometers from the comparative data. This added exposure to environmental conditions would have a significant effect on behavior due to activity differences between rodents in a light environment with background noise or a quiet, dark environment. Second, because an automated sample collection system was not used in these studies, it was necessary to manually exchange dialysate collection vials every 15 minutes throughout the session.

Although efforts were made to minimize disruption of the animal during this process, it was not possible to do so in every instance. Enclosing the chamber in a light and sound attenuating chamber with the collection vials mounted outside the chamber would address both the second and the first of these concerns. The third potential cause of behavioral disruptions was minor mechanical malfunctions of the microdialysis system. These malfunctions were expected and exposed weaknesses of the apparatus that required attention. The disruptions were usually brief and permanent repairs were found whenever possible. In most cases repairs included tightening set screws or replacing tubing connectors. Long term solutions for the third point included carefully inspecting and replacing tubing and connectors as required prior to starting a session.

Scores of focused stereotypy for Sprague Dawley and Fischer 344 rats throughout the sensitizing series of five d-amphetamine injections clearly show that behavioral sensitization had taken place for both strains (figure 2-18). On injection day six, amphetamine administration was followed 30 minutes later by 10.0 mg/kg clozapine. Comparison of this data with data from the previous injection of amphetamine plus saline clearly shows that the duration of focused stereotypy was increased for F344 rats receiving clozapine following saline treatment (figure 2-19). Although the increase in focused stereotypy duration was not as evident in Sprague Dawley rats, there was a transient decrease in focused stereotypy score immediately following

clozapine injection at 30 minutes. This was previously shown to occur in Sprague Dawley rats administered 10.0 mg/kg clozapine 30 minutes following amphetamine administration (Fowler et al., 2007) and will be considered more thoroughly in chapter 4.

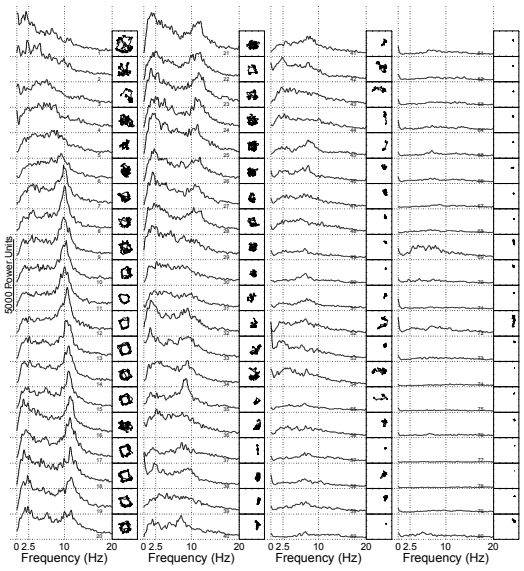
In summary, results from this chapter show that microdialysis and behavioral analyses can be performed simultaneously using a modified force-plate actometer. Because all compounds included in this study were detectable in prepared solutions, it is reasonable to believe that the methods used here could be modified to increase sensitivity for successful measurements in brain dialysate. The exception is clozapine which is likely unavailable in brain extracellular fluid. Other microdialysis methods have successfully been developed to detect amphetamine (Fuh et al., 2004) and dopamine (Zhang et al., 2006) using HPLC with tandem mass spectrometry detection. While feasible, attempting to measure these molecules simultaneously would require modification of the chromatographic conditions presented here and repeated trials in brain dialysate. Such an undertaking is beyond the scope of this study.

Figure 2-20. Power spectra and locomotor trajectory

Graphs on the following pages represent data derived from the force plate actometer for the two Fischer 344 and two Sprague Dawley rats that were included in this study. Graphs show power spectra and locomotor trajectory tracings for injections 1, 2, 4, and 6 for all rats included in this study. Graphs for injections 1 and 2 are on the top rows and injections 4 and 6 are on the bottom rows.

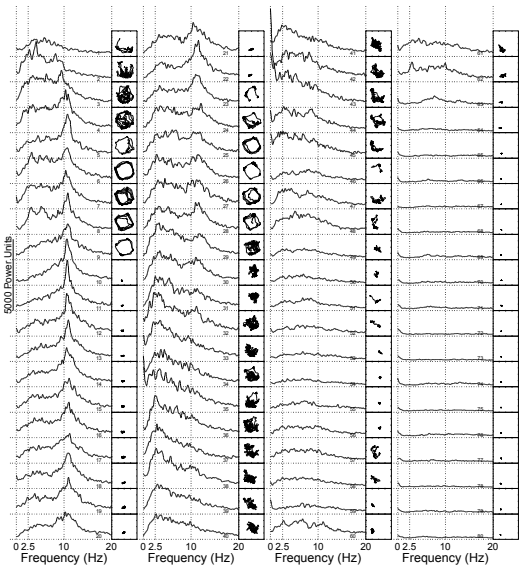
Sprague Dawley – Rat number 2 – Injection days 1, 2, 4, and 6

Power spectra and XY locomotion – 4 hour sessions



021120, hr 1 021120, hr 2 021120, hr 3 021120,hr 4

Injection 1



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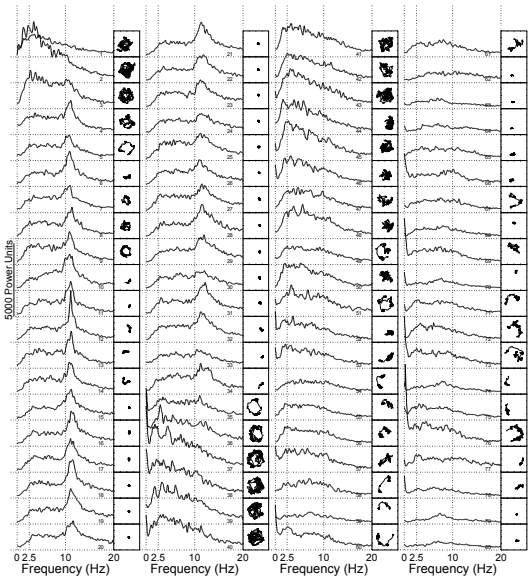
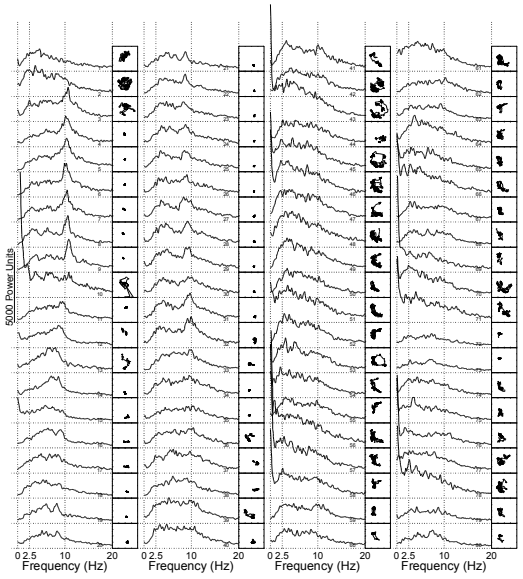
Injection 2



021202, hr 1 021202, hr 2 021202, hr 3 021202,hr 4

Injection 4

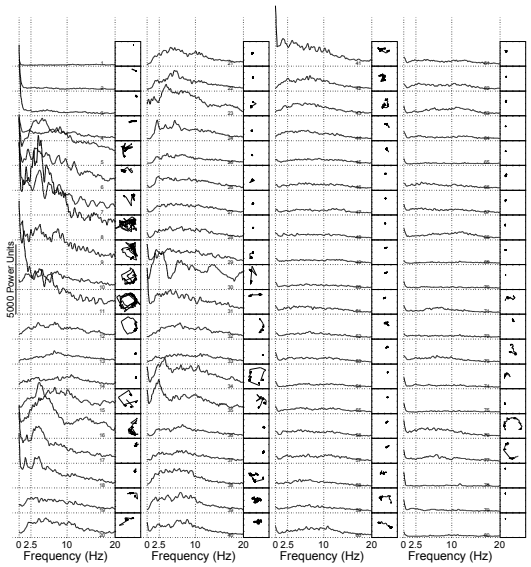


021210, hr 1 021210, hr 2 021210, hr 3 021210,hr 4

Injection 6

Fischer 344 – Rat number 3 – Injection days 1, 2, 4, and 6

Power spectra and XY locomotion – 4 hour sessions



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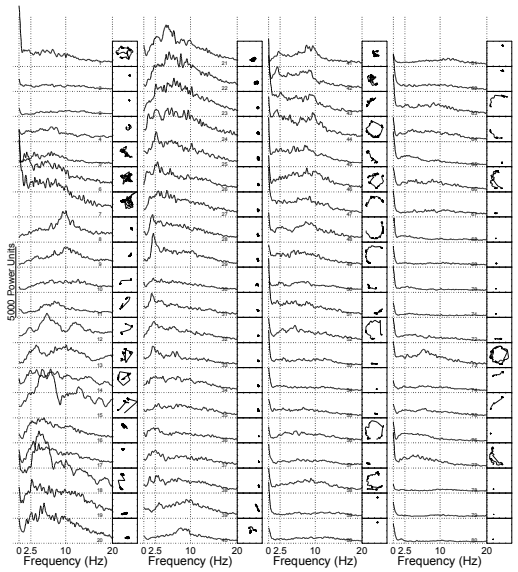
Injection 1



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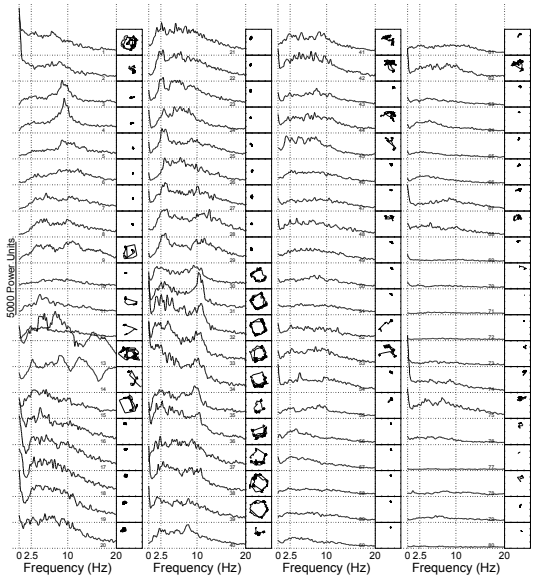
Injection 2



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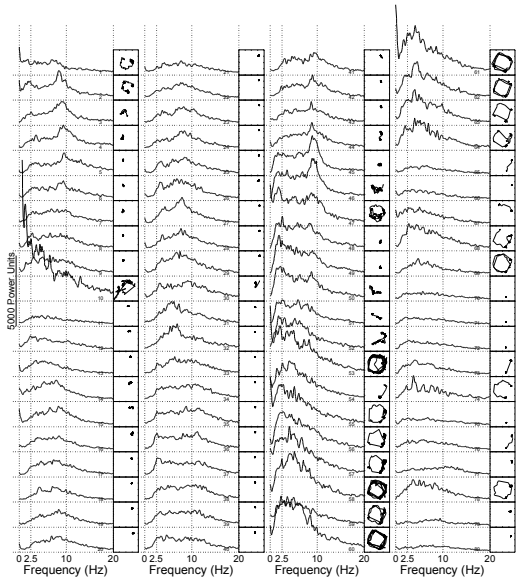
Injection 4

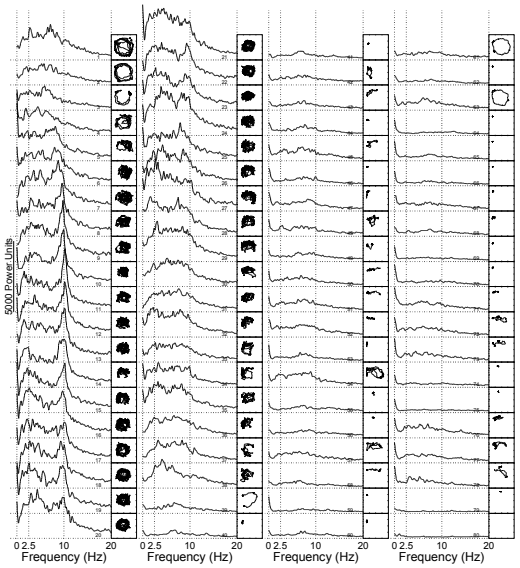


030205, hr 1 030205, hr 2 030205, hr 3 030205,hr 4

Injection 6

Sprague Dawley – Rat number4 – Injection days 1, 2, 4, and 6

Power spectra and XY locomotion – 4 hour sessions



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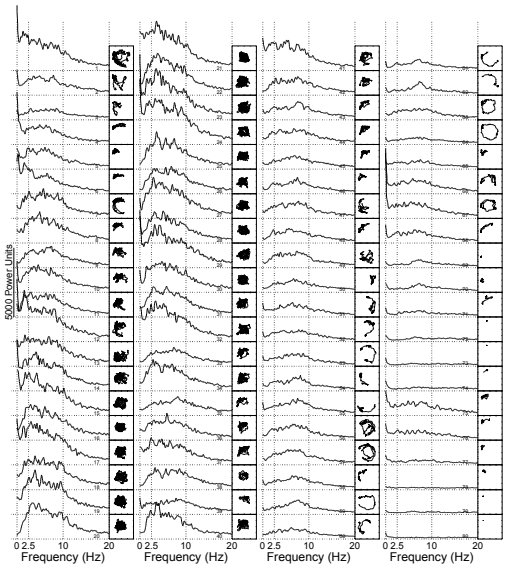
Injection 1



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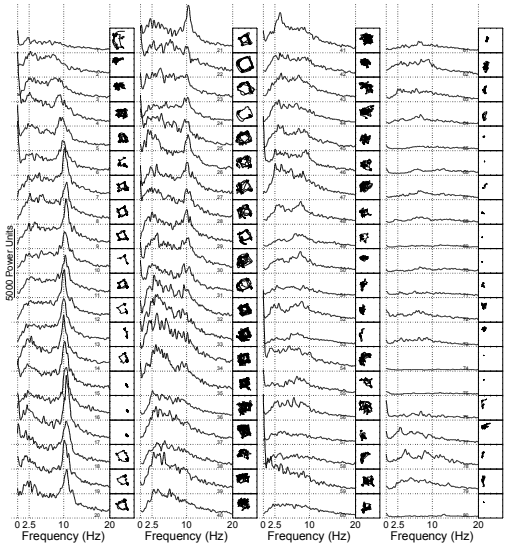
Injection 2



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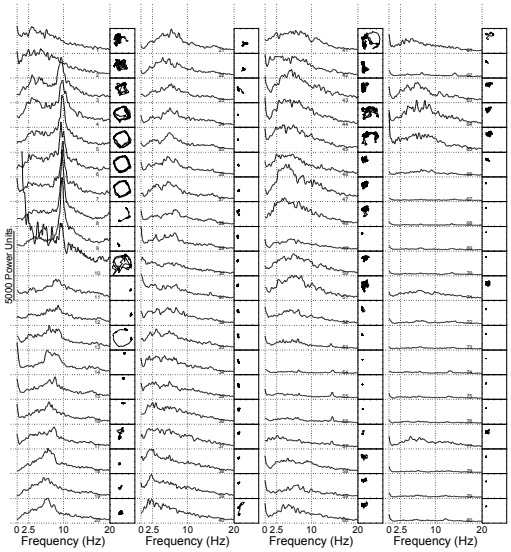
Injection 4

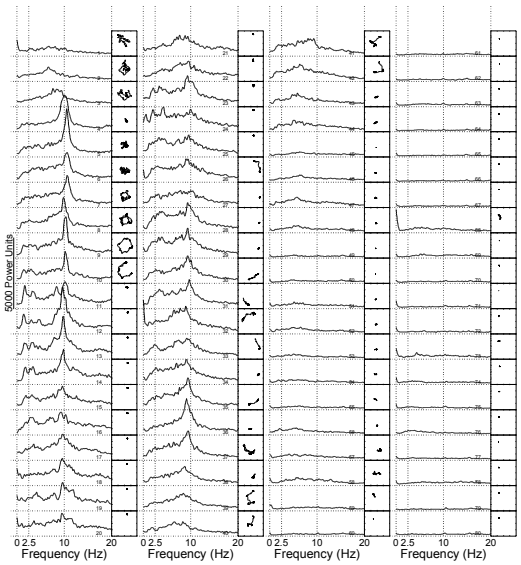


040215, hr 1 040215, hr 2 040215, hr 3 040215,hr 4

Injection 6

Fischer 344 – Rat number 5 – Injection days 1, 2, 4, and 6

Power spectra and XY locomotion – 4 hour sessions



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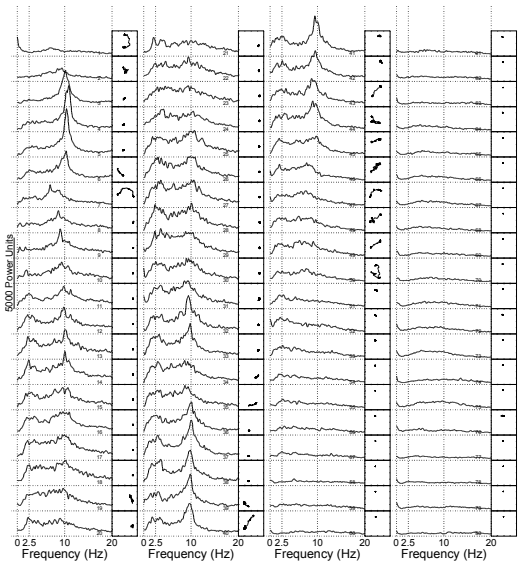
Injection 1



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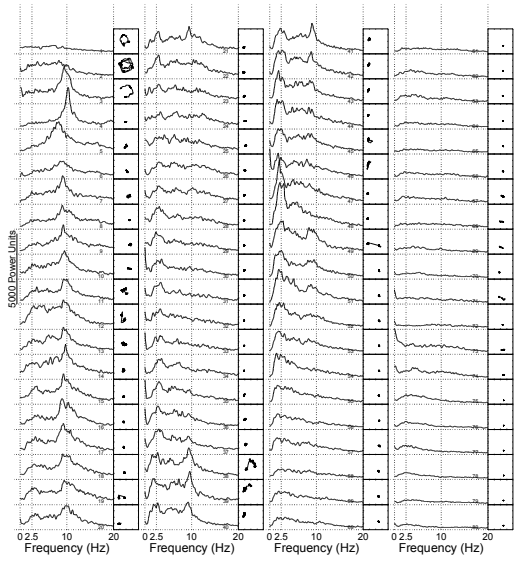
Injection 2



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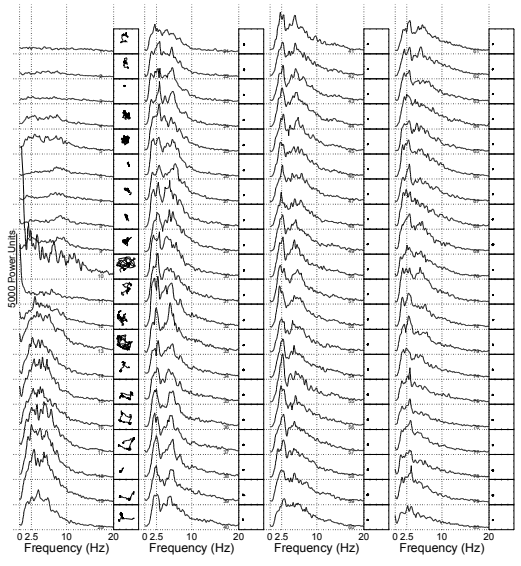
Injection 4



050216, hr 1 050216, hr 2 050216, hr 3 050216,hr 4

Injection 6

CHAPTER 3

Behavioral and Pharmacokinetic Analysis of F344 and SD rats

Introduction

Behavioral studies on Fischer 344 (F344) and Sprague Dawley rats show that these two strains differ in their response to amphetamine (Thasiyakorn et al., 2006). It is not clear what causes these differences. Studies have shown that amphetamine levels in plasma of Fischer 344 are different from Lewis rat strains suggesting a potential influence of strain on amphetamine concentrations (Clausing et al., 1996). The impact of brain striatum amphetamine concentration on behavior has never been studied in the context of behavioral sensitization or strain diversity. We have developed a method to analyze behavior and brain neurochemistry via microdialysis to measure brain amphetamine levels during a 4-hour behavioral session. This system will be used to determine whether measured behavioral differences result from an altered level of amphetamine reaching the brain.

The experiments in this chapter address hypotheses three, four and five and can be broken down into two primary parts. The first part focuses on amphetamine pharmacokinetics. By measuring amphetamine levels in striatum extracellular fluid via microdialysis we will establish whether there are significant differences in amphetamine uptake and elimination between F344

and Sprague Dawley rats during the first and sixth injection of a sensitizing series of d-amphetamine injections. At the same time we will establish whether there are pharmacokinetic differences between injection one and six within each strain resulting from behavioral sensitization associated with chronic amphetamine. The second part considers amphetamine pharmacokinetic results as they pertain to behavior. Specifically, part two addresses whether amphetamine levels are related to measured differences in behaviors between F344 and Sprague Dawley rat strains, and whether amphetamine levels are related to behavioral differences between sensitized and unsensitized rats within a strain. It also addresses whether changes in amphetamine levels over the course of a 4-hour session correspond to observed behaviors within the session. In other words, are the behavioral differences observed between strains, throughout the sensitization process, and over the course of a single session a result of pharmacokinetic or pharmacodynamic influences in terms of striatal amphetamine concentration? Different striatal amphetamine levels between strains or between the first and final injection of a sensitizing series would indicate that rats may be physiologically altered in the uptake or metabolism of amphetamine (pharmacokinetic). A finding that there are no significant differences in amphetamine levels in the striatum could support a pharmacodynamic cause for differences in behavioral response.

Sprague Dawley rats are outbred rats and are the maternal strain for inbred Fischer 344 and Lewis Strains (Klenerova, 2007). Despite this common genetic background, these strains have been shown to exhibit behavioral and morphological differences. Fischer 344 rats have been shown to be more sensitive to mechanical stimulation of their hindlimbs and have significantly lower von Frey threshold values than Sprague Dawley rats (Webb et al., 2003). Fischer 344 rats also have been shown to stand and locomote with their distal hindlimbs laterally displaced (Webb et al., 2003). These sensory and morphological features may have relevance in the current behavioral studies since d-amphetamine treated Fischer 344 rats have been observed to rest on their hindquarters while engaged in a lateral, repetitive stereotypical motion. Sprague Dawley rats display a repetitive vertical head movement while engaged in amphetamine-induced stereotypy. Sprague Dawley rats have also been suggested to have higher locomotor activity than Fischer 344 (Unis et al., 1991) although available data are inconclusive.

Evidence of differences in the behavioral response of Fischer 344 and Sprague Dawley rats to d-amphetamine treatment are shown in Figure 3-1. The graph shows stereotypy scores comparing Fischer and Sprague Dawley rats at a range of amphetamine doses. As seen in the figure, Fischer rats administered 2.5 mg/kg (column 2, row 3) express stereotypy and sensitization comparable to that seen in Sprague Dawley rats at 5.0 mg/kg (column 1, row 4). This indicates that Fischer rats are more sensitive to the

stereotypy-inducing effects of amphetamine than Sprague Dawley rats (Thisayakorn et al., 2006).

As previously stated, chronic or acute amphetamine administration may alter the penetration of molecules across the blood brain barrier (Sharma et al., 2006; Bowyer et al., 2006). It is reasonable to hypothesize that heightened behavioral effects may, at least, be partially attributed to higher levels of amphetamine in the brain. This could be true for animals receiving multiple doses that induce sensitization, or it may be that amphetamine penetrates the brain differently for different strains of rats or that the regional brain distribution of amphetamine is strain dependent.

Figure 3-1. Behavioral response of Fischer 344 and Sprague Dawley rats in response to various doses of d-amphetamine.

Graph shows stereotypy scores (y-axis) for Sprague Dawley (left) and Fischer 344 (right) rats at d-amphetamine doses of 1.25, 2.5, and 5.0 mg/kg IP. Fischer 344 rats were excluded from testing at 7.5 mg/kg due to self injurious behavior. At a dose of 1.25 mg/kg (row 2) F344 rats show a behavioral response similar to that of Sprague Dawley rats at 2.5 mg/kg (row 3). Sensitization is apparent in F344 rats at a dose of 2.5 mg/kg. A similar response is seen in Sprague Dawley rats at 5.0 mg/kg. Both strains show rapid sensitization at 5.0 mg/kg and 7.5mg/kg (Sprague Dawley rats only). (Data for graph courtesy of Krittiya Thisayakron)

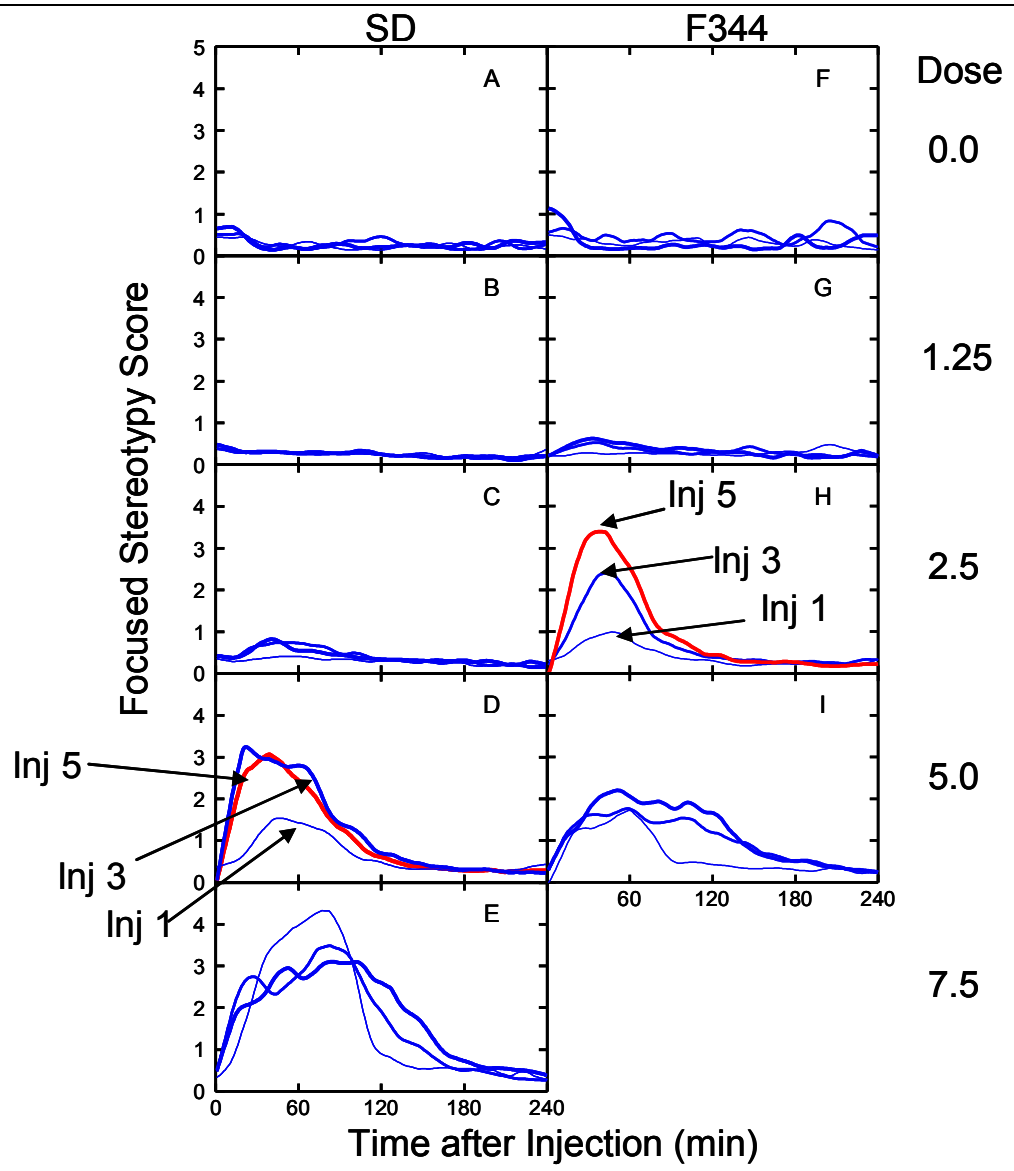

SD
F344
Dose
0.0
1.25
2.5
5.0
7.5
A
B
C
D
E
F
G
H
I
Inj 5
Inj 3
Inj 1
Focused Stereotypy Score
Time after Injection (min)
0
60
120
180
240

Figure 3-1

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats were purchased from Harlan. Male Fischer 344 rats were obtained from Charles River. Animals were received at the University of Kansas animal care facility at approximately 6-7 weeks of age and observed in quarantine for two weeks prior to being accessed. Experiments were performed over a three month period beginning approximately 1 month after arrival. As previously mentioned, animals were kept on a 12 hour light/dark cycle in standard plastic cages with non-aromatic wood chip bedding. Food and water were available *ad libitum* except while in experimental sessions.

Solutions

d-Amphetamine sulfate was purchased from Sigma and dissolved in 0.9% saline to a concentration of 5.0mg/kg for injection. For preparation of standard curves, a 1 uM stock solution was prepared and stored at -20º C. Standard curves were prepared from this stock immediately prior to each HPLC/MS MS analysis by serial dilution into artificial cerebral spinal fluid (130mM NaCl, 24mM $NaHCO_3$, 10mM Glucose, 1.5mM $MgSO_4.7H_2O$, 3.5mM KCl, 1.25mM $NaH_2PO_4.H_2O$, 2mM $CaCl_2.2H_2O$ adjusted to pH 7.4) containing

0.1% formic acid. DL-amphetamine-d_{11} is the stable isotope analogue of amphetamine used as an internal standard. DL-amphetamine-d_{11} was purchased from Sigma.

Experimental method

Sprague Dawley and Fischer 344 rats were received and the guide cannula implanted as described in chapter 2. The same procedures were followed as outlined in chapter 2 with a few exceptions. After the cannula implantation procedure, animals were allowed a one week recovery period before beginning a sensitizing series of six injections of 5.0 mg/kg d-amphetamine. Each of the six injections was separated by four days. Microdialysate and behavioral data was collected for injections one and six representing unsensitized and sensitized animals, respectively. For injections 2-5 amphetamine was administered in the animals' home cages. For the first and sixth treatment of the series, animals were placed in the force-plate actometer and artificial cerebral spinal fluid was perfused at 1µl/min until equilibrium was achieved. After equilibrium was confirmed, the animal was given an intraperitoneal injection of d-amphetamine and behavioral recording was immediately started. Consistent flow of dialysate was confirmed as described in chapter 2. Microdialysis and behavioral analysis was performed on a single modified force plate actometer as described in chapter 2.

Standard curves

Solutions of amphetamine and amphetamine-d_{11} were prepared immediately prior to each HPLC run by dissolving pure substance in 0.6 mM KH_2PO_4 (pH 2.7). The concentration of the internal standard was 100nM. For standard curve analysis, d-amphetamine was prepared and diluted by serial dilution to 512, 256, 128, 64, 32, 16, 8, 4, and 2 nM. Solutions were further diluted by mixing equal parts of each amphetamine concentration to the internal standard solution for final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, and 1 nM. The final concentration of internal standard was 50nM.

Behavioral quantification

Quantitative derivation of behavioral scoring is covered thoroughly in Fowler et al. (2001) and Fowler et al. (2003) and will be covered here briefly. Space used is the reciprocal of spatial confinement scores. Spatial confinement was calculated as the estimated area of the force plate used during a 1-minute period. Area was calculated as the square root of the product of the standard deviation of the X-coordinates and Y-coordinates that were occupied during each 1-minute period.

Power spectral measurements of vertical force changes were calculated by dividing force measurements over a 1 hour period into 180 contiguous segments of 20.48 seconds each (1024 samples). A fourier transform was performed on each data segment and the resulting power

spectral functions were expressed in terms of magnitude as a function of frequency. To obtain measures of rhythmicity over a 3 minute period, nine power spectra were averaged to yield a single function.

Decimated distance was calculated as the sum of the distances between successive centers of force coordinates from 256 equal sized sectors comprising the plate during a specified period. Graphs in this section reflect distance traveled in one minute periods.

Focused stereotypy was derived from power spectra combined with spatial confinement scores. When the rat was not locomoting, the area of the force plate that was occupied was low. A low level of locomotion combined with a high magnitude of vertical force oscillation reflects a high stereotypy score.

Pharmacokinetic computation

Pharmacokinetic analysis was performed using WinNonlin Professional, version 5.2 by Pharsight Corporation. This software was generously given to our lab through the Pharsight academic licensing program.

For determination of terminal elimination rate (lambda z) using a first order elimination paradigm, WinNonlin calculated the natural logarithm of the data points between Cmax and the final data point (total of 15 data points per animal). A regression line was calculated and the slope determined. Lambda

z (λ_z) is defined as -1*estimated slope and is estimated by linear regression of time vs. log concentration.

Half life values were based on the slope obtained from the lambda z values and calculated as the value at half the distance from Cmax to the final data point. The calculation for half life is:

$$\text{Half-life} = \ln(2)/\lambda_z$$

Percent elimination was calculated as the amount of drug eliminated during the 4 hour session. Percent elimination was based on the maximum concentration (at 30 minutes) and the final concentration at 240 minutes and was calculated as follows:

$$\% \text{ elimination} = 100 - ((\text{final concentration}/\text{Cmax})*100)$$

RESULTS

Pharmacokinetic analysis

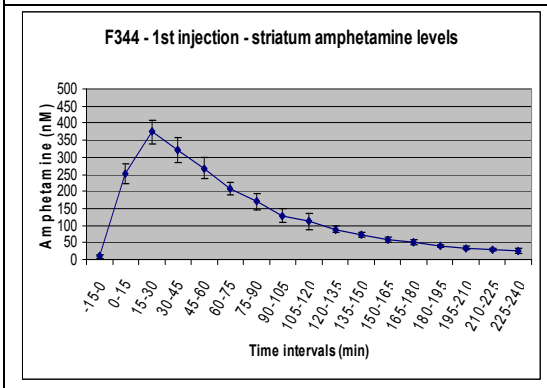
Of the sixteen animals that began the study, six Fischer 344 and seven Sprague Dawley rats provided useful data. One animal died in surgery, one animal dislodged the guide cannula following surgery, and one set of data had to be discarded due to mechanical failure. Of the remaining animals, analysis of striatum amphetamine levels over a 4 hour period revealed that all animals showed first order kinetics with a rapid absorption phase leading to a maximum concentration occurring between 15 and 30 minutes (figure 3-2). The elimination phase occurred over the remaining 3.5 hours with Sprague Dawley rats showing 95.6% elimination following the first injection and 93.1% elimination following the sixth injection. F344 rats showed 93.1% and 93.5% elimination following the first and sixth administration respectively (figure 3-3). Terminal elimination rates (lambda z) for Sprague Dawley rats were 0.014 for injection one and 0.016 min^{-1} for injection six. Terminal elimination rates for F344 rats were 0.016 and 0.013 for treatments 1 and 6 respectively (figure 3-4).

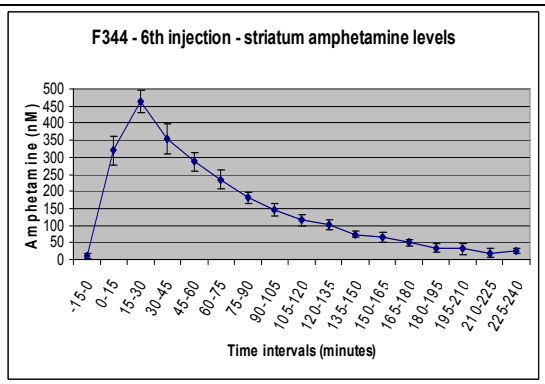
Half life values for F344 rats were 49.5 minutes on injection day one and 47.8 minutes on injection day 6. For Sprague Dawley rats values were 45.9 and 53.2 minutes for injections 1 and 6 respectively (figure 3-5). Values

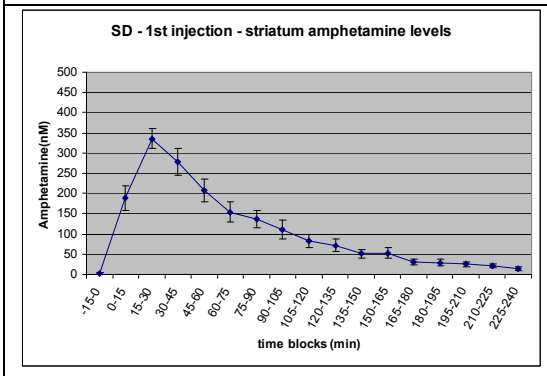
for maximum concentrations (Cmax) for F344 rats prior to sensitization were 370.6 nM while post-sensitization values were 405.0 nM. Pre- and Post-sensitization values for Sprague Dawley rats were 355.6 and 355.7 nM respectively (figure 3-6).

Figure 3-2. Average amphetamine levels during a four hour session for Sprague Dawley and Fischer 344 rats following the first and sixth injection of 5.0 mg/kg amphetamine.

Values are the average amphetamine levels for 6 Fischer 344 and 7 Sprague Dawley rats during a behavioral session. Flow rate was 1 µL/min and samples were collected every 15 minutes. Points represent amphetamine concentrations during each collection period. Brackets represent standard error of the mean. The top two graphs are Fischer 344 rats and the bottom two graphs are Sprague Dawley rats. The left side represents the first injection of a sensitizing series of six injections. The right side reflects injection 6. Graphs show that amphetamine followed first order kinetics with a maximum concentration (Cmax) at 30 minutes followed by a slower elimination phase.


F344 - 1st injection - striatum amphetamine levels
Amphetamine (nM)
Time intervals (min)


F344 - 6th injection - striatum amphetamine levels
Amphetamine (nM)
Time intervals (minutes)


SD - 1st injection - striatum amphetamine levels
Amphetamine(nM)
time blocks (min)

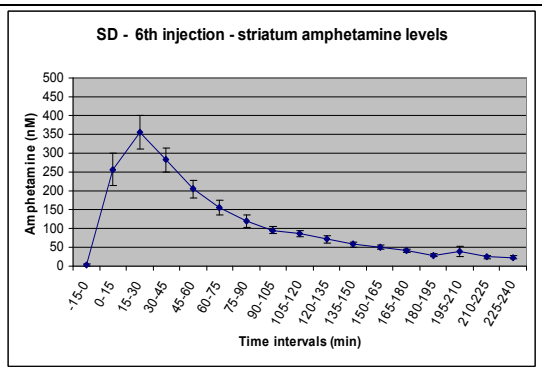

SD - 6th injection - striatum amphetamine levels
Amphetamine (nM)
Time intervals (min)

Figure 3-2

Figure 3-3. Percent elimination in response to amphetamine administration prior to behavioral sensitization and after behavioral sensitization in striatum of two rat strains.

Average elimination of drug from the striatum of 7 Sprague Dawley rats (SD) and 6 Fischer 344 rats (F344) following 5.0 mg/kg IP injection of amphetamine. Percent elimination represents values before (Inj 1) and after (Inj 6) amphetamine sensitization for both strains. Brackets represent the standard error of the mean for each group. Percent elimination was determined as percent of amphetamine eliminated over the remainder of a four hour session after reaching maximum drug concentration. No significant differences were found between Injection 1 and Injection 6 [$F(1,11)=0.772$, $p>0.05$], and there were no significant strain effects [$F(1,11)=1.981$, $p>0.05$].

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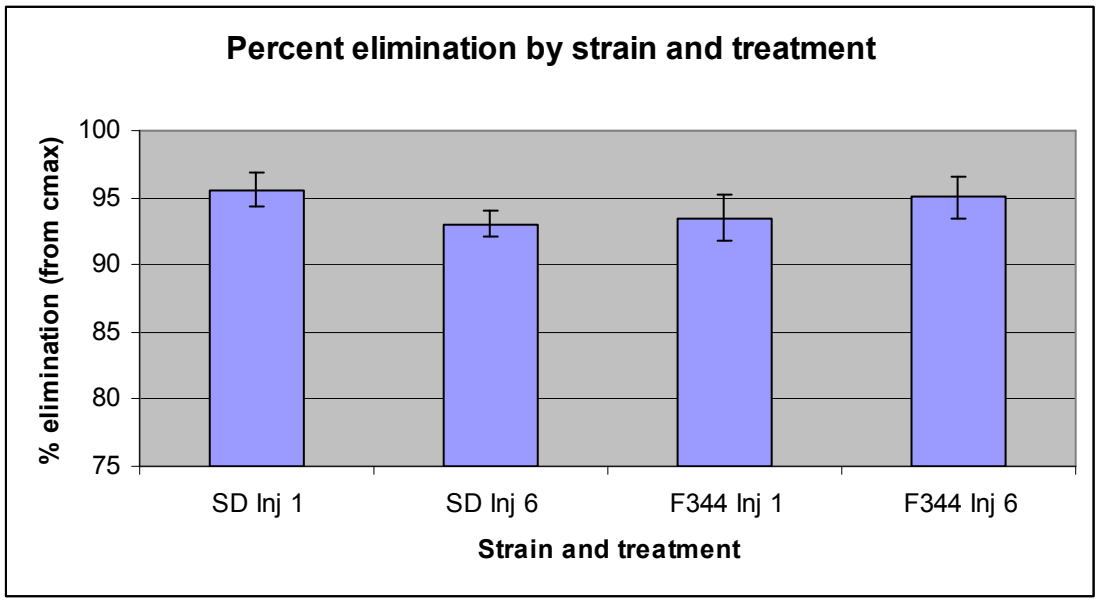

Percent elimination by strain and treatment
% elimination (from cmax)
100
95
90
85
80
75
SD Inj 1
SD Inj 6
F344 Inj 1
F344 Inj 6
Strain and treatment

Figure 3-3

Figure 3-4. Terminal rate constant in response to d-amphetamine administration prior to behavioral sensitization and after behavioral sensitization in striatum of two rat strains.

Average terminal rate constant (Lambda z) for elimination of drug from the striatum of 7 Sprague Dawley rats (SD) and 6 Fischer 344 rats (F344) following 5.0 mg/kg IP injection of amphetamine. Values represents Lambda z before (Inj 1) and after (Inj 6) amphetamine sensitization for both strains. Brackets represent the standard error of the mean for each group. Lambda z was determined as the slope of the natural log for all values between Cmax and the final data point. No significant differences were found between Injection 1 and Injection 6 [$F(1,11)=0.637$, $p>0.05$], and there were no significant strain effects [$F(1,11)=0.094$, $p>0.05$].

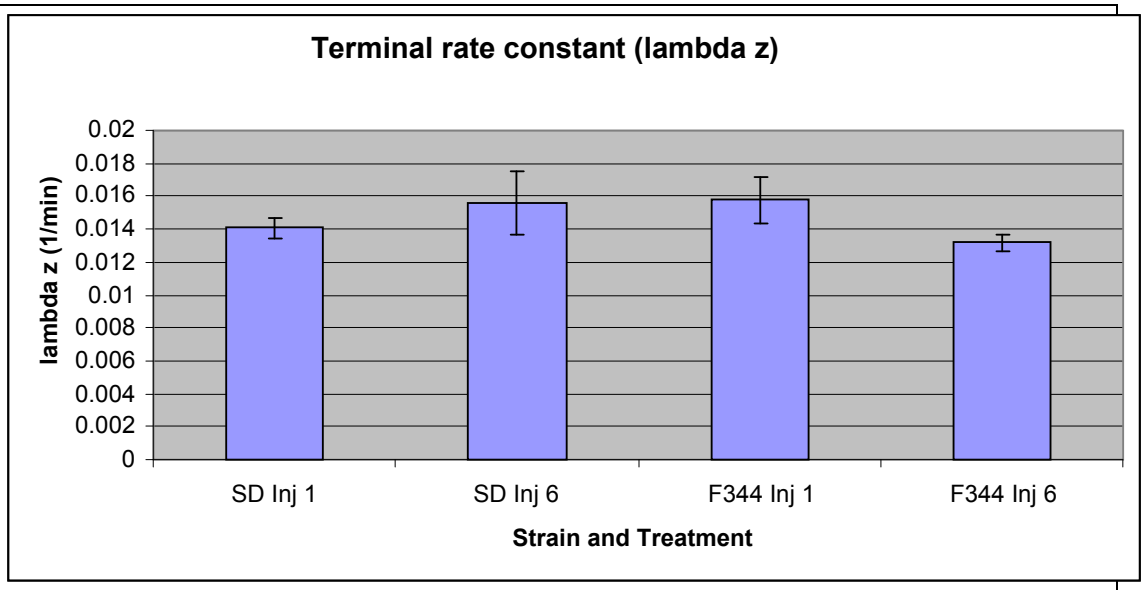

Terminal rate constant (lambda z)
lambda z (1/min)
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
SD Inj 1
SD Inj 6
F344 Inj 1
F344 Inj 6
Strain and Treatment

Figure 3-4

Figure 3-5. Half life of amphetamine prior to behavioral sensitization and after behavioral sensitization in the striatum of two rat strains administered 5.0 mg/kg d-amphetamine.

Half life values are based on the average values of 7 Sprague Dawley (SD) and 6 Fischer 344 rats (F344) following 5.0 mg/kg IP injection of d-amphetamine before (Inj 1) and after (Inj 6) amphetamine sensitization for both strains. Brackets represent the standard error of the mean for each group. Values were based on the average time for drug levels to reach half the estimated maximum concentration. Repeated measures analysis shows no significant differences between Injection 1 and Injection 6 [$F(1,11)=0.772$, $p>0.05$], and no significant strain effects [$F(1,11)=0.052$, $p>0.05$].

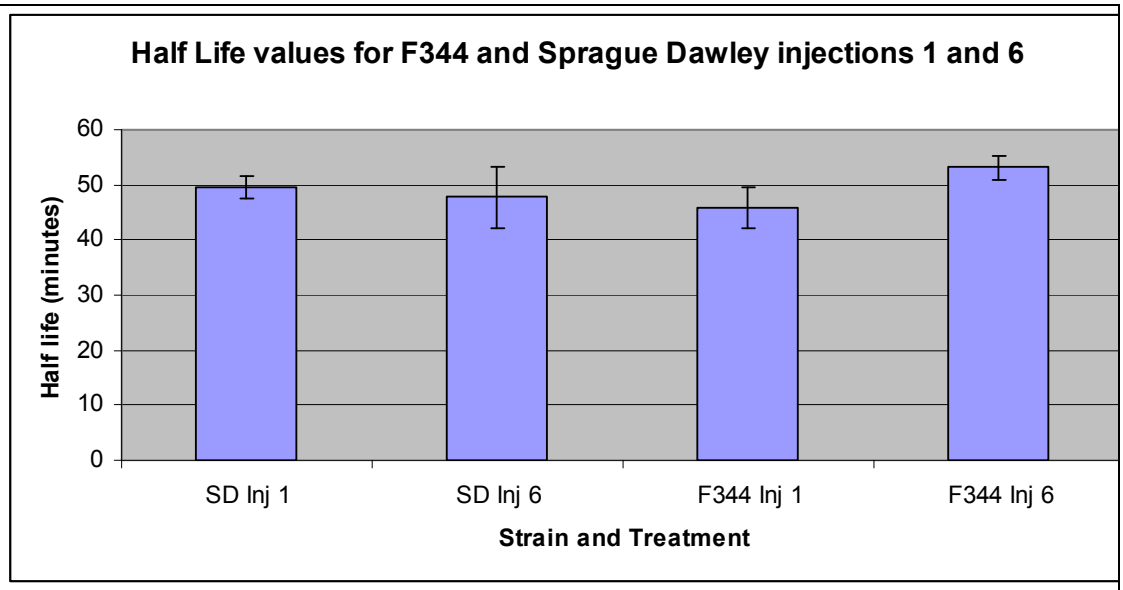

Half Life values for F344 and Sprague Dawley injections 1 and 6
Half life (minutes)
60
50
40
30
20
10
0
SD Inj 1
SD Inj 6
F344 Inj 1
F344 Inj 6
Strain and Treatment

Figure 3-5

Figure 3-6. Maximum amphetamine concentration in the striatum of two rat strains administered 5.0 mg/kg d-amphetamine prior to, and after behavioral sensitization.

Cmax values are the maximum measured concentration of amphetamine in dialysate collected over a 15 minute period for each subject averaged by strain and treatment. Strains include Fischer 344 (F344) and Sprague Dawley (SD) rats. Treatments include injections of 5.0 mg/kg d-amphetamine prior to behavioral sensitization (Inj 1) and after sensitization (Inj 6). Brackets represent the standard error of the mean for each group. Univariate repeated measures analysis shows that there are no significant differences between pre- and post-sensitization [$F(1,11)=4.265$, $p>0.05$], and there were no strain effects [$F(1,11)=2.693$, $p>0.05$].

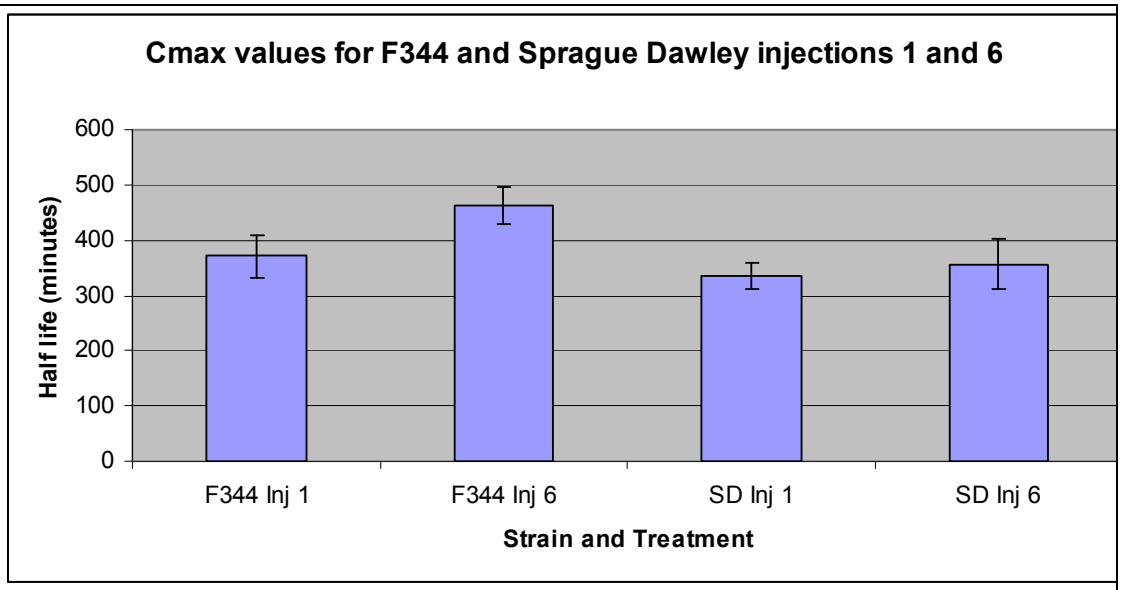

Cmax values for F344 and Sprague Dawley injections 1 and 6
Half life (minutes)
600
500
400
300
200
100
0
F344 Inj 1
F344 Inj 6
SD Inj 1
SD Inj 6
Strain and Treatment

Figure 3-6

Behavioral analysis

F344 and SD Injection 1 – Pre-sensitization

Behavioral analysis of the mean of all F344 and Sprague Dawley rats following the first injection show differences in several variables (figure 3-7). Analysis of figure 3-7 illustrates that Sprague Dawley rats showed more locomotor activity throughout the session with a maximum decimated distance over a one minute period of 1868 mm occurring between 0 and 30 minutes and falling gradually over the next 30-45 minutes (figure 3-7, decimated distance intervals 1-4). F344 rats displayed a sharper peak with lower amplitude from 15 to 20 minutes with levels retreating sharply over the next 15-30 minutes (15-min intervals 1-3).

Vertical force measurements (figure 3-7, “Spectrum”) highlight a difference between the expression of focused stereotypy of Fischer 344 and Sprague Dawley rats prior to sensitization. During frames 2 and 3 (15-45 min) F344 and Sprague Dawley rats both showed a distinct peak at about 10 Hz. This peak continued to be present in Sprague Dawley rats until frame 7-8. For F344 rats this peak is mostly nonexistent after the third 15 minute interval. Focused stereotypy (Figure 3-7, “Stereotypy Score”) prior to sensitization is also expressed differently among Sprague Dawley and F344 rats following the first amphetamine injection. Sprague Dawley stereotypy

scores increased gradually to a maximum level at one hour and remained at this maximum for approximately 15 minutes before gradually declining (stereotypy score intervals 4-5). Stereotypy scores for F344 rats increase more sharply reaching a plateau with similar amplitude at 30 minutes and lasting 60 minutes (intervals 2-6) before decreasing over a 15-30 minute period.

F344 and SD Injection 6 – Sensitized

Raw distance traveled during session 6 for F344 rats was 60% of the value of Sprague Dawley rats. Analysis of the locomotor activity measures "Space Used" and "Decimated Distance" in figure 8 show that F344 locomotor activity ceased within 15 minutes of amphetamine treatment and remained near zero for two hours (intervals 1-9) before some locomotor activity was restored. Observation of these parameters for Sprague Dawley rats also showed a decrease in locomotor activity in the beginning of the session, but the decrease was occurred at a slower rate and locomotor activity remained low for less time compared to F344 rats, before increasing over a 30 minute period starting at 90 minutes following amphetamine administration (Figure 3-8, intervals 1-6).

Power spectral analysis (Figure 3-8, "Spectrum") shows that F344 rats expressed vertical force activity leading to a 10 Hz peak during frames 2 and 3 (30 minute duration). Sprague Dawley rats showed sharp peaks at 10 Hz for a longer duration lasting 1.5 hours (frames 2-7).

Stereotypy scores of sensitized F344 rats sharply increased in less than 15 minutes following amphetamine administration and remained elevated for over 2 hours ("Stereotypy Score", intervals 1-10) before gradually decreasing over the remainder of the 4-hour session. Stereotypy scores for sensitized Sprague Dawley rats also increased rapidly but reached a value nearly double that of the F344 strain (intervals 1-6). Stereotypy scores for SD rats decreased sharply 1.5 hours into the session (intervals 6-8) and remained low for the remaining 2 hours of the 4 hour session. Figure 3-9 further illustrates the differences in expression of stereotypy between sensitized Fischer 344 and Sprague Dawley rats.

Behavioral sensitization

Behavioral sensitization was confirmed statistically for both rat strains by analyzing stereotypy scores at 4 timepoints representing 4 phases of amphetamine response during the 4 hour session (1) 15-30 minutes representing Cmax, (2) 60-75 minutes representing $T_{½}$, (3) 90-105 minutes and (4) 120-135 minutes (figure 3-10). Repeated measures analysis of variance of stereotypy scores at each of the four 15 minute periods confirm a significant difference between injection 1 and injection 6 ($F(1,2)=6.483$, $p<.01$). Strain had no effect on the observed differences between injection 1 and 6 indicating that both strains showed a sensitization effect.

Analysis of individual behavioral measures for the entire four hour session further illustrates that behavioral sensitization occurred in both strains following the sensitizing series of six amphetamine injections. Figure 3-11 shows that following sensitization, locomotor activity as measured by decimated distance scores, is lower for both strains during the stereotypy phase of the session in relation to values prior to sensitization. Locomotor activity was higher for both strains during the post stereotypy locomotor phase. Figure 3-11 also shows that in the first 15-30 minutes following amphetamine administration, locomotor activity rapidly decreased in sensitized animals while a temporary increase in locomotor activity was observed for unsensitized animals.

Observation of stereotypy scores for each strain of rat before and after sensitization shows that following the sixth injection of the series, sensitized Fischer 344 rats reached a maximum stereotypy score more rapidly and for a longer duration than unsensitized animals following the first injection (figure 3-12). Sprague Dawley rats showed only a moderate increase in duration of the stereotypy phase, but showed a large increase in amplitude of the stereotypy score (figure 3-13). Sensitization was further evident in the power spectral analysis that shows the intensity of vertical components of the rats' head movements. During the sixth session, sensitized F344 rats showed a somewhat larger, more focused 10 Hz peak during the 15-45 minute frames of the session as compared with the first injection of the series (3-14, top).

Sensitized Sprague Dawley rats showed an increase in peak amplitude, as well as a slight increase in duration following sensitization (figure 3-14, bottom).

Figure 3-7. Behavioral measures corresponding to striatal amphetamine concentration for Fischer 344 and Sprague Dawley rats during a four hour session prior to behavioral sensitization.

Graphs represent the means of six Fischer 344 rats, and seven Sprague Dawley rats during a four hour microdialysis session in the force plate actometer. Dialysate was collected from the striatum every fifteen minutes and amphetamine concentration (bottom row) was determined for each 15 minute interval. Power spectra (spectrum) are an average over 15 minute intervals corresponding to intervals in which dialysate was collected. Space used, stereotypy score, decimated distance, and absolute rotations are average scores over one minute intervals. LOWESS smoothing was applied with a tension of 0.10 for the entire range of 240 data points corresponding to 240 one-minute periods. Space used is a measure of spatial confinement and is the area of the plate used over each one-minute period. Stereotypy scores are based on power spectra obtained from activity in the vertical plane and mobility in the horizontal plane. High vertical activity and low horizontal activity results in a high score on stereotypy. Decimated distance is the distance traveled over each one minute period. Absolute rotations are the absolute value of cumulative rotations over each one minute period throughout the four hour session.

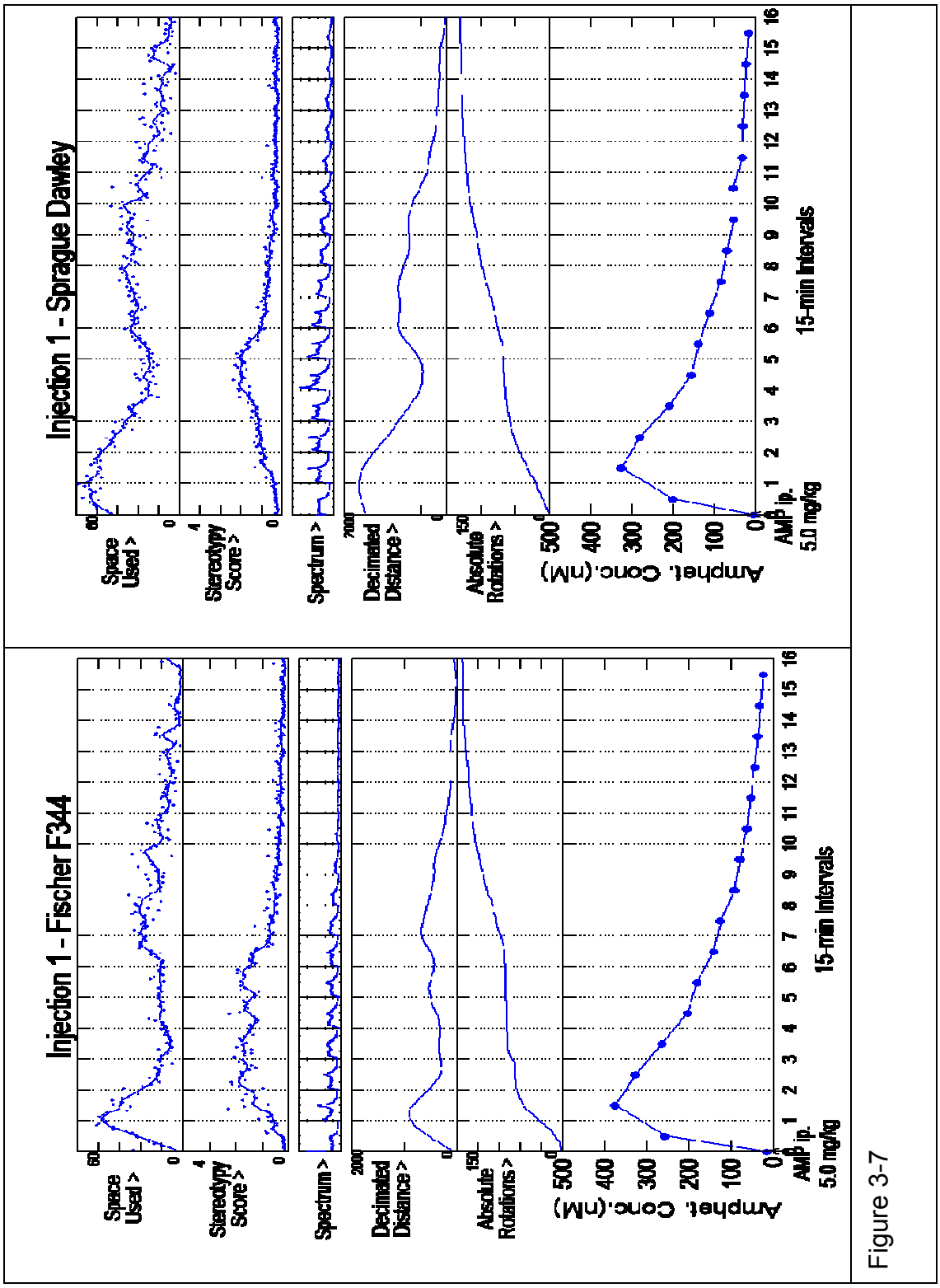

Injection 1 - Fischer F344
Injection 1 - Sprague Dawley
Space Used >
Stereotypy Score >
Spectrum >
Decimated Distance >
Absolute Rotations >
Amphet. Conc.(nM)
AMP ip.
5.0 mg/kg
15-min Intervals

Figure 3-7

Figure 3-8. Behavioral measures corresponding to striatal amphetamine concentration for Fischer 344 and Sprague Dawley rats during a four hour session after behavioral sensitization.

Graphs represent the means of six Fischer 344 rats, and seven Sprague Dawley rats during a four hour microdialysis session in the force plate actometer. Power spectra ("Spectrum") are an average over 15 minute intervals corresponding to the intervals in which dialysate was collected. Space used, stereotypy score, decimated distance, and absolute rotations are average scores over one minute intervals. LOWESS smoothing was applied with a tension of 0.10 for the entire range of 240 data points corresponding to 240 one-minute periods.

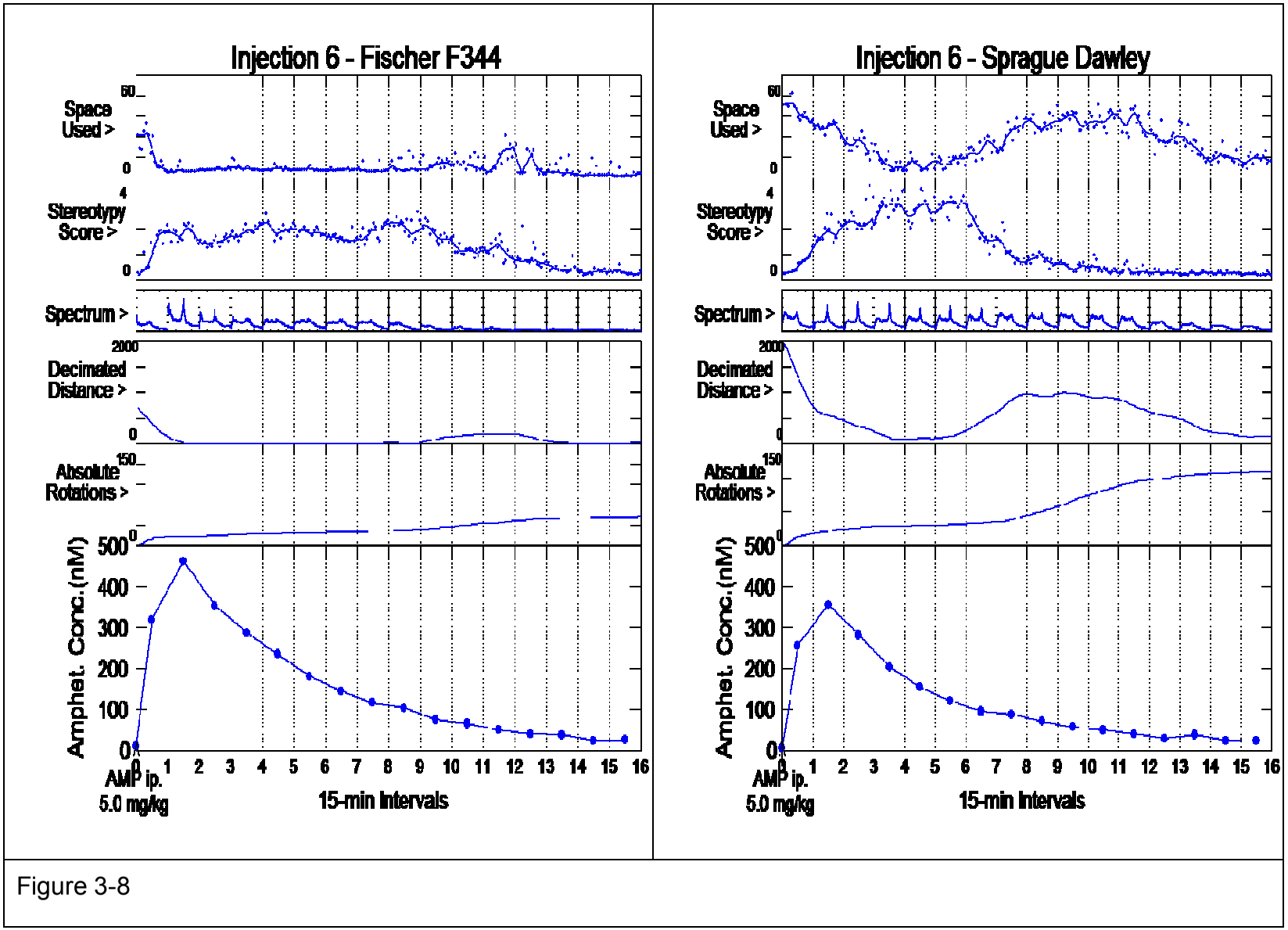

Injection 6 - Fischer F344
Space Used >
Stereotypy Score >
Spectrum >
Decimated Distance >
Absolute Rotations >
Amphet. Conc.(nM)
AMP ip.
5.0 mg/kg
15-min Intervals
Injection 6 - Sprague Dawley
Space Used >
Stereotypy Score >
Spectrum >
Decimated Distance >
Absolute Rotations >
Amphet. Conc.(nM)
AMP ip.
5.0 mg/kg
15-min Intervals

Figure 3-8

Figure 3-9. Focused stereotypy for sensitized Fischer 344 and Sprague Dawley rats over a four hour period following 5.0 mg/kg d-amphetamine administration.

Lines represent focused stereotypy of sensitized rats over a four hour period following treatment with 5.0 mg/kg d-amphetamine. Red lines represent stereotypy scores for Fischer 344 rats. Blue lines represent scores for Sprague Dawley rats. The figure shows that onset of focused stereotypy was rapid for both rats. Sprague Dawley rats had higher amplitude of stereotypy while Fischer 344 rats had a longer duration. Lines were based on average stereotypy scores for six Fischer 344 and seven Sprague Dawley rats over a range of 240 one-minute periods and plotted with a LOWESS smooth with a tension parameter of 0.10.

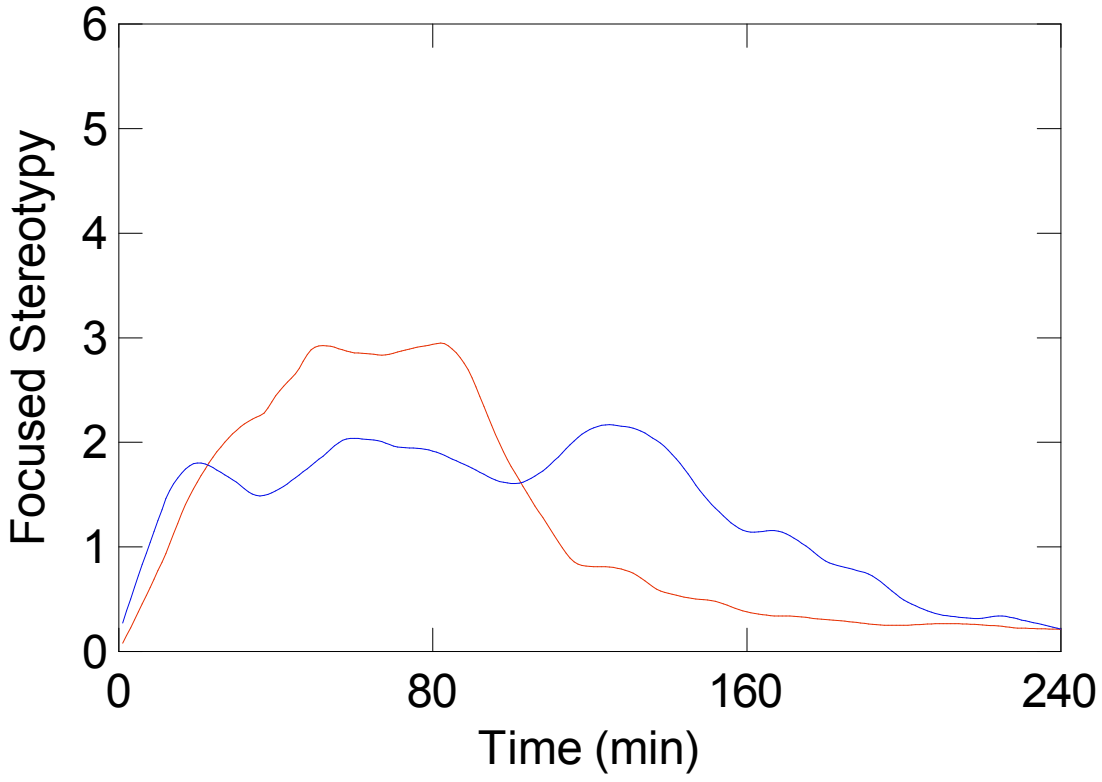


Figure 3-9

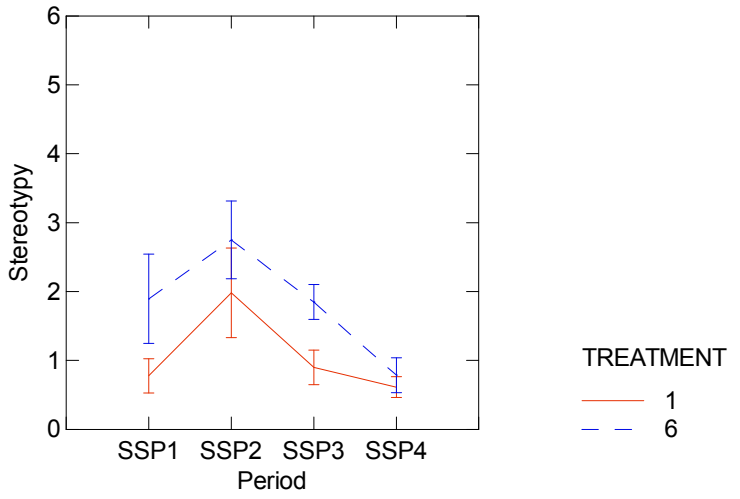
Fischer 344

Sprague Dawley

Figure 3-10. Plot of Stereotypy score for sensitized and unsensitized Fischer 344 and Sprague Dawley rats over 4 time periods during the drug terminal elimation phase.

Stereotypy scores are shown at 4 time periods (15-30 min, 60-75 min, 90-105 min, and 120-135 min) corresponding to periods of dialysate collection from the striatum of two rat strains. Sprague Dawley rats are shown in the upper box and Fischer 344 rats are shown in the lower box. Red, solid lines are values for unsensitized animals. Blue, dashed lines are values for sensitized animals. Brackets represent the standard error of the mean for six Fischer 344 and seven Sprague Dawley rats. Repeated measures analysis of log transformed stereotypy scores shows that there are significant differences between injection 1 and injection 6 [$F(1,11)=21.096$, $p<0.05$] and there is no main effect on strain [$F(1,11)= 0.672$, $p>0.05$] indicating sensitization occurred in both strains.

Sprague Dawley Stereotypy over four periods



Fischer 344 Stereotypy over four periods

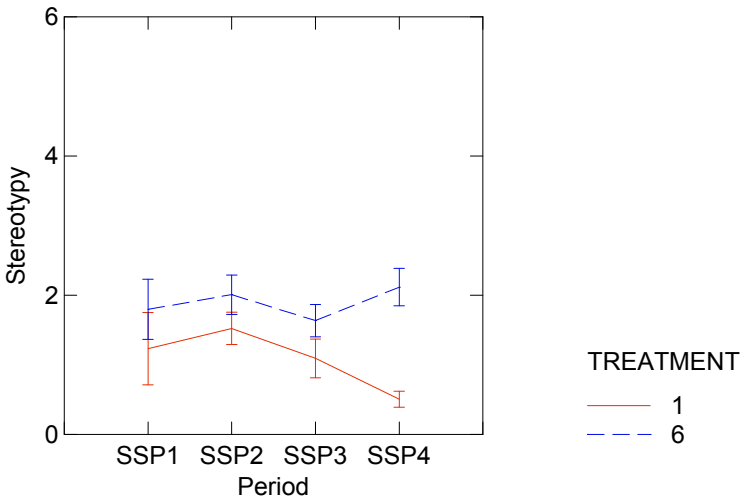
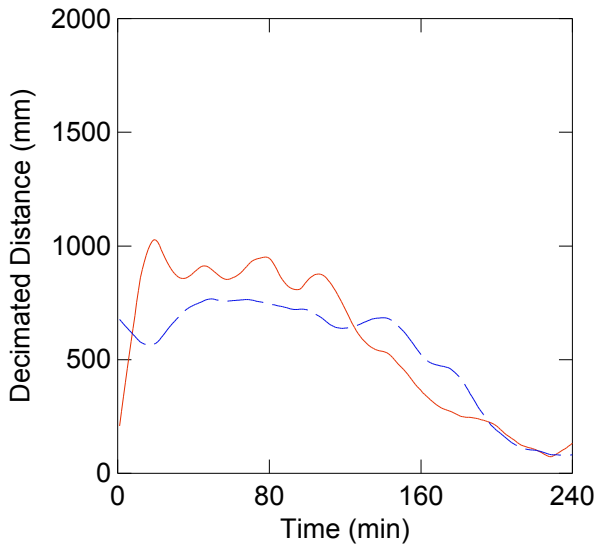
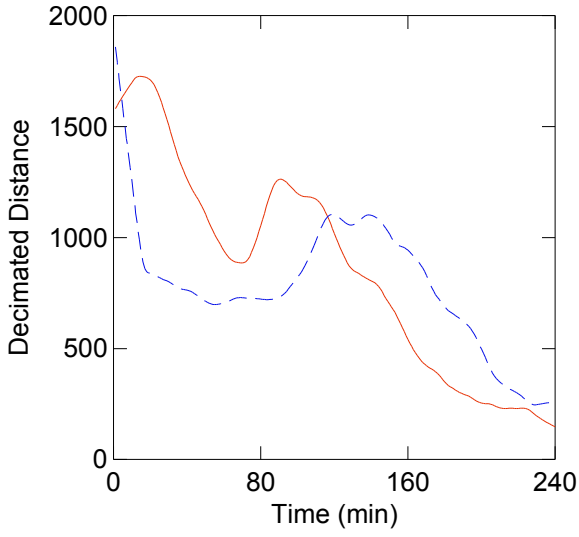


Figure 3-10

Figure 3-11. Plot of distance traveled for sensitized and unsensitized Fischer 344 and Sprague Dawley rats over a 4 hour behavioral session. Lines represent decimated distance scores (y-axis) of Fischer 344 and Sprague Dawley rats over a four hour period (x-axis) following treatment with 5.0 mg/kg d-amphetamine. The solid red line represents distance traveled following injection 1 of the series. The dashed blue line represents distance traveled following injection 6. The figure shows that in sensitized rats, locomotor activity was lower during the focused stereotypy phase and higher during the post stereotypy locomotor phase. Sensitized rats also showed an immediate decrease in activity following amphetamine injection. Unsensitized rats showed an initial spike in locomotor activity following amphetamine injection. Lines were based on average decimated distance scores for six Fischer 344 and seven Sprague Dawley rats over a range of 240 data points corresponding to 240 one-minute periods and were plotted with a LOWESS smooth with a tension parameter of 0.10



Sprague Dawley



Fischer 344

Figure 3-11

Figure 3-12. Focused stereotypy for Fischer 344 rats over a four hour period at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine.

Lines represent focused stereotypy scores of Fischer 344 rats over a four hour period following treatment with 5.0 mg/kg d-amphetamine. The red line represents stereotypy scores following injection 1 of the series. The blue line represents scores following injection 6. The figure shows that onset of stereotypy was more rapid and had a longer duration following the final injection of the series. Lines were based on average stereotypy scores for six Fischer 344 and seven Sprague Dawley rats over a range of 240 data points corresponding to 240 one-minute periods and were plotted with a LOWESS smooth with a tension parameter of 0.10

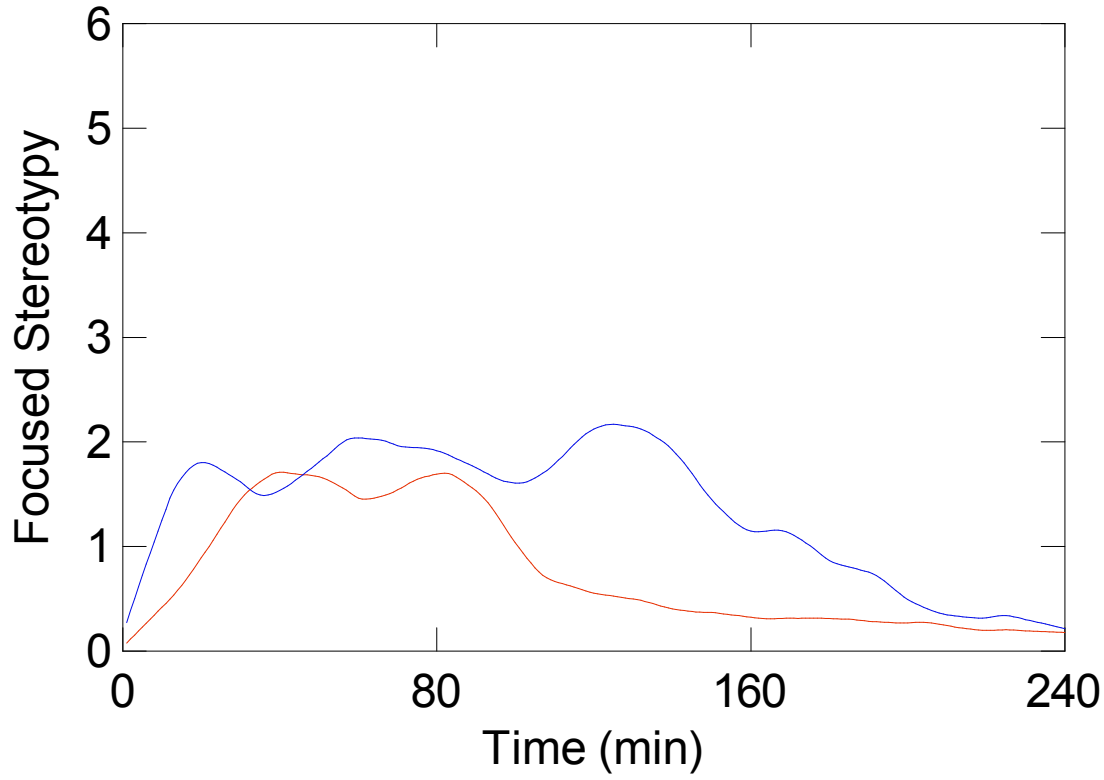


Figure 3-12 – Fischer 344

Sensitized

Unsensitized

Figure 3-13. Focused stereotypy for Sprague Dawley rats over a four hour period at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine.

Lines represent focused stereotypy scores of Sprague Dawley rats over a four hour period following treatment with 5.0 mg/kg d-amphetamine. The red line represents stereotypy scores following injection 1 of the series. The blue line represents scores following injection 6. The figure shows that onset of stereotypy was more rapid and had a higher amplitude following the final injection of the series. Lines were based on average stereotypy scores for six Fischer 344 and seven Sprague Dawley rats over a range of 240 data points corresponding to 240 one-minute periods and were plotted with a LOWESS smooth with a tension parameter of 0.10

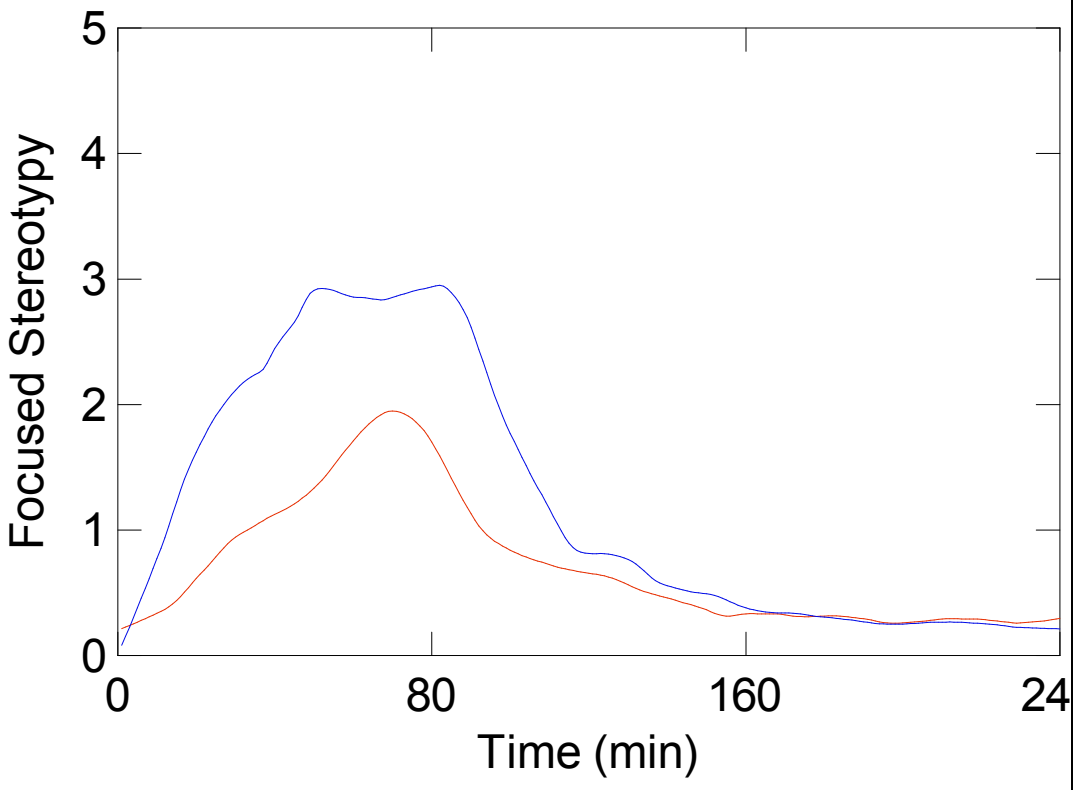


Figure 3-13 – Sprague Dawley

Sensitized _____

Unsensitized _____

Figure 3-14. Graphic of power spectra at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine in Fischer 344 and Sprague Dawley rats during a 4-hour session.

Values represent the power spectra, or vertical force activity in 15 minute intervals corresponding to intervals of microdialysis sample collection. The upper figure shows the average power spectra of six Fischer 344 rats for the first and sixth injection of a sensitizing series of 5.0 mg/kg d-amphetamine injections. For treatment one and six, Fischer 344 rats show a peak at about 10 Hz during frames 2 and 3 corresponding to 15-45 minutes. The 10 Hz peak is slightly visible in frames 5-7 indicating vertical head movements are more pronounced prior to sensitization. In sensitized Fischer rats (injection 6), the 10 Hz peak is sharper and more focused in frames 2 and 3 than in the corresponding frames for unsensitized animals. The lower figure shows power spectra of seven Sprague Dawley rats. A peak at about 10 Hz is visible during frames 2-8 in unsensitized rats (injection 1). For sensitized Sprague Dawley rats (injection 6), the peak becomes visible in the first frame and continues to be present as late as frame 12, indicating a more pronounced vertical component to the head movements associated with focused stereotypy in this strain. The 10 Hz peak is also sharper and more focused in sensitized Sprague Dawley rats as compared to corresponding peaks evident in unsensitized animals.

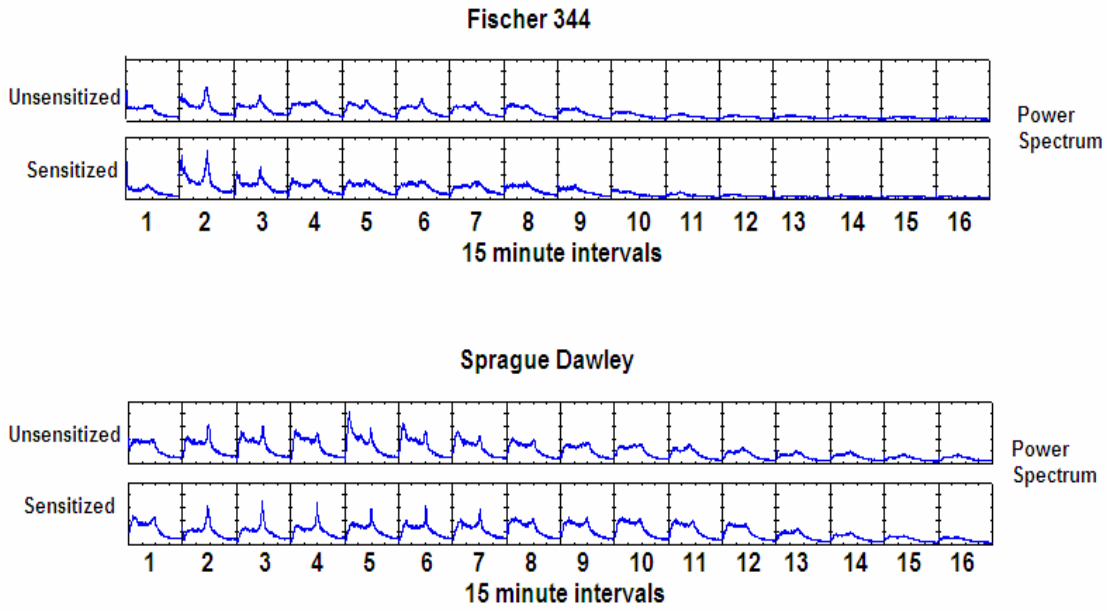

Fischer 344
Unsensitized
Sensitized
Power Spectrum
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
15 minute intervals
Sprague Dawley
Unsensitized
Sensitized
Power Spectrum
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
15 minute intervals

Figure 3-14

Amphetamine level and behavior

Comparisons of amphetamine concentration and stereotypy scores show that in the four 15 minute frames analyzed statistically (15-30 min, 60-75 min, 90-105 min, and 120-135 min) Sprague Dawley rats exhibited a relationship between amphetamine levels in the striatum and expressed focused stereotypy. Figure 3-15 shows that as brain amphetamine levels increased, stereotypy scores also increased during all 4 periods tested for injection 1 and injection 6. This pattern was not seen in Fischer rats where concentration appears to have no relationship to expressed stereotypy (Figure 3-16). Regression lines show that for injection 1 (top row), there was a slight increase in stereotypy score as amphetamine levels rose during periods 1 and 3. But these levels fall during the second and fourth periods. For injection 6, regression lines are opposite in that they fall during the first and third period and rise during the second and fourth period (Figure 3-16).

Spatial confinement is a sensitive measure of horizontal activity in non-locomoting as well as locomoting rats and was used to show relationships between activity and amphetamine concentrations. Spatial confinement scores are represented in figures 3-17 and 3-18 as “Radius”. Figure 3-17 shows that for the measure of spatial confinement in Sprague Dawley rats, the radius occupied by the animal during each of the four periods decreases

with increased amphetamine levels. This is not true for injection six in which regression lines during the first two periods are nearly flat while the third and fourth periods show an increase or decrease, respectively (figure 3-17). Figure 3-18 shows that F344 rats are the opposite, such that regression lines indicate no relationship to radius occupied during the first amphetamine treatment. For the first frame (figure 3-18 - Period 1, Treatment 1) a clear downward regression is seen. For periods 2 and 3, there is an upward trend, while in period 4 a tight grouping shows an overall downward regression. For injection 6 (sensitized rats) figure 3-18 shows that all lines trend upward but the radius occupied is very low for all frames (treatment 6, periods 1-4).

Sprague Dawley rats display a large amount of variability in spatial confinement during nearly every period and for both injections (figure 3-17 “Radius”). However, Fischer rats express a great amount of variability in spatial confinement only during the first injection with very little variability in the sixth injection following behavioral sensitization (figure 3-18). The sixth injection also shows that as amphetamine levels increase, the measured horizontal activity of the animal also increases as shown by a positive slope of radius used versus amphetamine levels during each of the four periods.

Analysis of striatal amphetamine concentration and focused stereotypy scores for the four periods previously identified (figure 3-19) show that expression of stereotypy is not related to amphetamine level within a session. First order elimination of amphetamine is apparent in both F344 and Sprague

Dawley rats (figure 3-19, left frames). There are no significant differences between amphetamine concentrations prior to behavioral sensitization (injection 1) or after sensitization has occurred (injection 6). The corresponding scores for focused stereotypy to the right of the concentration curves show that for Fischer rats stereotypy scores remain high throughout the four periods during which amphetamine concentration is decreasing with sensitized animals showing higher stereotypy scores for all frames examined. Similarly, Sprague Dawley rats show that during the amphetamine elimination focused stereotypy remains elevated. Both sensitized and unsensitized Sprague Dawley rats show a peak during period two (corresponding to striatal amphetamine concentration at $T_{½}$) but focused stereotypy for sensitized animals was higher than unsensitized animals for the first three periods before converging with unsensitized animals during the fourth period.

Figure 3-15. Relationship between stereotypy scores and striatal amphetamine concentrations at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine for Sprague Dawley rats over 4 time periods during the drug terminal elimation phase.

Rows represent the first and sixth injection of a sensitizing series of 5.0 mg/kg d-amphetamine in Sprague Dawley rats. Columns represent each of four time periods following drug administration (15-30 min, 60-75 min, 90-105 min, and 120-135 min). For each frame, the x-axis is drug concentration in the striatum and the y-axis is stereotypy score (SS). Points represent the values for individual subjects. Regression lines have a positive slope during all periods for injection 1 and injection 6.

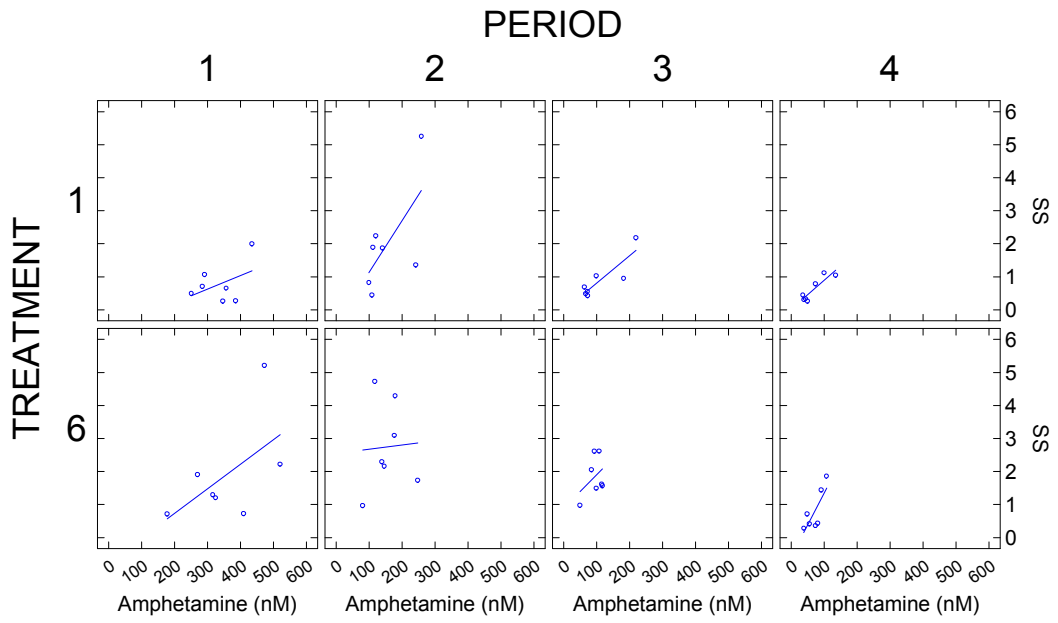


Figure 3-15 – Sprague Dawley - Stereotypy

Figure 3-16. Relationship between stereotypy scores and striatal amphetamine concentrations at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine for Fischer 344 rats over 4 time periods during the drug terminal elimation phase.

Rows represent the first and sixth injection of a sensitizing series of 5.0 mg/kg d-amphetamine in Fischer 344 rats. Columns represent each of four time periods following drug administration (15-30 min, 60-75 min, 90-105 min, and 120-135 min). For each frame, the x-axis is drug concentration in the striatum and the y-axis is stereotypy score (SS). Points represent the values for individual subjects. No obvious relationships can be seen between amphetamine concentration and focused stereotypy

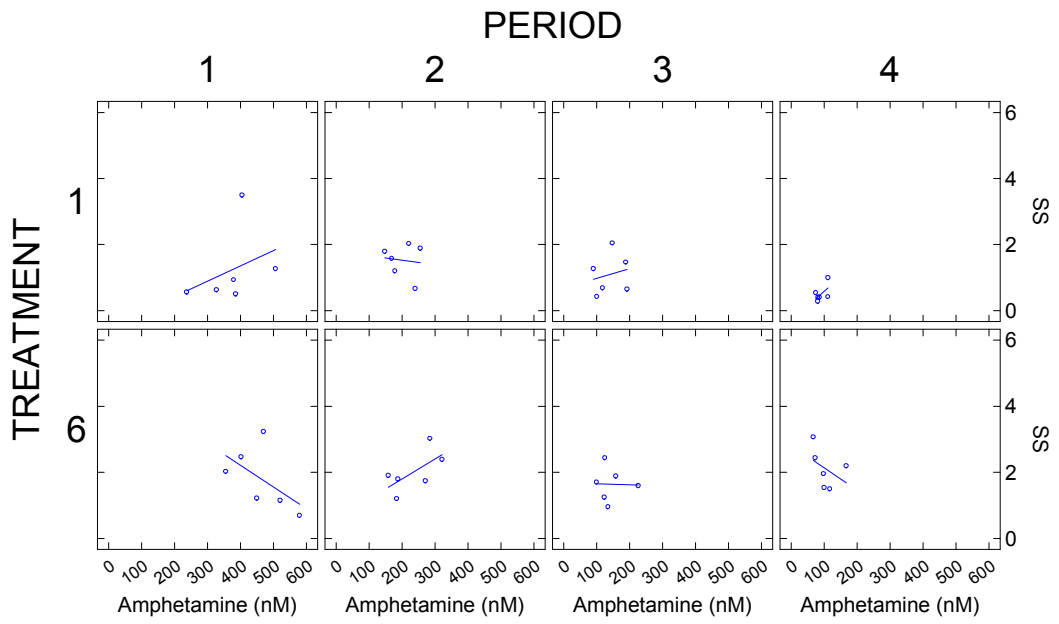


Figure 3-16 - F344 - Stereotypy

Figure 3-17. Relationship of spatial confinement and striatal amphetamine concentrations at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine for Sprague Dawley 344 rats over 4 time periods during the drug terminal elimation phase.

Rows represent the first and sixth injection of a sensitizing series of 5.0 mg/kg d-amphetamine in Sprague Dawley rats. Columns represent each of four time periods following drug administration (15-30 min, 60-75 min, 90-105 min, and 120-135 min). For each frame, the x-axis is drug concentration in the striatum and the y-axes are inverted scores of spatial confinement (Rad). Points represent the values for individual subjects. Regression lines of values of radius occupied (inverse of spatial confinement) and drug levels show that as amphetamine concentrations increase rats occupy less space on the plate for all four periods. Following the sixth injection (treatment 6), animals do not show an obvious trend or relationship between amphetamine concentration and radius occupied.

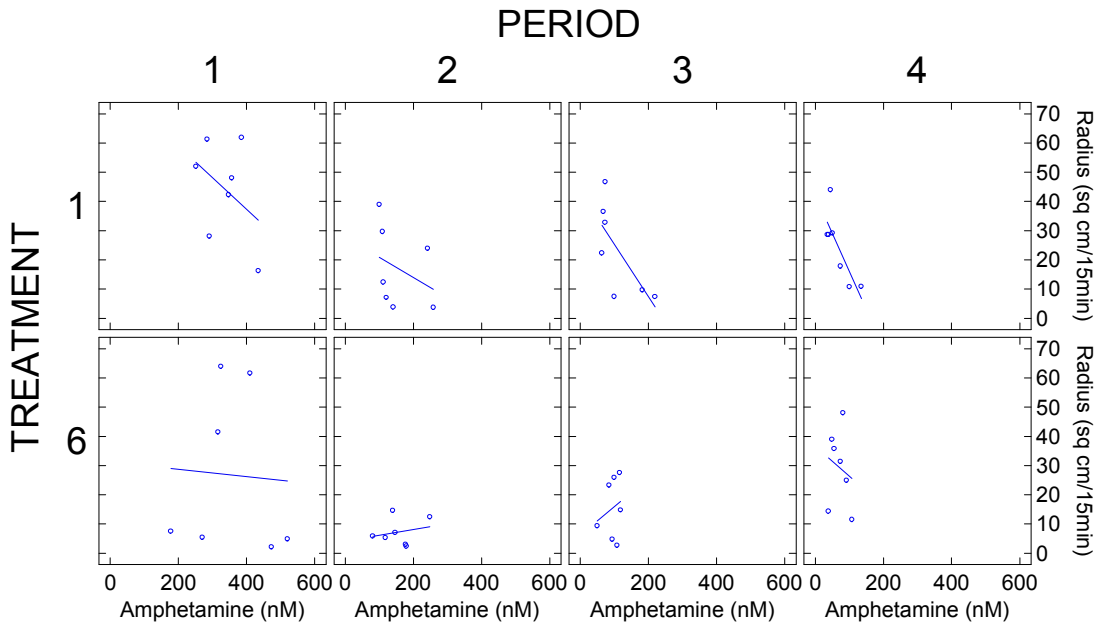


Figure 3-17 - SD - Space used

Figure 3-18. Relationship of spatial confinement and striatal amphetamine concentrations at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine for Fischer 344 rats over 4 time periods during the drug terminal elimation phase.

Rows represent the first and sixth injection of a sensitizing series of 5.0 mg/kg d-amphetamine in Fischer 344 rats. Columns represent each of four time periods following drug administration (15-30 min, 60-75 min, 90-105 min, and 120-135 min). For each frame, the x-axis is drug concentration in the striatum and the y-axes are inverted scores of spatial confinement (Rad). Points represent the values for individual subjects. For injection one of the series, regression lines indicate that there is no relationship between values of radius occupied and drug levels in the striatum. Following the sixth injection (treatment 6), regression lines indicate that a positive relationship may exist between drug concentration and radius occupied. However, these values also show that scores of radius occupied (inverse of spatial confinement) are very low and show very little variability compared with values prior to sensitization.

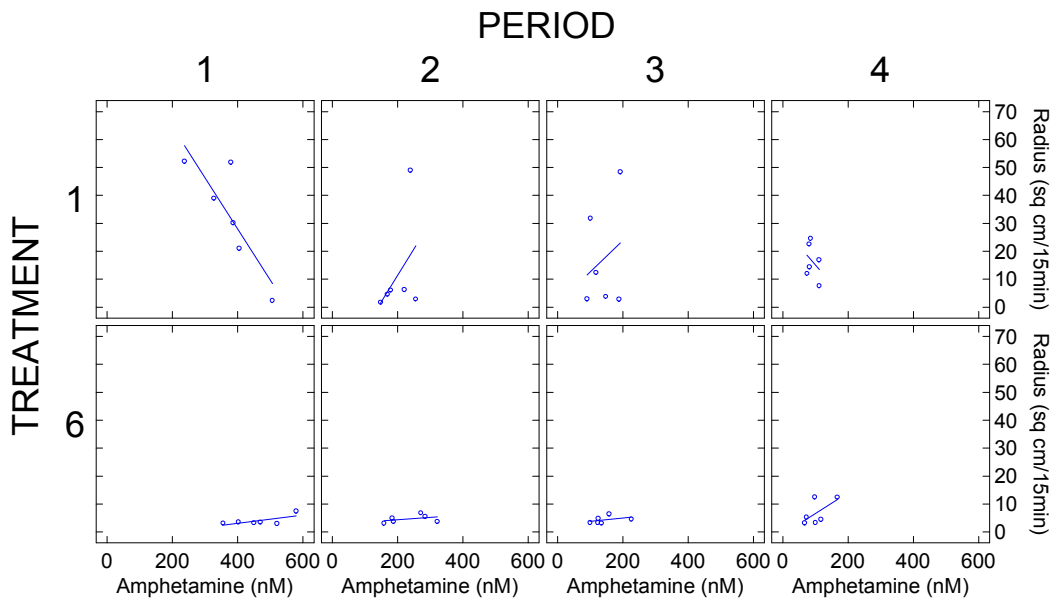
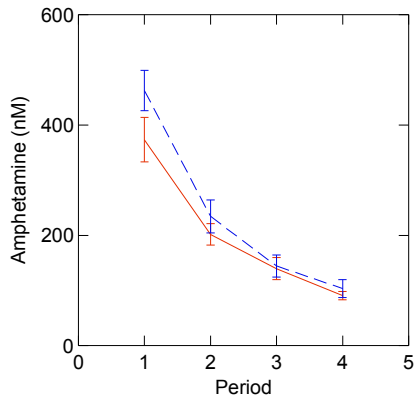
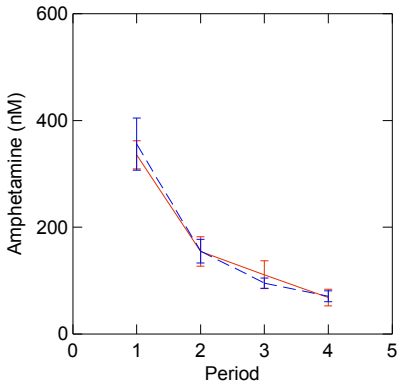
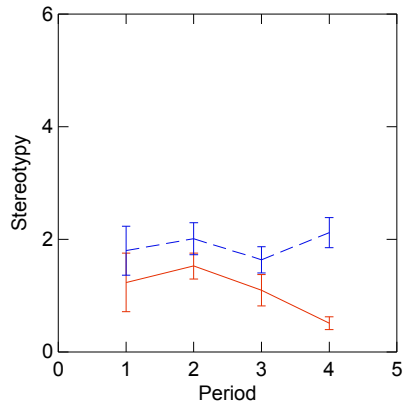
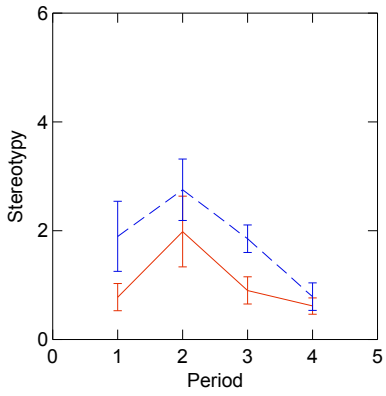


Figure 3-18 – F344 – Space used

Figure 3-19. Amphetamine concentration in the striatum and corresponding stereotypy scores at the beginning and end of a sensitizing series of six injections of 5.0 mg/kg d-amphetamine for Fischer 344 and Sprague Dawley rats over 4 time periods

Columns include concentration and stereotypy scores for Fischer 344 and Sprague Dawley rats. The upper frames include amphetamine concentration data at 4 time periods (15-30 min, 60-75 min, 90-105 min, and 120-135 min) corresponding to periods of dialysate collection from the striatum of two rat strains. The lower frames are stereotypy scores of the corresponding time frames. Sprague Dawley rats are shown in the left column and Fischer 344 rats are shown in the right column. Red, solid lines are values for the first injection of a sensitizing series of injections (Treatment 1). Blue, dashed lines are values for the last treatment of the series (Treatment 6). Brackets represent the standard error of the mean for six Fischer 344 and seven Sprague Dawley rats. Amphetamine shows a logarithmic downward slope consistent with first order elimination for both strains of rats before and after sensitization,. Stereotypy scores do not correspond to amphetamine level data. Stereotypy scores for Fischer 344 rats remain elevated throughout the four periods during which amphetamine levels are decreasing. Sprague Dawley rats reach a peak amphetamine score during the timeframe corresponding to the point at which amphetamine levels dropped to half their maximum concentration ($T_{½}$).

Fischer 344	Sprague Dawley
	
	

DISCUSSION

The results of this study show that there are significant behavioral differences between Sprague Dawley and Fischer 344 rats that cannot be explained by amphetamine concentrations in the striatum. The first part of this study was to establish whether there are significant kinetic differences in amphetamine uptake and elimination between F344 and Sprague Dawley rats during the first and sixth injection of a sensitizing series of injections. Pharmacokinetic analysis of amphetamine levels in dialysate collected from the striatum revealed that all subjects followed first order kinetics with a rapid absorption phase leading to a maximum drug concentration occurring between 15 and 30 minutes. Analysis of drug elimination revealed that there were no differences between Sprague Dawley and Fischer 344 rats in absolute drug elimination or the elimination rate (lambda z) prior to, or following a sensitizing series of six amphetamine injections. Similarly, half-life values showed no significant differences between strains or treatment groups. Values of maximum concentration (Cmax) were slightly higher following sensitization for both Sprague Dawley and F344 rats. The difference in Cmax values between injection 1 and injection 6 approached significance ($p=0.063$), but did not meet the *a priori* criterion for statistical significance.

The second part of the present study addresses behavior as it relates to amphetamine pharmacokinetics. Analysis of behavioral results suggests

that even before sensitization took place F344 and SD rats showed differences on several behavioral measures. Sprague Dawley rats were more active in terms of locomotor activity as measured by distance traveled and scores of spatial confinement. Power spectral analysis also shows that Sprague Dawley rats exhibited higher amplitude, longer-lasting vertical component at 10 Hz than F344 rats. For Sprague Dawley rats, this vertical component was present throughout the focused stereotypy phase of the session and represents the vigorous vertical head movements associated with focused stereotypy in Sprague Dawley rats. In F344 rats, the 10 Hz peak declined after only two 15 minute periods at a time when the rat was still engaged in focused stereotypy. This pattern was present both before and after sensitization and is likely due to F344 rats switching from a vertical to a more lateral head movement as the stereotypy intensifies. Higher sensitivity of F344 rats is also evident from focused stereotypy scores. Both before and after sensitization, F344 rats achieved a peak stereotypy score more rapidly than SD rats. The duration of stereotypy is also longer for F344 rats.

As stated, this study confirms previous findings that Fischer 344 and Sprague Dawley rats differ in their behavioral response to 5.0 mg/kg d-amphetamine both prior to, and following amphetamine sensitization. However, the results show that the between-strain behavioral differences cannot be explained by amphetamine levels in the striatum. Pharmacokinetic analysis shows that there are no significant differences in the absorption or

elimination of amphetamine in the striatum between these strains and at these time periods. Analysis of data from individual subjects as shown in figures 13-16 illustrate that small variations in the amount of amphetamine can be found between subjects. Variability in amphetamine levels between each subject is likely a result of variations inherent to intraperitoneal injections. Considering the observed range of amphetamine concentrations in this study, one might predict that increasing levels of amphetamine in the striatum would lead to higher focused stereotypy scores and to lower scores on horizontal activity as represented by space used. Indeed, for Sprague Dawley rats, increasing amphetamine levels show some relationship to increasing stereotypy for both injection 1 and 6. However, these apparent effects of amphetamine levels on behavior were limited to scores of focused stereotypy and only in Sprague Dawley rats. Supporting data showing influences on spatial confinement showed no consistent relationships for either strain and treatment number (sensitization) did not play a role. From the lack of significant strain differences between amphetamine pharmacokinetics in the striatum and evidence that behavioral scores are not greatly influenced by small variations in amphetamine levels we can conclude that the observed behavioral differences between Sprague Dawley and Fischer 344 rats are not directly due to effects of amphetamine concentration in this brain region.

Results confirm that F344 and SD rats showed behavioral sensitization to amphetamine following a series of six injections of 5.0 mg/kg d-amphetamine. In these studies, sensitization was expressed in both strains by a significant change in expression of focused stereotypy. An additional question is whether both rat strains show the same degree of sensitization. Sensitization was expressed differently between the strains. Sprague Dawley rats showed an increase in amplitude of focused stereotypy and only a mild increase in duration. Fischer 344 rats also showed an increase in amplitude, but a larger difference in stereotypy duration.

Because this study compared behavioral effects concurrently with amphetamine concentration, it is conclusive that amphetamine levels do not explain observed behavioral differences between Sprague Dawley and Fischer 344 rats. Lack of significant differences in amphetamine pharmacokinetic data between injection 1 and injection 6 indicate that for both Sprague Dawley and Fischer 344 rats, amphetamine concentration in the striatum played little or no role in behavioral sensitization. Because amphetamine levels were comparable between strains throughout the 4-hour behavioral session, it can also be concluded that IP injection effects and penetration of the blood brain barrier is likely not different between these strains. It is also evident that blood brain barrier permeability to amphetamine does not change significantly throughout the sensitization process. However, because amphetamine levels were observed to be higher (though not

significantly) for both strains following the sensitizing series of injections, it is possible that some physiological change may occur in amphetamine uptake into the brain. Repeating this study with a larger sample size and with a range of amphetamine doses may be able to detect if differences actually exist. Also, because levels of amphetamine were measured only in the striatum, it cannot be ruled out that differences may be observed in other brain regions.

CHAPTER 4

Effect of whole brain clozapine concentration on amphetamine-induced behaviors

Introduction

The current chapter addresses potential interactions of the atypical antipsychotic drug clozapine and d-amphetamine in rats. The experiments in this chapter address hypothesis number six and attempt to answer the following questions: 1) Are whole-brain clozapine levels higher in amphetamine treated sensitized rats as compared to saline treated controls? 2) Are observed differences in the behavioral response linked to differences in brain clozapine levels?

Clozapine is a highly effective atypical antipsychotic that has been shown to be superior in patients suffering from severe chronic schizophrenia with poor previous treatment response (Azorin et al., 2001; Conley et al., 1999). Although there is some debate over the distinction between typical and atypical antipsychotic drugs, the main points as outlined by Meltzer (2000) are that atypical antipsychotic drugs produce an antipsychotic effect in most patients without producing significant extrapyramidal side effects common in older drugs. Pharmacologically, typical antipsychotic drugs such as haloperidol bind tightly to the dopamine D2 receptor, even more tightly

than dopamine, with relatively low binding at other receptor types (Seeman, 2002). Clozapine has a more promiscuous binding profile with a higher ratio of serotonin type 2 (5-HT2)-receptor binding to D2 binding (Worrel et al., 2000; Leysen et al., 1993). It was proposed by Meltzer (1989) that the interaction between serotonin (5-HT) and dopamine (DA) systems may play a critical role in the mechanism of action for atypical antipsychotics because potent 5-HT2A receptor antagonism together with relatively weak D2 receptor antagonism can differentiate most atypical antipsychotics from typical antipsychotics. However Kapur and Seeman (2001) claimed that the effectiveness of atypical antipsychotic drugs was due to lower affinity at the D2 receptor and proposed that an antipsychotic effect could be produced by appropriate modulation of the D2 receptor alone. Seeman (2002) went on to claim that the binding affinity of clozapine to 5-HT2a receptors was not sufficient to elicit a significant effect at therapeutic doses.

Disturbance in normal dopaminergic transmission has long been suggested to play a role in the symptoms of schizophrenia. Reduced activity in the mesocortical dopamine pathway has been linked to the negative symptoms of schizophrenia (apathy, social withdrawal, poverty of thought), while hyperactivity of the mesolimbic dopamine system is responsible for positive or psychotic symptoms (Lieberman, 2004). Amphetamine is a dopamine releaser and has been shown to have enhanced effects on dopamine release in neuroleptic-free schizophrenic patients (Laruelle et al,

1996; Abi-Dargham et al., 2004). The excitatory effect of amphetamine depends on forebrain inputs to DA neurons and requires activation of adrenergic α1 receptors (Shi et al, 2000). Prazosin, a selective α1 noradrenergic antagonist, has been reported to have behavioral effects similar to clozapine in the prepulse-inhibition startle paradigm (Bakshi and Geyer 1997). These results suggest that clozapine inhibits DA transmission by at least two different and additive mechanisms: binding to DA receptors and inhibiting a subset of excitatory inputs to DA neurons. Previous evidence suggests that these inputs may be derived directly or indirectly from the prefrontal cortex (PFC).

As was previously stated, clozapine has been shown to delay operant responding by amphetamine treated, sensitized rats. Table 4-1 shows a comparison of amphetamine treated sensitized rats given saline, raclopride (specific D2 antagonist), the typical antipsychotic drug haloperidol, or clozapine. Rats were trained on a four hour operant task to press a lever to receive milk as a reward. As can be seen from the table, compared to saline, clozapine delays the return to lever pressing following the stereotypy phase in a dose dependant manner (2.5mg/kg, 5.0mg/kg, and 10.0mg/kg). Haloperidol and the D2 receptor antagonist hasten the return to lever pressing (Osterhaus et al., 2004) indicating a role of the D2 receptor in goal-oriented motor behavior.

Clozapine is primarily metabolized by the CYP1A2 and CYP3A4 subfamily of cytochrome p-450 enzymes. However, there is evidence that the CYP2D6 enzyme, which is primarily responsible for amphetamine metabolism, is also involved. This potentially competitive interaction must be considered as a possible factor in the observed prolongation of amphetamine-induced behaviors in rats. In support of this finding, and the potential for metabolic interactions, this chapter will address whether clozapine concentrations are higher in the presence of amphetamine.

Table 4-1. Administering clozapine to rats engaged in amphetamine-induced stereotypy delayed return to lever pressing compared to compounds with higher D2 receptor antagonism, raclopride and haloperidol.

Animals were trained to lever press under a combination variable interval and fixed ratio schedule to receive condensed milk as a reward. 5.0 mg/kg amphetamine disrupted lever pressing during the focused stereotypy phase. The selective D2 receptor antagonist raclopride, the typical antipsychotic haloperidol or the atypical antipsychotic drug clozapine were administed 30 minutes following amphetamine administration. Values on the left show the drug treatment each group received. Values on the right show the relative time in minutes that the animal returned to lever pressing compared to rats receiving amphetamine/saline. Positive numbers indicated that the treatment caused the animal to resume lever pressing earlier than amphetamine/saline treated controls. Negative numbers indicate that the treatment delayed the animal's return to lever pressing. Results show that that risperidone and haloperidol allow the animal to return to lever pressing earlier than saline alone while clozapine delayed return to lever pressing in a dose dependant manner.

Drug Treatment and dose (mg/kg)	Mean (SEM) change In time (min) of lever pressing relative to 5.0 amph/vehicle
Veh/Veh	Not Applicable
Amp 5.0/Veh	0
Amp 5.0/Rac 0.05	+28.3 (5.5)
Amp 5.0/Rac 0.1	+28.0 (6.2)
Amp 5.0/Rac 0.2	+27.0 (4.9)
Amp 5.0/Rac 0.5	+28.3 (9.1)
Amp 5.0/Hal 0.08	+23.3 (4.1)
Amp 5.0/Clz 2.5	-11.7 (5.4)
Amp 5.0/Clz 5.0	-26.7 (3.9)
Amp 5.0/Clz 10.0	-88.3 (15.1)

Table 4-1

MATERIALS AND METHODS

Solutions

Acetonitrile, potassium phosphate monobasic, 85% phosphoric acid, hexane, sodium hydroxide, and sulfuric acid were purchased from Fisher. Clozapine, d-amphetamine sulfate and Isoamyl alcohol were purchased from Sigma. The internal standard olanzapine was obtained from Lilly Corporation, and sterile, 0.9% saline was obtained from AmTech.

Experimental design

16 Fischer 344 and 16 Sprague Dawley rats were used for this study. All 32 rats received a series of six intraperitoneal (IP) injections of 5.0 mg/kg d-amphetamine. Each of the six injections was separated by four days. On each injection day, rats were placed in the force-plate actometer chamber for a 4 hour session immediately after receiving amphetamine. Behavioral data were collected for the entire 4 hour session. For the first five injection days, an IP injection of saline was given 30 minutes into the session as a control for the clozapine injection that was given on injection day six. On the sixth injection day, 8 Sprague Dawley and 8 F344 rats were given an IP injection of 0.9% saline (1.0 ml/kg) as a control. The remaining 16 animals were

administered d-amphetamine (5.0 mg/kg). A force-plate actometer session was begun immediately following drug or saline administration. All animals received an IP injection of 10.0 mg/kg Clozapine 30 minutes following amphetamine administration. Animals were removed from the chamber after 135 minutes at the time when clozapine levels were thought to be most divergent (in the presence of amphetamine compared to no amphetamine). Animals were immediately decapitated and brains were removed, weighed, frozen on dry ice and stored at -80º prior to clozapine extraction procedures.

Behavioral apparatus

8 force-plate actometers were used for analysis of behavior. A single computer was used to collect data from four actometer chambers. Recordings were taken at 50 samples per second. Actometers were constructed with a plexiglass encasement and a square plate 30 centimeters across as previously described in detail (Fowler et al., 2001). Each actometer was enclosed in a sound and light attenuating chamber.

Clozapine Extraction Method

A liquid-liquid extraction procedure was modified from Avenoso et al. (2000) for recovering clozapine from rat brain. Rat brains were thawed and homogenized in 2 mL 0.9% saline and immediately centrifuged at 3500 g for 20 minutes. The supernatant was added to a tube containing 20 µl of 1 µg/ml

Olanzapine (internal standard). The mixture was then made alkaline by adding 1 ml of 2 M NaOH and extracted with 6 ml hexane-isoamyl alcohol (98:2 v/v). The sample was shaken (by inversion) for 35-45 minutes then separated by centrifugation at 2500g for 15 minutes. The upper organic layer was placed in a separate tube and 200 µl of 0.025 M H_2SO_4 was added. The solution was then vortex mixed for 45 seconds and centrifuged for 15 minutes at 2500g. The upper organic layer was discarded and the aqueous layer was injected directly into the HPLC column. All samples were run in duplicate with an injection volume of 20 µL. This extraction method was previously shown to yield 91% recovery of total clozapine (Avenoso et al., 2000).

HPLC

High Performance Liquid Chromatography (HPLC) was carried out on a Shimadzu Model LC 10 Advp with a dual reciprocating pump and a SPD 10Avp UV-VIS detector set at 254 nm. The System controller was a Model SCL 10Avp. The method was modified from D'Arrigo et al., (2006). Mobile phase A consisted of water-acetonitrile 80:20 vol/vol containing 0.6 mM KH_2PO_4. Solution B was water-acetonitrile 20:80 containing 0.6 mM KH_2PO_4 adjusted to pH 2.7 with H_3PO_4. The optimal gradient was determined to be a 15 minute cycle beginning at 90% A for 4 minutes sloping to 80% A over 4 minutes. Flow was held at 80% A for 3 minutes before returning to 90% A.

Chromatographic separation was carried out on a Waters Spherisorb S5 C6 column (250mm X 4.6mm ID) at a flow rate of 1 ml/min.

Standard Curve

Solutions of clozapine and olanzapine were prepared immediately prior to each HPLC run by dissolving pure substance in 0.6 mM KH_2PO_4 (pH 2.7). The concentration of the internal standard olanzapine was 100nM. For standard curve analysis, clozapine was prepared and diluted by serial dilution to 512, 256, 128, 64, 32, 16, 8, 4, and 2 nM. Solutions were further diluted by adding equal parts of each clozapine concentration to the internal standard solution for final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, and 1 nM. The final concentration of internal standard was 50nM. Brain levels calculated from the standard curve were based on ng clozapine divided into absolute brain weight.

RESULTS

Figure 4-1 shows whole brain clozapine levels in Sprague Dawley and Fischer 344 (F344) rats treated with saline 30 minutes prior to clozapine treatment, and Sprague Dawley rats treated with 5.0 mg/kg d-amphetamine 30 minutes prior to receiving 10.0 mg/kg clozapine. Clozapine data for F344 rats treated with amphetamine prior to clozapine was unavailable due to chromatographic instrument failure and was not included in these results. No significant differences in brain clozapine concentrations were found between Sprague Dawley rats treated with amphetamine and those treated with saline in place of amphetamine. F344 rats treated with saline prior to clozapine were not significantly different from Sprague Dawley rats receiving the same treatment.

Figure 4-2 shows power spectra of vertical force variations for Sprague Dawley rats during three time periods. The first time block is from 10 to 30 minutes following the initial amphetamine treatment. The first 10 minutes were excluded due to a high amount of locomotor activity resulting from placing the animal in the chamber after administering the injection. Including this initial exploration period could erroneously mask the amphetamine-induced head movements associated with stereotypy. The second time block was 60-90 minutes following amphetamine treatment and represents a period

of time when amphetamine-treated sensitized animals are usually fully engaged in focused stereotypy. The final time block was 90-120 minutes. This is the period of time during which vertical head movements often subside and locomotor activity resumes.

The two graphs in the upper row of figure 4-2 are from injection day 5. On this day, animals received 5.0 mg/kg amphetamine followed 30 minutes later by saline. These animals serve as behavioral controls since previous experience has shown that amphetamine-induced behaviors do not significantly change following the fifth of a sensitizing series of amphetamine injections. The two graphs on the bottom row represent the same animals treated on the sixth treatment day. On the sixth injection day, one group of animals was treated with amphetamine and the other was treated with saline. Both groups were treated 30 minutes later by an IP injection of 10.0 mg/kg clozapine. The graphs on the left represent the group of animals that received amphetamine prior to clozapine on injection day 6 (A + C group). The graphs on the right represent animals that received saline prior to clozapine on injection day 6 (S + C group). The amphetamine treated animals from days 5 and 6 all show a peak at about 10 Hz during the first time period (10-30 minutes). This rhythmic peak is characteristic of an animal engaged in the head movements associated with focused stereotypy. The behavioral control animals (inj 5, top row) that received amphetamine, but did not receive clozapine also show a lower amplitude, but distinguishable 10 Hz

peak during the second time period (60-90 minutes). The 10 Hz peak is largely nonexistent during the third time period (90-120 minutes) of treatment 5 indicating that the stereotypical head movements subsided and the animals became engaged in less rhythmic, locomotion types of behavior. The rats that received clozapine following the initial amphetamine injection on injection day 6 (lower right) showed a clear peak at 10 Hz during the first time block indicating that the rats were initially engaged in focused stereotypy. Following the first time block, at which time the animals received clozapine, the peak slowed to about 8 Hz indicating that the stereotypical head movements were slowed by clozapine treatment. Furthermore, this rhythm is clearly distinguishable during the second and third time periods. The presence of the head movements during the third period is in contrast to the control group (inj 5) which showed little or no peak during this period. This indicates that the amphetamine-induced stereotypical head movements continued for a longer duration in clozapine treated animals than in the same animals receiving saline during the previous injection. In clozapine control rats that received saline instead of amphetamine followed by 10.0 mg/kg clozapine (lower left graph) no distinguishable peaks are present.

Locomotor activity throughout the session is shown in figure 4-3 as a measure of decimated distance over 1 minute time blocks. Graphs on the left represent sensitized rats on injection day 5 that received 5.0 mg/kg amphetamine followed 30 minutes later by saline. These animals show a

characteristic decrease in locomotor activity during the stereotypy phase of amphetamine treatment. This period of decreased locomotion is reasonably consistent for both groups of animals. Columns on the right show the same animals on injection day 6 after receiving either amphetamine or saline, followed 30 minutes later by 10.0 mg/kg clozapine. Animals that received amphetamine prior to clozapine treatment showed a transient increase in locomotion following clozapine administration at 30 minutes. This locomotion is likely not just an artifact of injection since no such increase occurred in saline treated controls on day 5 (left). Although clozapine brain data was not available for the amphetamine treated group, the behavioral data indicates that locomotor activity also increases for a short period of time following clozapine administration at this 10.0 mg/kg dose.

Figure 4-1. Whole brain clozapine levels 100 minutes following IP injection of 10.0 mg/kg clozapine in rats pretreated with 5.0 mg/kg d-amphetamine or saline vehicle.

Bars show clozapine concentration in ng/g brain tissue. The bar on the left shows clozapine levels in a Sprague Dawley rat treated with amphetamine 30 minutes prior to clozapine. The middle and right bar are Sprague Dawley and Fischer 344 rats (respectively) treated with saline vehicle 30 minutes prior to clozapine injection. Brackets represent standard error of the mean. Rats were treated with 5.0 mg/ kg amphetamine immediately prior to starting behavioral recording in the force plate actometer. 30 minutes following the start of the session rats were administered 10.0 mg/kg clozapine. At 130 minutes following the start of the session rats were removed, decapitated and the brains removed and frozen. Analysis of variance indicates no significant differences in clozapine concentration between rats receiving amphetamine versus saline prior to clozapine ($p>0.05$).

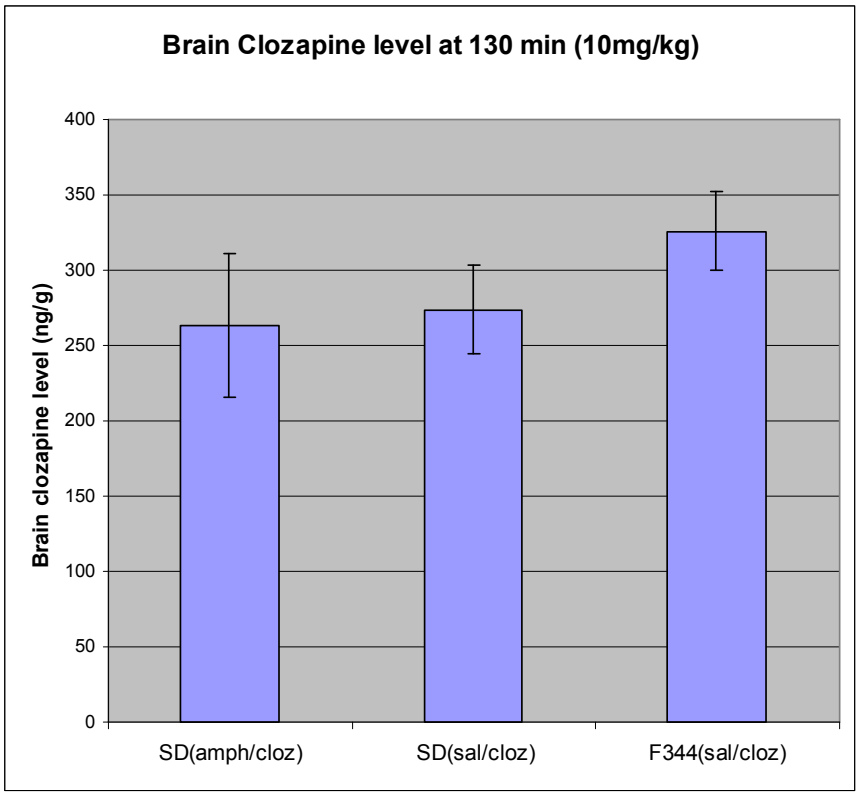

Brain Clozapine level at 130 min (10mg/kg)
Brain clozapine level (ng/g)
400
350
300
250
200
150
100
50
0
SD(amph/cloz)
SD(sal/cloz)
F344(sal/cloz)

Figure 4-1

Figure 4-2. Effect of clozapine on amphetamine-induced variations in power spectra of vertical force during three time blocks for sensitized Sprague Dawley rats.

Figure 4-2 shows power spectra of vertical force variations for Sprague Dawley rats during three time periods. Red lines represent the first time block from 10 to 30 minutes following the initial 5.0 mg/kg amphetamine treatment. Blue lines represent the second time block 60-90 minutes following amphetamine treatment. The green line represents 90-120 minutes following amphetamine treatement. The top graphs are data from sensitized rats receiving amphetamine at the start of the session followed by saline vehicle 30 minutes later. The bottom graphs are the same animals 4 days later. Animals on the lower left received saline at the beginning of the session followed by 10.0 mg/kg clozapine 30 minutes later. Animals on the lower right received amphetamine followed 30 minutes later by clozapine. Amphetamine treated rats show a distinctive shift to a lower frequency following clozapine treatment. Also apparent from the graph is the presence of frequency peaks during the third period in clozapine treated animals indicating a lengthening of the period of time the animal is engaged in stereotypical head movements.

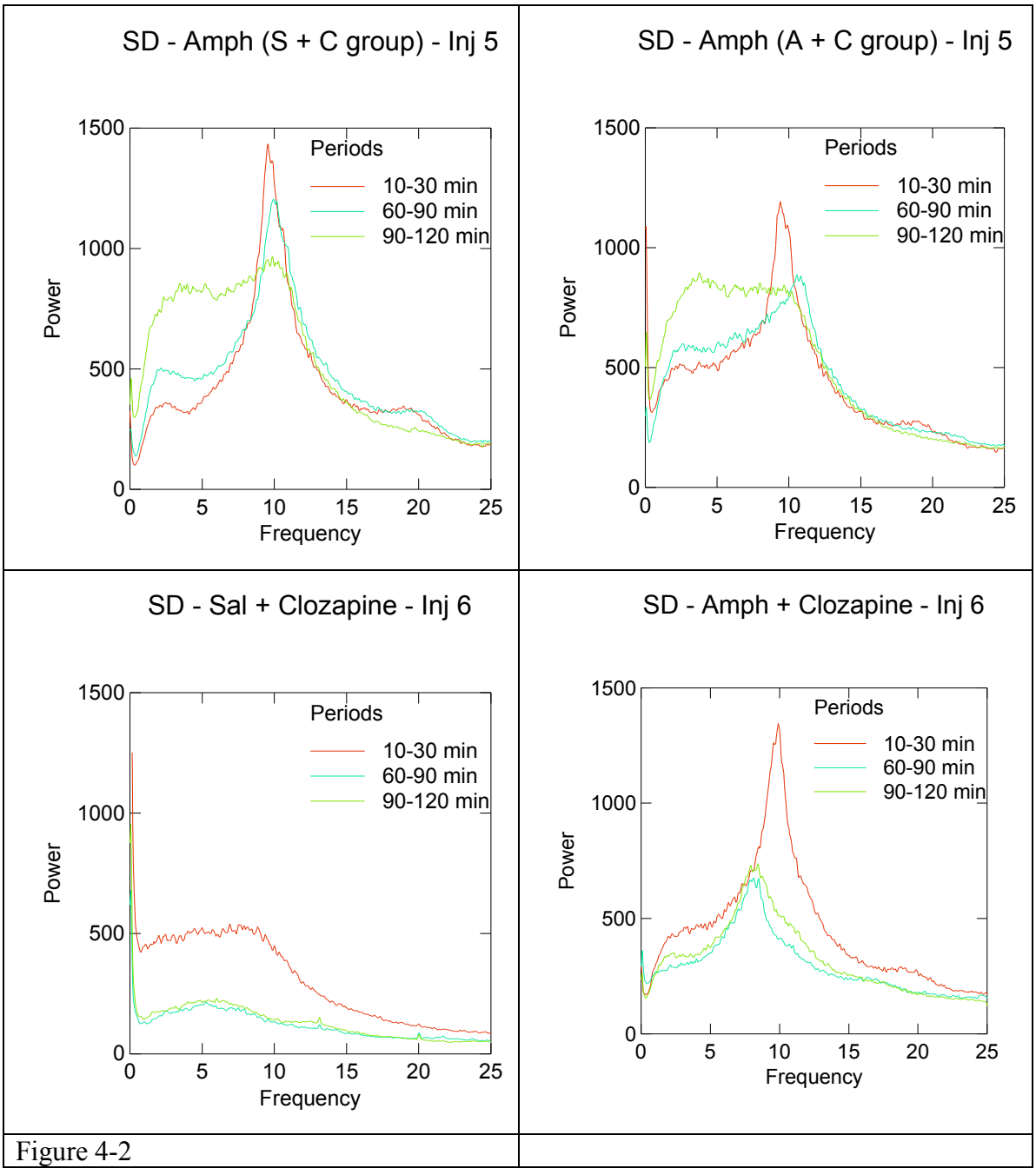

SD - Amph (S + C group) - Inj 5
SD - Amph (A + C group) - Inj 5
SD - Sal + Clozapine - Inj 6
SD - Amph + Clozapine - Inj 6
Periods
10-30 min
60-90 min
90-120 min
Power
Frequency
0
500
1000
1500
0
5
10
15
20
25

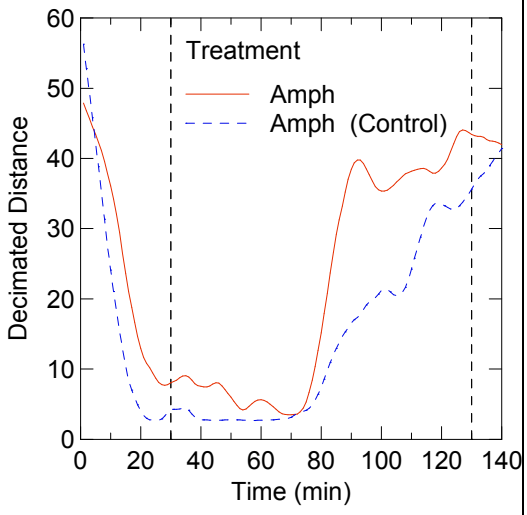
Figure 4-2

Figure 4-3. Effect of clozapine on amphetamine-induced locomotor activity for sensitized Sprague Dawley and Fischer 344 rats.

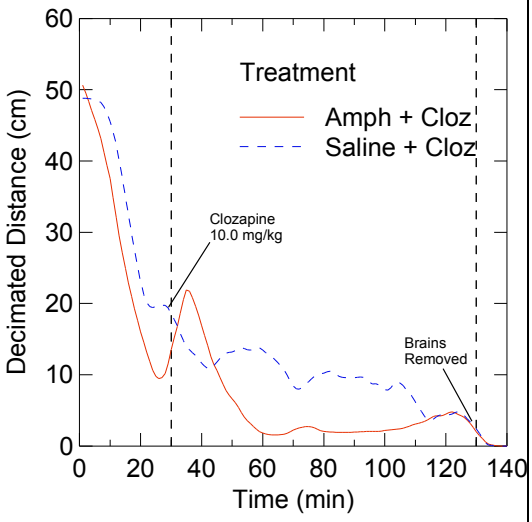
Upper graphs represent Sprague Dawley rats. Lower graphs represent Fischer 344 rats. Graphs on the left are from injection day five and represent amphetamine induced locomotor activity in sensitized animals with no clozapine treatment. Graphs on the right are data from animals that received either 5.0 mg/kg amphetamine or saline vehicle 30 minutes prior to 10.0 mg/kg clozapine on injection day 6 (four days later). Lines represent decimated distance in 1 minute time intervals. Solid red lines are data from animals in the experimental group that received amphetamine 30 minutes prior to saline on injection day 5 (left) and amphetamine prior to clozapine four days later on injection day 6 (right). Dashed blue lines are data from control animals that received amphetamine followed by saline on injection day 5 (left), and saline followed by clozapine on injection day 6. Brains were removed for analysis at 130 minutes following the start of the session on injection day 6. All rats show a characteristic decrease in locomotor activity during the stereotypy phase of amphetamine treatment. Amphetamine treated rats show a transient spike in locomotor activity following clozapine treatment (right).

Figure 4-3

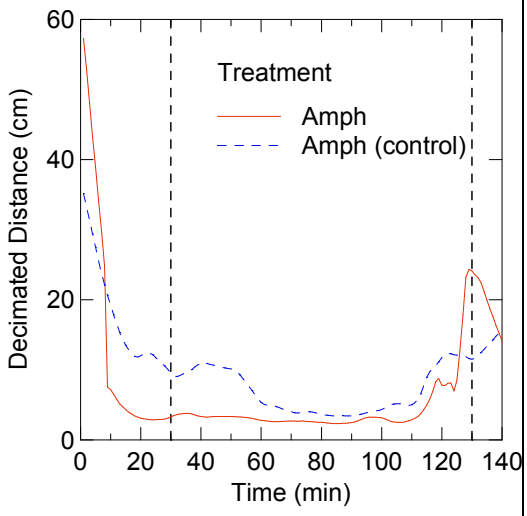
SD – Injection 5 – Mean scores
5.0mg/kg Amph + 1ml/kg Saline



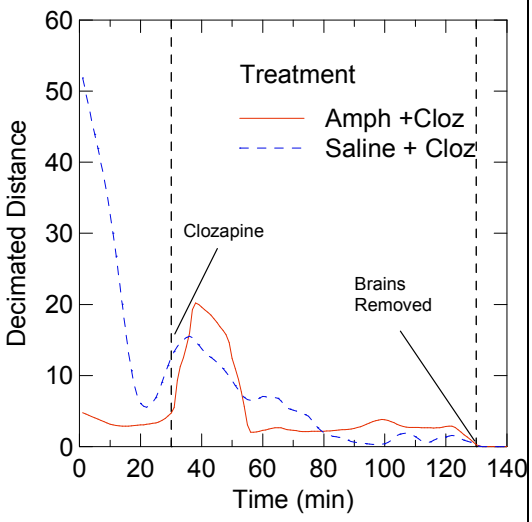
SD – Injection 6 – Mean scores
Saline or Amph + 10.0mg/kg Cloz.



F344 – Injection 5 – Mean scores
5.0mg/kg Amph + Saline



F344 – Injection 6 – Mean scores
Saline or Amph + 10.0mg/kg Cloz.



DISCUSSION

Results of whole brain analysis of clozapine levels at 100 minutes following IP injection of 10.0 mg/kg clozapine indicate that clozapine's effects on amphetamine-induced behaviors do not appear to be a result of competitive metabolic processes as was hypothesized. No differences in whole brain clozapine levels were seen between animals administered saline or amphetamine prior to clozapine. Also, there does not seem to be a strain effect on clozapine metabolism in the absence of amphetamine. Fischer 344 rats and Sprague Dawley rats given clozapine in the absence of amphetamine show no differences in clozapine levels at the time point measured. This indicates that clozapine's capacity to increase the duration of amphetamine-induced behaviors is likely due to direct effects on neurotransmitter receptor systems in the brain.

A clue to these effects is apparent from the results of locomotor activity measures. An interesting finding is the transient increase in locomotor activity immediately following clozapine administration in amphetamine treated rats. As was previously stated, this increase in activity is not likely an injection artifact since the effect was not seen when the same animals were administered saline instead of clozapine at 30 minutes during the previous

treatment 4 days prior (figure 4-3). The observed increase in locomotion following clozapine administration was followed by an exaggerated decrease in locomotor activity to levels lower than saline/clozapine treated controls (figure 4-2, column 2, 40-80 minutes). This indicates that it is not the sedative effect of clozapine alone, but rather the interaction of clozapine with amphetamine that is causing these effects. This effect was observed in both Sprague Dawley and F344 rats. Research in this lab previously reported a transient decrease in focused stereotypy scores in amphetamine-treated rats following administration of 10.0 mg/kg clozapine but not in lower doses (Fowler et al., 2007).

It is well established that clozapine, like most atypical antipschotics binds to multiple receptors (Wilffert et al., 2005; Leysen et al., 1993; Meltzer, 1989). Because amphetamine functions as a dopamine releaser, it is possible that the initial increase in locomotion following clozapine administration is a result of D2 receptor antagonism. Because clozapine has a relatively low affinity for dopamine D2 receptors, effective D2 antagonism requires that clozapine levels must be present at high concentrations (Seeman, 2002). Oral doses of clozapine show only negligible binding at doses below 10 mg/kg in rats (Kapur, 2000; Barth, 2006). It is plausible that an intraperitoneal injection of 10.0 mg/kg clozapine is a sufficiently high concentration that transient occupation of the D2 receptor by clozapine temporarily interrupted the effects of amphetamine. Furthermore, because

clozapine is rapidly metabolized (Baldessarini, 1993) the concentration of clozapine rapidly falls below the threshold for efficacious D2 receptor binding. At this point, the stereotypy inducing effects of amphetamine are able to resume and clozapine binding at other receptors is left to yield a greater influence on behavior.

It was previously reported that clozapine slows the rhythmic head movements associated with amphetamine-induced focused stereotypy and prolongs stereotypy in Sprague Dawley rats (Fowler et al., 2007). Comparison of power spectra of vertical force for time blocks 1-3 support that finding (figure 4-4). Following clozapine administration at 30 minutes in the amphetamine treated group, the 10 Hz rhythm present during time block 1 shifted to a lower frequency (about 8 Hz) during time blocks 2 and 3 (60-120 minutes). This may be related to the sedation effect that has been reported in atypical antipsychotics (Miller, 2000). It was hypothesized that this effect may originate from α1 adrenergic receptor blockade since Clozapine and Olanzapine have higher affinity at these receptors than most other atypical antipsychotics and have been shown to have a greater sedative potential (Ahnaou et al., 2003). Prazosin, an alpha 1 noradrenergic receptor antagonist was also shown to slow amphetamine-induced head movement rhythm in a dose dependent fashion (Fowler et al., 2007) indicating potential noradrenergic involvement in this phenomenon.

The presence of rhythmic vertical forces during time block 3 in amphetamine/clozapine treated animals indicates a prolongation of stereotypical head movements since no peak was present during this time frame in amphetamine-treated rats with no clozapine (figure 4-2). It was hypothesized (Fowler et al., 2007) that the prolongation of focused stereotypy may be partially due to drug elimination kinetics. While amphetamine levels were not measured in this study, the lack of significant differences in clozapine levels between animals receiving amphetamine or saline 30 minutes prior to clozapine treatment indicate that clozapine does not significantly compete with amphetamine for metabolic resources. This finding suggests that the prolongation of vertical head movements and stereotypy probably have a different cause.

The findings offered in this section do not support the hypothesis that amphetamine-clozapine interactions contribute to slowing or prolongation of stereotypical head movements in Sprague Dawley rats. Because clozapine has a broad range of neurotransmitter systems with which it interacts, further exploration of the individual interactions of these systems with amphetamine-induced behaviors should be explored to help explain the observed behavioral effects.

CHAPTER 5

CONCLUSIONS

The purpose of the present studies was to simultaneously measure drug concentration and the associated behavioral response in a single experiment. This was accomplished using two techniques. First, a force-plate actometer was equipped with a microdialysis device to collect extracellular fluid from the striatum of rats. An HPLC-tandem mass spectrometry method was developed for the quantification of analytes collected from this device. Using this system, it was determined that amphetamine, amphetamine metabolites and clozapine could be detected at limits of quantitation in the low nanomolar to picomolar range. The analytical method was unable to achieve a level of sensitivity required for monitoring brain levels of the catecholamine dopamine. Behavioral data that were obtained from the modified actometer/microdialysis apparatus was determined to be comparable to data previously obtained from actometers without the modification. The apparatus was then used to investigate the relationship between amphetamine levels and the behavioral response in two different rat strains (F344 and Sprague Dawley). Behavioral and amphetamine pharmacokinetic data that was obtained allowed us to analyze how amphetamine levels relate to behavioral differences between strains,

between sensitized and unsensitized rats, and behavioral changes that occur within a session.

The second technique used in these studies measured whole brain clozapine levels in amphetamine or saline treated rats. The method involved liquid-liquid extraction of clozapine from brain followed by HPLC-UV analysis. This method was chosen due to ineffective measures of clozapine in brain dialysate obtained from the previously developed microdialysis technique. Clozapine levels represented one time point (130 min) following measurements of the behavioral response. While this technique did not have the power that microdialysis affords, we were able to obtain data showing previously observed behavioral responses to clozapine and that metabolic interference of amphetamine with clozapine does not significantly contribute to these behaviors.

In summary, results obtained from these experiments show that amphetamine levels do not have a significant impact on previously observed behavioral differences between rat strains, between sensitized and unsensitized rats and behavioral changes that occur within a session. Furthermore, metabolic interactions of clozapine with amphetamine do not appear to significantly contribute to the behavioral response of clozapine with amphetamine treated rats. These findings implicate a pharmacodynamic rather than a pharmacokinetic contribution to the observed behaviors which is

significant to previous and ongoing studies of behavioral pharmacology involving these compounds.

In the following section, the original hypotheses introduced in chapter 1 will be restated. An analysis of findings will be summarily presented along with concluding remarks and future directions.

Hypothesis #1: Brain levels of amphetamine, amphetamine metabolites, dopamine and the atypical antipsychotic drug clozapine can be collected and quantified from an ambulatory animal over a 4-hour period.

A method was successfully developed to separate and quantify dopamine, clozapine, amphetamine, and the amphetamine metabolites p-hydroxyamphetamine (OHamp), norephedrine (nor), and p-hydroxynorephedrine (OHnor). Of these molecules, only amphetamine provided useful data from *in vivo* samples obtained from brain dialysate. Dopamine was detectable, but the lower limit of quantitation was found to be 50 nM (figure 2-5) which is higher than basal levels of dopamine previously found in the striatum (Chen, 2004) limiting its usefulness in this paradigm.

Clozapine had very low limits of quantitation but could not be detected at quantifiable levels in brain dialysate. Previous studies in mice receiving 10.0 mg/kg clozapine show that whole brain concentrations of clozapine were in the low μM range (Osterhaus et al., 2003) which is 1000-fold higher than

detectable limits found here. This suggests that the inability to detect clozapine was not due to low brain levels of clozapine, but rather to clozapine's absence from dialysate. This is supported by a study showing that clozapine is largely protein bound (Zhou et al., 2005) and thereby unavailable in brain dialysate.

The amphetamine metabolites were not detectable in all brain dialysate samples. NOR and OHNor were completely undetectable while OHAmp could be detected transiently and at levels near the lower limits of quantitation. Results of data that were obtained from OHAmp show that average levels may be increasing over the course of sensitization (figure 2-11). The involvement of OHAmp has been implicated in the behavioral augmentation associated with chronic amphetamine administration (Dougan et al., 1986, 1987). However, the physiological impact of the low levels found here is not likely to be relevant compared to the large behavioral changes that occur throughout the sensitization process. Kaminskas et al. (2002) showed that OHAmp levels found in the striatum were 10-fold lower than blood levels and suggested that such levels were not sufficient to have an impact on dopamine release. Another study by Simpson, (1980) reported that the rate of biotransformation of amphetamine into OHAmp in rats was slower than the rate of elimination. This would suggest that OHAmp (and perhaps NOR and OHNor) is cleared from the brain more rapidly than it is produced and is thereby present only at very low levels.

Hypothesis one is partially supported by experimental results from this study. All analytes tested are clearly quantifiable using the HPLC-tandem mass spectrometry method. Of these compounds only amphetamine provided useful data from brain extracellular fluid at a dose of 5.0 mg/kg. Clozapine is likely not detectable using this method. Modification of the chromatographic method might improve resolution of amphetamine metabolites and provide sufficient detection levels to monitor dopamine in brain dialysate. While success was achieved on some level using this system, it is apparent that additional development is required to maximize functionality.

Hypothesis #2:

Behavioral data obtained from rats with a microdialysis probe surgically implanted in the striatum and tethered to a microdialysis system are comparable to behavioral data previously obtained from untethered animals that did not undergo surgical probe implantation.

Results show that rats with a microdialysis probe surgically implanted in the striatum that were subjected to microdialysis testing had a comparable behavioral response in the force plate actometer as rats that were in an enclosed force plate actometer chamber that did not undergo microdialysis testing. Criteria for successful behavioral measures included the presence of a peak at about 10 Hz (representing stereotypical head movements) and

suppression of locomotor activity during the first 1-2 hours following amphetamine administration. These criteria were chosen since they are the basis for calculating focused stereotypy scores.

Previous results measuring behavior on the force plate actometer have shown that Sprague Dawley rats administered IP injections of 5.0 mg/kg amphetamine showed a 10 Hz peak that typically lasted into the second hour following treatment (figure 2-16, top). Suppression of locomotor activity typically occurs during this period of time while the animal is engaging in stereotypical head movements. Figure 2-16 (bottom) verifies that Sprague Dawley rats subjected to microdialysis testing expressed these behavioral attributes. Fischer 344 rats also show suppressed locomotor activity during the focused stereotypy phase. The 10 Hz peak that is characteristic of Sprague Dawley rats is broader and with lower amplitude in Fischer 344 animals. Figure 2-17 compares the results of Fischer 344 rats involved in this study that were undergoing microdialysis testing with data from rats that were not.

Scores of focused stereotypy were calculated and showed that sensitization took place for both rat strains (figures 2-18 and 2-19). Behavioral measures of animals receiving 10.0 mg/kg clozapine following amphetamine administration clearly showed characteristic lengthening of focused stereotypy duration and a transient decrease in focused stereotypy immediately following 10.0 mg/kg clozapine administration previously

reported in Sprague Dawley rats at this dose (Fowler et al., 2007). Figure 2-20 shows results from injection days 1, 2, 4 and 6 for all rats tested using the present method.

Overall, these data support hypothesis number two by showing that while some behavioral disruptions occurred throughout the session, the basic characteristics mentioned above are present in most graphs and for all animals. This indicates that surgical implantation of the probe and exposure to the microdialysis apparatus did not inexorably mask these characteristics.

Future directions for hypotheses 1 and 2

In this study, high resolution behavioral measures using the force plate actometer were combined with microdialysis to simultaneously measure behavior and brain neurochemistry. Measuring multiple analytes would improve the power of the current method. In the experiments presented here, the chromatographic and mass spectrometry instrument was shared and therefore not available for extensive exploratory method development. Further development of the chromatography and mass spectrometry methods introduced here may enhance detection of dopamine and the amphetamine metabolites to quantifiable levels. HPLC-mass spectrometry methods have recently been published for the determination of multiple neurotransmitters including dopamine, serotonin, norepinephrine, acetylcholine, and gamma-aminobutyric acid (GABA) achieving limits of detection in the femtomolar

range (Zhang et al., 2006; Hows et al., 2004). Monitoring drug levels and the neurochemical response to drugs via microdialysis while simultaneously measuring behavior could reveal which neurotransmitters are involved in the behavioral response. Improving detection limits would also allow smaller sample sizes leading to improved temporal resolution.

Electrochemical detection is a more accessible alternative to mass spectrometry. Detecting catecholamines in brain dialysate is routinely done using HPLC with electrochemical detection (Peaston and Weinkove, 2004). Though electrochemical detection would not provide quantification for the range of molecules potentially measurable by mass spectrometry, reliable methods are available with detection levels sufficient for monitoring dopamine and dopamine metabolites in the striatum.

The primary weaknesses of the behavioral testing method presented here were behavioral disruptions caused by the presence of the experimenter, and disruptions caused by malfunction of microdialysis equipment. Placing the actometer in a light and sound attenuating chamber would minimize disruptions caused by noise and activity from the surrounding environment. Such a chamber would require modifications in the form of access ports for tubing and the tethering mechanism plus greater accessibility for additional animal handling that is required for connecting the animal to the apparatus. Mechanical malfunctions have largely been eliminated through regular replacement of tubing and tubing connectors. The tethering

mechanism was modified such that weak connections have been replaced with more robust hardware and epoxy as required. Reducing restrictions on animal mobility imposed by the microdialysis harness and tethering system would improve behavioral data. The interplay between freedom of movement for the animal and maintaining instrument integrity limits the degree to which behavioral impairment can be completely alleviated. The integrity of the probe and tubing is paramount to successfully and reliably monitoring brain extracellular fluid.

Hypothesis #3: The heightened behavioral response of Fischer 344 rats to amphetamine compared to Sprague Dawley rats is due to higher levels of amphetamine in the striatum.

No significant differences were found between Fischer 344 and Sprague Dawley rats on measurements of percent elimination, elimination rate constant, and half life of amphetamine. Results of cmax for Fischer 344 rats indicate that maximum amphetamine concentrations after sensitization are elevated compared to maximum concentrations of Sprague Dawley rats (figure 3-6), however the difference failed to reach significance. Behavioral differences between these strains were confirmed in that sensitized Fischer 344 rats have an elevated duration of focused stereotypy compared to sensitized Sprague Dawley rats (figure 3-9). Suppression of locomotor activity as measured by scores of decimated distance and spatial

confinement was greater for Fischer 344 than Sprague Dawley rats (figure 3-8, “Decimated distance”, “Space Used”). Although dose effects curves were not available from this study, previous results showed a greater behavioral response throughout the sensitization process for Fischer 344 rats compared to Sprague Dawley rats receiving the same dose (figure 3-1).

These data indicate that hypothesis three was not supported based on the current experimental conditions. Because there were no differences in extracellular amphetamine pharmacokinetics in the striatum between F344 and Sprague Dawley rats, the discrepancy in behavioral response between these strains does not originate from brain amphetamine levels.

Hypothesis #4: Measured behavioral changes that occur throughout the sensitization process are due to changes in amphetamine pharmacokinetics.

Amphetamine pharmacokinetic analysis reveals that there are no significant differences between the first and sixth injection of a sensitizing series of injections on measures of percent elimination, elimination rate constant, half life, or maximum concentration. Behavioral sensitization was confirmed statistically for both rat strains by analyzing stereotypy scores at 4 time points representing 4 phases of amphetamine response during the 4-hour sessions (figure 3-9).

The lack of pharmacokinetic differences between injection one and injection six, coupled with significant behavioral differences indicate that a behavioral response occurred for both strains in the absence of significant amphetamine pharmacokinetic changes in the striatum. This finding does not support the hypothesis that amphetamine concentration and metabolism in the striatum play a direct role in the sensitization response of Fischer 344 and Sprague Dawley rats.

Hypothesis #5: There is no relation between the intensity and duration of the behavioral response to amphetamine and amphetamine concentration in the striatum.

Hypothesis five was based on previous studies suggesting that there is no simple quantitative relationship between relative extracellular dopamine concentrations and specific components of amphetamine induced behaviors (Kuczenski and Segal, 1994; Sharp et al., 1987). Amphetamine levels have been shown to closely mimic dopamine levels in the caudate putamen and nucleus accumbens (Kuczenski et al., 1997). Results from this lab suggest that during a single session, amphetamine induced behaviors persist or even increase during the period of time that dopamine and amphetamine brain levels are reportedly decreasing. This study shows that amphetamine levels in the striatum follow first order kinetics with a rapid absorption phase reaching maximum levels between 15 and 30 minutes (figure 3-2). Figure 3-

18 shows results of four 15-minute periods during the amphetamine elimination phase (top) and the associated stereotypy scores (bottom) for F344 and Sprague Dawley rats. It is evident from these graphs that scores of focused stereotypy do not follow the amphetamine elimination pattern. Focused stereotypy remains level (figure 3-18, Fischer 344) or increases to a peak level (figure 3-18, Sprague Dawley) during the time that amphetamine concentrations are sharply decreasing.

These findings support the hypothesis that amphetamine in the extracellular fluid of the striatum does not directly contribute to expressed behaviors over the course of a single session.

Future directions for hypotheses 3, 4, and 5

In behavioral pharmacology, accurate dosing is paramount to the accurate assessment of behavioral response. In this study intraperitoneal injections were exclusively used as the route of administration of drugs. As a result of the inherent variability of this method, a great deal of variation in drug concentration was seen between individual rats within a group. Unintended dosing variability could be largely eliminated by administering drug intravenously (IV). IV infusion could also be used to maintain constant drug levels and to alter drug levels throughout a session to monitor the behavioral and neurochemical response. Reverse microdialysis is a technique in which a substance is introduced into the extracellular space via the

microdialysis probe. This allows a predetermined concentration of a substance to be administered to a specific region. In addition to the local administration of the drug, this technique permits the simultaneous sampling of the extracellular levels of endogenous compounds. Accurately manipulating drug concentrations throughout a session while simultaneously measuring catecholamine release and the behavioral response could reveal how pharmacological interactions affect behavior.

The studies in this section only looked at amphetamine release in the striatum. Enhanced dopamine overflow in the nucleus accumbens (Vezina, 2004) and dopaminergic and glutamatergic interactions in the medial prefrontal cortex have also been implicated in amphetamine response and sensitization effects. Expanding the current method to include these regions could reveal the level to which these areas are directly involved. Placement of multiple probes could give simultaneous information about the neurchemical response and interaction of these regions.

Microdialysis is advantageous in that a wide variety of compounds can be collected and analyzed from a single sample. Temporal resolution of this technique depends on the required volume of analyte for effective analysis, and the sensitivity of the detection method. Commonly, the temporal resolution of microdialysis is more than 10 minutes between samples. Behavioral measures obtained from the force plate actometer have a resolution of 50 samples or more per second. When combining measures of

behavioral and neurochemical response, the sampling intervals required of microdialysis severely limits measurements of behavioral and neurochemical interactions. Cyclic voltammetry allows measurements of neuron firing and dopamine release at resolutions comparable to the force plate actometer (Greco et al., 2006). Using this method, the dopaminergic association with subtle behaviors observed within a session can be quantified.

Hypothesis #6: Clozapine metabolism is altered by concurrent administration of clozapine and amphetamine in rats.

Hypothesis six was based on previous reports showing that clozapine administration lengthened the duration of amphetamine induced focused stereotypy in rats (Fowler et al., 2007). Amphetamine is primarily metabolized by the CYP2D subfamily of cytochrome p450 enzymes. This enzyme has been reported to also have some level of involvement in clozapine metabolism raising the possibility of competitive interactions between amphetamine and clozapine. In this study sensitized rats were administered either 5.0 mg/kg amphetamine or vehicle followed 30 minutes later by 10.0 mg/kg clozapine. At 130 minutes following the first administration of either drug or vehicle, brains were removed and clozapine levels were tested. Results showed that whole brain clozapine levels in rats receiving amphetamine prior to clozapine were not statistically different from

rats receiving vehicle prior to clozapine. These data imply that the presence of amphetamine did not alter clozapine metabolism as was hypothesized.

Future directions for hypothesis 6

Clozapine levels were only measured at a single time point. To definitively determine whether there is a drug interaction, clozapine levels at multiple time points should be measured. Also, clozapine's effects on amphetamine kinetics were not measured. While several metabolic pathways including CYP2D are involved in clozapine metabolism, amphetamine is almost exclusively metabolized by the CYP2D enzyme. This implies that amphetamine metabolism may be altered by the presence of clozapine even though clozapine levels are not changed by amphetamine. Inhibited amphetamine elimination could result in the prolonged behavioral response found in this study and others. Using the microdialysis method developed in chapters 2 and 3, the effect of clozapine administration on amphetamine pharmacokinetics can be measured.

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