Characterization of Impulsive-Like Behavior Produced by Developmental Deltamethrin Exposure: Role of Dopaminergic Dysfunction

by

MICHELE MARIE TAYLOR

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ABSTRACT OF THE DISSERTATION

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Dissertation Director:

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Attention-deficit hyperactivity disorder (ADHD) is characterized by impulsivity, hyperactivity and attention deficits, all of which are thought to arise from catecholaminergic dysfunction. Recent studies suggest pesticide exposure may contribute to the incidence or severity of ADHD. Here, we provide evidence that pesticide exposure may be involved in the pathogenesis of the disorder. We hypothesized that developmental exposure of mice to deltamethrin (DM) would produce impulsive-like behavior through alterations in dopaminergic function. Implementing an operant behavioral paradigm, we demonstrated that DM causes impulsive-like behavior as evidenced by deficits in waiting behavior and the capacity to inhibit responding or to refrain from responding during delays in reinforcement that was ameliorated by treatment with the common ADHD therapeutic, methylphenidate (MPD). In correlation with these data, MPD was quantified in murine plasma and tissue via electron spray ionization/ion trap mass spectrometry. MPD was found at detectable levels in both matrices and remained so for at least 2 hrs, a time frame consistent with reversal by MPD of DM-

induced behavioral alterations. To determine the contribution of dopamine receptor subtypes D1 and D2 on impulsive-like behavior, we utilized a fixed-interval (FI) schedule of reinforcement together with dopamine receptor antagonist treatment. Developmental DM produced a response pattern similar to that of children diagnosed with ADHD. Once baseline performance was established, D1- and D2-receptor antagonists SCH 23390 and eticlopride (ETI) were administered. Each returned some of DM-induced FI performance alterations to control levels, suggesting a role for both subtypes. To determine whether there is a relationship between receptor density alterations and consequent behavioral dysfunction following developmental DM exposure, we used quantitative autoradiography to determine the density of the D1- and D2-like dopamine receptors in the striatum (STR). We found changes in D1 but not D2-like dopamine receptor density and homogenate binding as a result of DM-exposure. Since there has been a dramatic increase in the amount of pyrethroid pesticides used recently and exposure of pregnant women to pyrethroids has been confirmed, it is important to determine the mechanism by which they affect the dopamine system and whether pyrethroid exposure might contribute to the pathogenesis of ADHD.

DEDICATION

I am grateful to my parents, Cassandra Ann and Colin Gordon Taylor, for instilling in me a strong work ethic. Their steadfast support carried me through when quitting would have been the far easier path to pursue. I dedicate this dissertation to my two sons, Trevor Nathaniel and Colin Philip Johnston.

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ABBREVIATIONS

ADHD	Attention-Deficit Hyperactivity Disorder
DA	Dopamine
DAT	Dopamine transporter
DOPAC	3,4-dihydrophenylacetic acid
DM	Deltamethrin
D1	Dopamine receptor subtype 1
D2	Dopamine receptor subtype 2
ETI	Eticlopride
FC	Frontal cortex
FI	Fixed-interval
FR	Fixed-ratio
FRWAIT	Fixed-ratio wait for reward
GD	Gestation day
HVA	Homovanilic acid
IRT	Interresponse time
LONGWAIT	Longest wait time
MPD	Methylphenidate
NA	Nucleus accumbens
NAc	Nucleus accumbens core
PFC	Prefrontal cortex
PND	Postnatal day

PRP	Postreinforcement pause
RATE	Rate of responding
RESETS	Number of fixed-ratio component resets
RESPSR	Number of responses emitted per reinforcer
RR	Run rate
SPE	Solid phase extraction
SN	Substantia nigra
VTA	Ventral tegmental area
WAITSR	Number of reinforcers earned for waiting
WAIT-FRTIME	Ratio of wait time over time to complete a fixed ratio
5HT	Serotonin

CHAPTER 1:

GENERAL INTRODUCTION

Growing concern over the developmental neurotoxicity of pesticides has led the US Environmental Protection Agency (EPA) to restrict the application of many of the traditional pesticides used for home pest eradication. In turn, this has resulted in increased exposure of other pesticides such as pyrethroids (Power et al., 2007), which are often thought of as safe alternatives to some of the traditional classes of insecticides. Pyrethroid pesticides are synthetic derivatives of natural pyrethrum, a mixture of naturally occurring chemicals found in certain chrysanthemum flowers. Pyrethrum was first recognized as having insecticidal properties around 1800 in Asia when it was used to kill ticks and various insects such as fleas and mosquitoes (ATSDR, 2003). Six individual chemicals in the pyrethrum extract have active insecticidal properties and these compounds are called pyrethrins. The six pyrethrins are pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmolin II. Pyrethrins are often used in household insecticides and products to control insects on pets or livestock. Pyrethroids are manufactured chemicals that are very similar in structure to the pyrethrins, but are often more toxic to insects and to mammals, and last longer in the environment than pyrethrins (ATSDR, 2003). Over 1,000 synthetic analogs and derivatives of the pyrethrins have been developed, yet only around a dozen are commonly used in the United States. All pyrethroids have in common an acid moiety, a central ester bond, and an alcohol moiety. The acid moiety contains two chiral carbons, allowing for pyrethroids to exist as stereoisomers. Some compounds also contain a chiral carbon on the alcohol moiety, allowing for three chiral carbons and totaling eight distinct stereoenantiomers. Many of

the toxic effects of pyrethroids are stereospecific, implicating specific binding sites for each.

Formation of synthetic structural analogs to this basic structure are designed to maximize insecticidal potency and photostability but in doing so, may also result in pyrethroid activity to non-target species (Gammon, 1985; Gray, 1985; Naumann, 1998; Soderlund et al., 2002; Valentine, 1990; Vijverberg et al., 1990). A common modification to the basic structure is the addition of an α-cyano group triple bonded to the alcohol moiety. Addition of an α-cyano group is considered a watershed moment in the history of synthetic pyrethrin analogs due to their exceptional insecticidal properties which ushered in hundreds of similar modifications (Elliot et al., 1978; Gammon et al., 1981; Soderlund et al., 2002; Zerba, 1999). It has been shown that the α-cyano group increases, by an order of magnitude, the acute lethality in rodents (Lawrence et al., 1982; Soderlund et al., 2002; Valentine, 1990). Newer pyrethroid compounds such as deltamethrin, α-cypermethrin, binfenthrin and esfenvalerate, have higher concentrations of active stereoisomers and much greater oral LD50's in rats than the older pyrethroids (Elliot et al., 1978; Katsuda, 1999; Naumann, 1998; Soderlund et al., 2002). The toxic potency of newer pyrethroids has increased, allowing for less product to be applied to achieve adequate pest control, thereby reducing the hazard posed to non-target species.

Technical-grade pyrethroids are typically formulated with up to 99% inert ingredients that improve storing, application, handling, or effectiveness. The EPA maintains a list of all inert ingredients found in pesticide products and categorizes them according to four classes (1) inert ingredients of toxicological concern; (2) potentially toxic inert ingredients; (3) inert ingredients of unknown toxicity; and (4) inert ingredients

of minimal concern (EPA, 2003). It does not however, specify which ingredients are contained in any particular formulated product, nor does federal law require the pesticide label to list the inert ingredients unless they are class 1 inert ingredients. Pyrethroids are often formulated with synergists such as MGK-264, piperonyl butoxide, piperonyl sulfoxide or sesamex, which increase their effectiveness.

On August 3, 1996, 104th Congress enacted significant changes to the Federal Insecticide, Fungicide, and Rodentidice Act (FIFRA), governing the sale and use of pesticide products and the Federal Food, Drug, and Cosmetic Act (FFDCA), which limits pesticide residues on food. The Food Quality Protection Act (FQPA), enacted as public law 104-170, developed guidelines for evaluating exposure to pesticides as a class when they share a common mechanism of action (FQPA, 1996). It also requires that all potential routes of exposure such as inhalation, dietary ingestion, indirect ingestion and dermal absorption via food, air, water, pets, soil, and dust be considered (FQPA, 1996). There are about twenty pyrethroids that are currently registered for use in the United States, but less than twelve are frequently used (EPA, 1991). In 2008, ten pyrethrins, pyrethroids and synergists, all registered prior to 1984, underwent risk management Reregistration Eligibility Decisions (REDs) In 2010, EPA began reevaluating all pyrethrins, pyrethroids and synergists which were not subject to re-evaluation under the reregistration program. Deltamethrin (DM), a type II pyrethroid, has been registered for use on golf courses, ornamental gardens, lawns, pet collars and crops such as cotton, corn, cereals and soybeans (ATSDR, 2004). It is a colorless, odorless chemical with a molecular weight of 505.2. The melting point is 101-102º C and the partition coefficients

are 6.1, 1.5 x 10-8 and 1.2 x 10-4 (Log K_{ow}, vapor pressure [mm Hg at 25° C] and Henry's Law constant [atm-m3/mol at 25º C], respectively

PYRETHROID NOMENCLATURE AND MODE OF ACTION

Structure-activity relationship studies of several pyrethroids resulted in the characterization of two syndromes, type I and type II (structures of selected pyrethroids in each class are depicted in Figure 1) (Verschoyle et al., 1980; Verschoyle et al., 1972). Type I pyrethroids are pyrethrin derivatives that do not include a cyano group and may elicit aggressive sparring, increased sensitivity to external stimuli and fine tremor progressing to whole-body tremors and prostration (type I or T syndrome). Type II pyrethroids, on the other hand, are pyrethrin derivatives that include a cyano group (depicted in Figure 1 as a carbon atom triple bonded to a nitrogen atom) at the 3-phenoxybenzyl alcohol moiety. Type II pyrethroids may elicit pawing and burrowing, profuse salivation and coarse tremor which progresses to a sinuous writhing motion referred to as choreoathetosis (CS syndrome). This classification system is useful but refers to doses of pyrethroids that result in overt neurotoxicity, not low-dose or developmental neurotoxicity, and does not encompass all of the pyrethroids, some of which produce toxic signs that relate to both T and CS syndromes (Soderlund et al., 2002; Verschoyle et al., 1980). Therefore, it is questionable whether this classification system, based on very high (near-lethal) doses is relevant to human exposures.

The primary mode of action in both target (insects) and non-target (mammals) species is disruption of voltage-sensitive sodium channels (VSSC). Pyrethroids slow the opening (active state) and closing (inactive state) of VSSC's. The membrane potential

becomes hyperpolarized and smaller depolarizing events can cause the channel to open and once open, they remain open longer because delayed inactivation allows more sodium ions to pass through (Figure 2). Type II pyrethroids delay the inactivation of VSSC's longer than the type I. In fact, type II pyrethroids hold open the channel for such a protracted period of time and the membrane potential becomes so depolarized that an action potential is no longer possible. This is referred to as a depolarization-dependent block. Type I pyrethroids on the other hand, cause a repetitive firing pattern in which they open the channel only long enough to result in back to back firing of action potentials (repetitive discharge) (Shafer et al., 2005). The effects of type I and II pyrethroids on channel opening and closing are thought to be responsible for the differing effects elicited in the T and CS syndromes (Ray, 2001). Adding to the complexity, mammalian VSSC's are comprised of one α and two β subunits with myriad potential combinations possible ten α (Ogata et al., 2002) and four β (Isom, 2002) subunits have been identified that express in a tissue-, region-, and time-specific manner). This may influence the resultant impact of the two pyrethroid types of channel open and close times (Shafer et al., 2005).

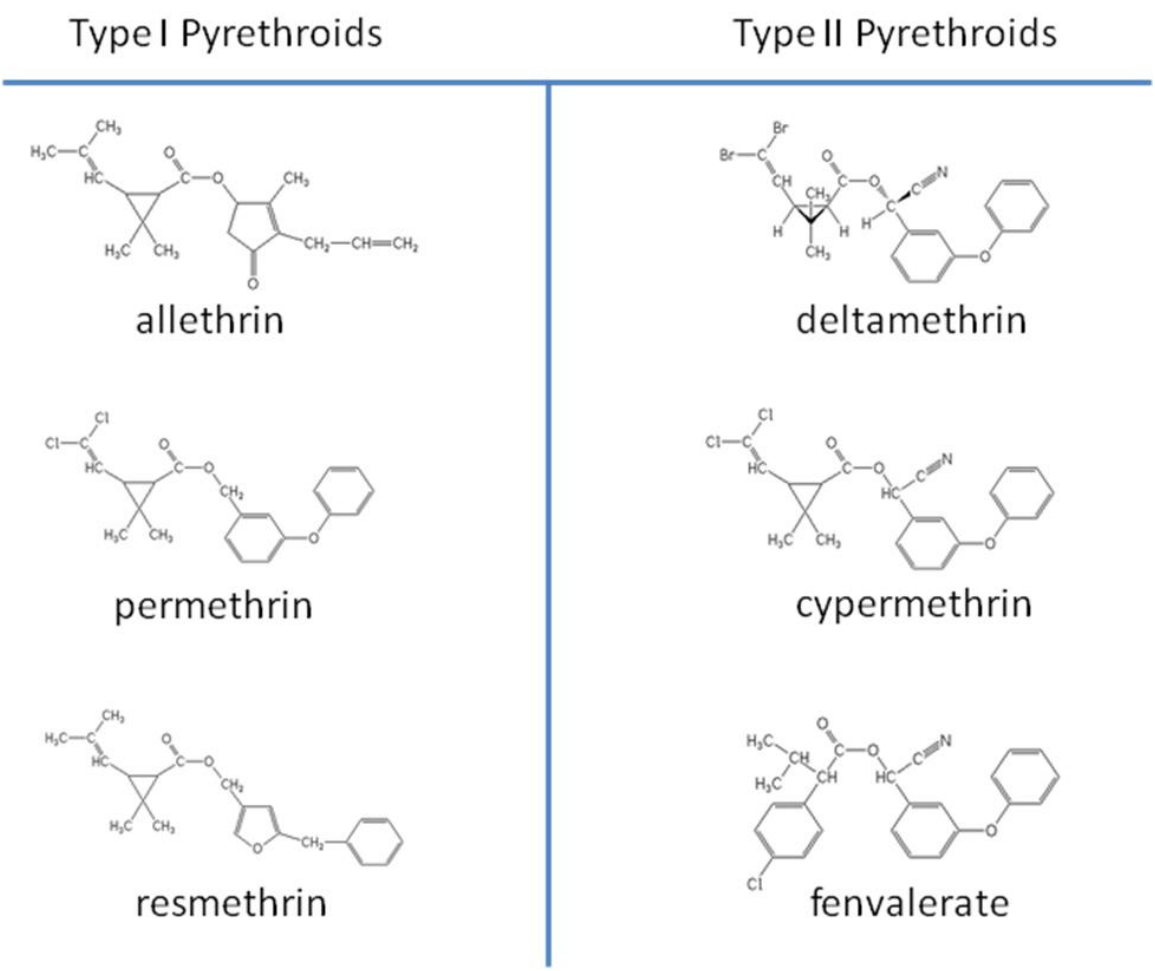

Figure 1. Chemical structures of selected Type I and II pyrethroids. Type I pyrethroids (left panel) do not have a cyano group and may elicit tremors. Type II pyrethroids (right panel) contain a cyano group and may elicit sinuous writhing (choreoathetosis) and excessive salivation.

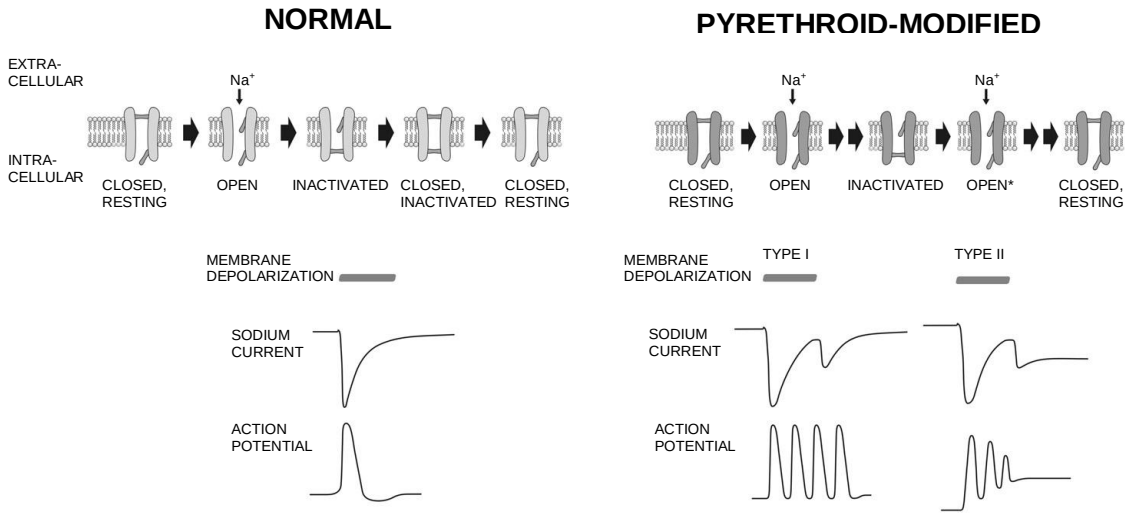

Figure 2. Impact of pyrethroids on sodium channels. Depicted are sodium channels, whole cell currents and action potentials under normal and pyrethroid-modified conditions (Shafer et al., 2005).

DEVELOPMENTAL TOXICITY OF PYRETHROIDS

It has been suggested that sensitivity to pyrethroids is age-related to a greater degree than other pesticide classes (Shafer et al., 2005). The acute lethality of DM and cypermethrin is 16- and 17-fold higher, respectively, in neonatal than in adult rats which is possibly due to lower metabolic capacity (Sheets, 2000). Pretreatment of young rats aged 8, 16 and 21 days with piperonyl butoxide (a monooxygenase inhibitor) or tri-o-totyl-phosphate (an esterase inhibitor) does not alter the lethality of either cypermethrin or DM (Cantalamessa, 1993). However, the lethality of both pyrethroids increased significantly in adult rats pretreated with the esterase but not the monooxygenase inhibitor. It was concluded that neonatal rats are more sensitive to pyrethroid toxicity as a result of incomplete development of the liver enzymes responsible for catalyzing their metabolism and that ester hydrolysis of pyrethroids is likely responsible for detoxification by the adult liver (Cantalamessa, 1993). Cismethrin and permethrin (both type I pyrethroids) did not produce age-dependent toxicity (Sheets, 2000). Age-dependent sensitivity to pyrethroids may also be related to dose, given that it only manifests at high doses. Acute oral doses of type I (cismethrin and permethrin) and type II (DM and cypermethrin) pyrethroids were administered to both adults and neonates to either a lethal or low dose to determine whether neonates are more sensitive, and if so, whether existing acceptable residue levels provide adequate protection (Sheets, 2000). There was no evidence that pups were more sensitive than the adults either at lethal or low doses of the type I pyrethroids. However, the pups were more sensitive to the lethal dose of both DM and cypermethrin (Sheets, 2000). Whole-brain levels of DM indicate that immature detoxification systems are overwhelmed at high doses. It was argued that

young animals are not more sensitive than adults to pyrethroids so existing tolerance levels are sufficient and no additional uncertainty factor is necessary. Such data are useful to inform decisions related to the FQPA and additional studies which compare young and adult animals for potential age-dependent sensitivities only strengthen these decisions (Shafer et al., 2005).

Shafer et al (2005), reviewed the available developmental pyrethroid effects literature and summarized test compound, species, dosing regimen, vehicle and major findings. Allethrin (d-allethrin, bioallehtrin, and S-bioallethrin) and permetrin were the only type I pyrethroids that were subject to peer review as having potential developmental neurotoxic action. The published reports from developmental studies for type II pyrethroids included cypermethrin, fenvalerate, cyhalothrin and DM. Rodents were the only animal models examined (13 studies using rats; 9 studies using mice). Rodents are the typical subject of choice in order to either compare within laboratory historical controls or to directory replicate the findings of another laboratory. More recent studies have examined the developmental effects of pyrethroids in zebrafish (DeMicco et al., 2010; Gu et al., 2010; Jin et al., 2010; Jin et al., 2011; Xu et al., 2010). Still, very few studies to date have examined the developmental neurotoxic effects of pyrethroids.

Persistent neurochemical and behavioral changes following developmental pyrethroid exposure have been reported. Most studies examining neurochemical changes following developmental pyrethroid exposure have focused on the muscarinic acetylcholine receptor (reviewed by Shafer et al., 2005) but the catecholaminergic system has also been investigated. For instance, increased adult striatal 3,4-

dihydroxyphenylacetic acid (DOPAC) levels have been reported following developmental DM (Lazarini et al., 2001) but not a fenvalerate-containing commercial product (Moniz et al., 1999). Increased striatal ^{3}H-spiroperidol binding on PND21 was found following exposure to fenvalerate, but only when the exposure occurred gestationally. When the exposure occurred during lactation, binding decreased. These data suggest that the dopamine system may be perturbed following developmental pyrethroid exposure. However, the data so far lack consistency.

The behavioral data have been equally inconsistent. Mice postnatally exposed to pyrethroids from PND10-16 exhibit increased motor activity and failure to habituate (Ahibom et al., 1994; Eriksson et al., 1991; Eriksson et al., 1993; Eriksson et al., 1990). However, Muhammed and Ray (unpublished data, reviewed by Shafer, et al., 2005) yielded motor activity increases in some but not all cohorts tested. Gestational exposure to DM from GD5-21 resulted in decreased motor activity (Husain et al., 1992) but when exposure occurred postnatally (PND22-37), spontaneous locomotor activity increased (Husain et al., 1994). Lazarini et al. (2001) found locomotor behavior in an open field test did not change but decreased immobility time in a forced swim test following gestational DM exposure (GD5-15). Briefly, rats were subjected to two trials during which they were placed in inescapable acrylic cylinders that were filled with water. Immobility time (floating) is recorded during the second trial. The forced swim test is commonly used to test antidepressant efficacy as they decrease immobility time. Developmental DM treatment did not alter open field locomotor activity but did decrease immobility time in the forced swim test which suggests there was not a locomotor impairment per se but increases anxiety (Lazarini et al., 2001). Exposure to fenvalerate

(type II) during GD18 and PND2-5 did not impact open field activity or avoidance behavior (Gomes et al., 1991). There are several limitations of these experiments that may explain these discrepancies. For instance, several studies did not maintain the litter as the unit of measure; using more than one pup from a litter and thereby increasing the probability of a type I error (rejecting a null hypothesis when, in fact, it's true (Aziz et al., 2001; Gupta et al., 1999; Husain et al., 1992; Imamura et al., 2002). Several studies used technical grade pyrethroids (Eriksson et al., 1990; Gupta et al., 1999; Imamura et al., 2002) while others used commercial mixtures (Aziz et al., 2001; Gupta et al., 1999; Husain et al., 1992). It has also been demonstrated that the vehicle and route of exposure can impact the effects of DM on motor activity (Crofton et al., 1995). The timing of exposure (gestational versus lactational) may also contribute to the inconsistencies. Future research should use a systematic approach which facilitates comparisons across laboratories.

Given the lack of data describing the developmental neurotoxicity of pyrethroids, consideration must given when designing future studies to address several data needs. Shafer et al. (2005) suggested developing a biologically-based dose-response (BBDR) model similar to the one used for the developmental neurotoxicity of perchlorate. BBDR's are a risk assessment tool used to elucidate the chemicals' mode of action and the tissue dosimetry (Andersen et al., 2001). If the mode of pyrethroid action in an animal model is shown to also be operative in humans, extrapolations from animals to humans are strengthened (Cohen et al., 2004; Meek et al., 2003; Sonich-Mullin et al., 2001). Two important components of BBDR's are (1) physiologically based pharmacokinetic (PBPK) models, which connect target tissue doses with exposure levels

(Andersen et al., 2001) and (2) physiologically based pharmacodynamic (PBPD) models, which quantitatively elucidate the mode of action (Andersen et al., 2001; Conolly, 2002). The implementation of PBPD models may elucidate data gaps by identifying adverse outcomes (alterations in motor activity) and linking them to specific mode of actions (disruption in ion channel function). In addition to pharmacokinetic and pharmacodynamic data from animal models, more information regarding human exposure is needed. Most of the human exposure data were obtained from studies conducted in Germany of applicators in agriculture, greenhouse and indoor home applications (Berger-Preiss et al., 2002; Hardt et al., 2003; Leng et al., 2003). The authors of the German studies suggest that exposure to pyrethroids in the general population is through the diet (Heudorf et al., 2001; Schettgen et al., 2002) but no direct evidence supported this conclusion. Lu et al. (2006) concluded that exposure to children is likely not through the diet but from residential application of pyrethroids. They collected urinary samples and household pesticide use information of children in Seattle, Washington. During the first phase of the study, the children consumed conventional diets exclusively. During the second phase of the study, they exchanged the conventional food items with organic ones. Detectable levels of all five pyrethroid metabolites were found. The most frequently detected metabolite was 3-PBA, followed by trans- and cis-DCCA, FPBA and DBCA. Very few samples had detectable levels of DBCA (the DM-specific metabolite). Introducing an all organic diet did not impact metabolite levels but did correspond to self-reported residential pyrethroid use. This data suggests that pyrethroid exposure is not due to diet alone.

Relatively few studies have evaluated the potential developmental neurotoxicity of pyrethroids. However, it has been demonstrated that pyrethroid pesticides, such as DM, has an acute oral LD50 of 12mg/kg in weanling rats compared to 80mg/kg in adult rats (Sheets, 2000). In terms of DM exposure, the margin of exposure (MOE) has been calculated by dividing estimated human exposure levels by the no observed effect level (NOEL). The NOEL for acute dietary exposure (of 1 day or single exposure) is 1mg/kg/day and the estimated exposure (99^{th} percentile) in the U.S. is 0.000728mg/kg/day, resulting in a calculated MOE of 1,373. For children aged 1-6 years old, the estimated exposure level is 0.001855mg/kg/day resulting in a calculated MOE of 539. Both MOE's are greater than 100 and the EPA states there is no cause for concern if total exposure calculated for the 99^{th} percentile yields an MOE equal to or greater than 100. Significant levels of the pyrethroid metabolite 3-phenoxybenzoic acid (3-PBA) have been detected in the urine of pregnant women, infants and children (Berkowitz et al., 2003; Heudorf et al., 2004; Schettgen et al., 2002). While, PBA is a possible metabolite of numerous pyrethroid pesticides such as permethrin, cypermethrin and sumithrin, it does not confirm exposure to any particular pyrethroid. (Berkowitz et al., 2003). However, the detection of 3-PBA in urinary samples in a New York City cohort of pregnant women was notable given the fast clearance rate of pyrethroids. In addition, it correlated well with self-reported use of pyrethroid pesticides and may also reflect the widespread use of sumithrin to combat West Nile Virus in New York City during the same timeframe (Health, 2002).

PYRETHROIDS AND DOPAMINE FUNCTION

Research has shown that DM alters catecholamine, and in particular, dopamine function. For instance, in vivo exposure of mice to DM results in robust increases (70%) in maximal DA uptake (Kirby et al., 1999) and DAT levels (Elwan et al., 2006). Increased DA release in the striatum of freely moving rats acutely treated with DM has also been reported (Hossain et al., 2006). Male and female offspring of rats exposed prenatally to DM had higher striatal levels of DA (females only) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Since there has been a recent dramatic increase in the amount of pyrethroid pesticides used and exposure of pregnant women to pyrethroids has been confirmed (Whyatt et al., 2003; Whyatt et al., 2007), it is important to determine the mechanism by which pyrethroids affect the dopamine system.

There are three major catecholamine pathways in the brain of behavioral relevance. These include the: 1) mesolimbic (reward center), 2) nigrostriatal (motor function) 3) and mesocortical (attention and memory) systems. Cell bodies of the mesolimbic system originate in the ventral tegmental area (VTA) then project to the nucleus accumbens (NA), the amygdala, the septal area and the hippocampus. The NA is referred to as the brains' reward region and is believed to be responsible for the rewarding effects of alcohol and drugs (Cooper et al., 2003). The septal area and the hippocampus are involved in spatial learning and memory formation (Kandel, 2000). The nigrostriatal system originates in the substantia nigra (SN) and its axons project to the striatum, which is composed of two structures: the caudate and the putamen (Kandel, 2000). This system controls motor function (Jucaite et al., 2005). The mesocortical

system has cell bodies in the VTA as well; however, this pathway terminates in the prefrontal cortex. These neurons have an excitatory effect on the frontal cortex and affect formation of short-term memory, planning and problem solving. Thus, the mesocortical pathway is critical to complex cognitive function. The mesolimbic and mesocortical systems work in concert (mesocorticolimbic system) to initiate and maintain goal directed and reward mediated behaviors, including cognitive sets (Kandel, 2000).

Mechanistically, the mesolimbic dopamine system appears to play an integral role in the mediation of impulsive behavior. Selective lesions to the nucleus accumbens core induce persistent impulsive choice in rats (Cardinal et al., 2001). Prior to lesioning, the rats were given a choice between a small, immediate and large, delayed reward and there was no difference in choice between the groups. Then, one group of rats was lesioned in the nucleus accumbens core, the anterior cingulated cortex and medial prefrontal cortex. Lesions in the nucleus accumbens core produced a robust and persistent deficit in the rats' ability to choose the delayed reinforcer compared to the sham lesioned group (Cardinal et al., 2001). Although the dopamine system was not specifically targeted in this lesion study, the effects observed may have a dopaminergic origin. Indeed, it has been shown that microinjection of N-ethyoxycarbonyl-2-ethoxy-1,2-dihydroquinline (EEDQ), a non-specific dopamine antagonist, into the nucleus accumbens produces a dramatic reduction in fixed interval (FI) response rates (Cory-Slechta et al., 1998; Cory-Slechta et al., 1997). The rate of responding increases 2-3 days following the injection. This timeframe is consistent with the turnover of dopamine receptor proteins, suggesting a role of the dopamine system in FI rate reduction (Cory-Slechta et al., 1998; Cory-Slechta et al., 1997). To further test the role of dopamine in FI rate reduction, dopamine

was injected into the same region. They found increased FI response rates at low doses and decreased response rates at higher doses, which further suggested that dopamine activity in the accumbens is intricately involved in FI performance and is implicated in impulsive-like behavior. These effects appear to be selective for the nucleus accumbens in that EEDQ injected into the dorsomedial striatum did not impact FI rates (Cory-Slechta et al., 1997). Administration of D1 and D2 dopamine receptor antagonists (SCH23390 and eticlopride respectively) both prevented the effects of EEDQ when administered to the nucleus accumbens but not the striatum (Cory-Slechta et al., 1997). In contrast, impulsive-like behavior of rats performing on a delayed reward task increased following SCH23390 but not eticlopride (van Gaalen et al., 2006). Amphetamine-induced reductions in impulsivity were attenuated by pretreatment with eticlopride but not SCH23390 (van Gaalen et al., 2006) in these rats. These data further implicate a role for dopamine in the mediation of impulsive-like behavior, particularly in the nucleus accumbens region. Thus, environmental factors that alter the proper functioning of the mesolimbic dopamine system may lead to impulsive-like behaviors.

In a pilot experiment, conducted in our lab, mice developmentally exposed to low levels of DM exhibited increased rate of responding on an FI schedule of reinforcement (Fig. 3). These effects demonstrated a gender effect as male but not female response rates increased.

Research Objectives

This proposal seeks to characterize the behavioral effects of developmental DM exposure and examine potential mechanisms responsible for these behavioral effects. Preliminary data from this laboratory have demonstrated that mice exposed during development to low levels of the pyrethroid pesticide DM exhibit elevated DAT levels, hyperactivity which is reduced by methylphenidate, and behavioral deficits in the Y-maze. Epidemiological studies also found that children with elevated levels of pyrethroid metabolites in their urine were more than twice as likely to be diagnosed with ADHD, which is characterized by the behavioral effects observed in mice developmentally exposed to DM. Based on these findings, we hypothesized that developmental exposure to DM produced hyperlocomotion and impulsive-like behavior through alterations in dopaminergic function. The specific aims of this research were to:

(1) determine the contribution of dopamine receptor subtypes on hyperactivity and impulsive-like behavior in mice developmentally exposed to DM

(2) determine the neuroanatomical location and DM-induced changes in dopamine transporter and receptor levels

(3) determine whether perinatal DM exposure in mice results in impulsive-like behavior that is ameliorated by methylphenidate treatment

(4) characterize the pharmacokinetic profile of methylphenidate in murine tissue

CHAPTER 2:

MATERIALS AND METHODS

Materials

Adult C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, MA). Analytical grade (purity ≥ 99%) deltamethrin (Lot #371-38A) was obtained from ChemService Inc. (West Chester, PA) and stored in canisters within a drawer to prevent exposure to UV light. SCH 23390 and eticlopride were obtained from Sigma (purity ≥ 98%). Methylphenidate hydrochloride (purity ≥ 99%) (Ritalin®) (Lot #026K1133) was purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich. The radioligands for DA D1- and D2-like receptors ([^{3}H]-SCH2390 and [^{3}H]-YM 09151-2 respectively) and DAT ([^{3}H]-WIN 35,428) were purchased from NEN Life Science Products (Boston, MA).

Developmental Exposure

During mating, one male was housed in a cage with two females. Gestation day 0 (GD0) was determined by the presence of a vaginal plug. Each dam was then given 0 (n=8) or 3mg/kg DM (n=11) every 3 days from GD0 to weaning on postnatal day 22 (PND22). DM was dissolved in corn oil and 1ul/g body weight was mixed with peanut butter and given to the dams only. Control dams received unadulterated peanut butter. This method was chosen over oral gavage in order to avoid stress to the dam during dosing.

Fixed-Interval

Behavioral testing was conducted in operant chambers (Med Associates, Inc., St. Albans, VT) that were housed in light-and sound-attenuated enclosures equipped with fans for ventilation. Each of two response levers was mounted on the front wall of the operant chamber 2.2cm above the grid floor. A force requirement of 2 g was required to break a photobeam, thereby recording a response. Only left lever responses produced reinforcement; responses on the right lever were recorded but were of no programmed consequence. Food reinforcers (20 mg food pellets, Bio-Serv, Frenchtown, NJ) were delivered via a food trough that was centered between the two levers (2.5cm apart). Mice were trained to press one of two levers in an operant chamber via an autoshape program. All of the mice acquired the lever press response by the third day. No treatment related effects in shaping were observed. Next, a FI 60-sec schedule of reinforcement was implemented under which a 20 mg food pellet was delivered following the first lever press occurring after at least 60-sec had elapsed. Lever presses emitted prior to the completion of 60-sec were of no programmed consequences. Behavioral sessions were conducted between 9:00am and 5:00pm, 7 days a week, and were 20min in duration.

Prior to antagonist testing, mice were acclimated to intraperitoneal (i.p.) injections. Saline was administered via i.p. injection immediately following baseline sessions 31-35. All subsequent injections were administered 15 min prior to behavioral testing. Each dose of antagonist was preceded by: 1) a rest day (on which no injection was given) and 2) a saline day (on which saline was administered). Thus, each drug administration was separated by two intervening days. SCH 23390 was administered to male (0, .0125, .025, .0375mg/kg) and female (0, .00625, .0125, .025mg/kg) mice via i.p.

injection 15 min prior to behavioral testing. It was determined during testing that .025mg/kg would be the highest dose tested in the female mice and that .00625mg/kg SCH 23390 would be administered as the low dose. This was based on the observation that .025mg/kg SCH 23390 resulted in a 70% decrease in rate and a 771% increase in IRT in the controls. Males exposed to the same dose had a 57% decrease and 110% increase in control rate and IRT respectively (data not shown). It should be noted that, in general, the female mice were more sensitive than the males to the injection process and became increasingly difficult to handle throughout the remainder of the experiment. The D2 antagonist eticlopride (0, .025, .05 and .1mg/kg) was administered to both male and female mice 15 min prior to behavioral testing.

Autoradiography

Adult male offspring (aged 18-25 weeks) were decapitated and brains excised and immediately frozen in powdered dry ice and stored at -80 °C until sectioning for determination of D1 and D2-like dopamine receptors by autoradiography (n= 7 for control and DM). The brains were sectioned at 10um on a cryostat set at -20°C. Cortical sections from the prefrontal cortex (1.94mm from Bregma to include infralimbic, cingulate, and prelimbic cortices), striatum (1.10mm from Bregma to include nucleus accumbens and dorsal striatum), and midbrain (-2.92mm from Bregma to include substantia nigra and ventral tegmental area) were mounted on pig gelatin subbed glass slides and stored at -80 °C until assay. Two to three sections were mounted per slide and on average, nine slides were prepared for each region. For determination of dopamine receptor levels, we used the D1-like receptor antagonist 3H-SCH 23390 and the D2-like

receptor antagonist 3H-YM 90151-2 according to the method of Richfield et al. (1989). The slides were slowly thawed from -80˚ to room temperature. The incubation buffers for D1 (25mM Tris [pH 7.5], 100mM NaCl, 1mM $MgCl_2$) and D2 (25mM Tris [pH 7.5], 120mM NaCl, 1mM MgCl) were prepared; half was kept at 25˚C (warm buffer used during incubation) and the other half at 4˚C (cold buffer used for post incubation wash). Immediately prior to assay, ascorbate was added to both warm and cold buffers. Tritiated antagonists were then added to the warm buffer and counted by aliquoting 100uL of tritiated buffer into 3mL of scintillation fluid and measuring on a scintillation counter. Once the buffer was counted and desired concentrations were achieved, it was divided into three separate glass dishes (about 150mL each). Blank, non-tritiated buffer (containing fluphenazine [3uM] and sulpiride [10uM] for D1 and D2 respectively) was prepared and placed in a fourth, separate, dish. Slides were incubated until equilibrium (150min for DA D1-like and 240min for DA D2-like). All incubations were conducted at a concentration of tritiated ligand equal to the K_d of that ligand for the specific receptor in the rat striatum (0.55nM for DA D1-like and 0.1nM for DA D2-like) (Richfield, 1987). Following incubation, the slides were washed (10min for DA D1-like and 60min for DA D2-like) in cold buffer, then dried using a cool stream of air. For autoradiographical analysis, dried slides were put in X-ray cassettes with 14C-labeled plastic standards calibrated with tritiated brain paste sections. The slides and standards were exposed to Kodak Biomax MS film for 1 week and the film developed in Kodak D19 for 3min then fixed with Kodak rapid fix for 3.5min. To assist in verification of structures, the Franklin and Paxinos mouse brain atlas was used. All quantitative binding data were determined directly from film densities, utilizing a video-based image analysis system (MCID Elite,

Imaging Research Inc., Ontario, Canada). Total binding was determined by averaging together two adjacent sections per subject. Nonspecific binding accounted for <10% of the total binding.

Homogenate Binding

D1-like and D2-like dopamine receptor levels were determined by binding of the specific antagonist ^{3}H-SCH23390 and ^{3}H-YM-09151-2 essentially as described by Schulz and co-workers (2007) and Niznik and co-workers (2003), respectively, with modifications to reduce the assay volume to 200 µl. Striatal tissue samples were homogenized in 50mM Tris-HCl containing 150 mM NaCl and 5mM KCl with a glass mortar using a Wheaton motorized tissue grinder and a Teflon pestle. Homogenates were centrifuged at 48,000 x g for 10 minutes and the supernatant discarded. The pellet was resuspended in the same buffer by homogenization and washed twice more by centrifugation to yield crude membrane preparations. Binding assays were conducted with a single concentration of 1 nM for 1 hr at 25^{o}C. Non-specific binding was determined by the inclusion of 10 µM cold SCH23390 or eticlopride for D1 and D2 assays, respectively. For all binding assays, incubations were terminated by rapid vacuum filtration onto GF/B filter plates and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the total binding (no unlabeled antagonist included) minus non-specific binding (unlabeled antagonist included), and expressed as fmol/mg protein.

Fixed-Ratio Waiting-for-Reward

Behavioral testing was conducted in operant chambers (Med Associates, Inc., St. Albans, VT) that were housed in light-and sound-attenuated enclosures equipped with fans for ventilation. Each of two response levers were mounted on the front wall of the operant chamber 2.2cm above the grid floor. A force requirement of 2 g was required to break a photobeam, thereby recording a response. Only left lever responses produced reinforcement; responses on the right lever were recorded but were of no consequence. Food reinforcers (20mg food pellets, Bio-Serv, Frenchtown, NJ) were delivered via a food trough that was centered between the two levers (2.5cm apart). A Dell Optiplex GX1 computer was programmed with Med State Notation to control behavioral contingencies and data collection.

Male and female offspring of mice exposed to 0 or 3mg/kg DM were trained to press one of two levers in an operant chamber for 45min a day until the lever press response had been acquired. Once responding reliably, the mice were transitioned to a fixed-ratio 1 (FR1) schedule of reinforcement, in other words, every lever press resulted in pellet delivery. The response requirement was gradually incremented according to daily performance. Once all of the mice reached asymptote (predetermined based on pilot data as FR25), a FR waiting-for-reward paradigm was implemented. It provided free food deliveries after the completion of a FR25, with the time between deliveries systematically increasing after each successive food delivery. The starting time interval was 5 seconds and increased by 5 seconds following each free food delivery. Any intervening response reset the FR requirement.

Response rates gradually increased across the initial sessions. The base value during the initial 3 sessions was 2 sec, which incremented every 2 seconds, a value which generated "free" pellets at a rate that exceeded the subjects' retrieval and consumption rate. Consequently, pellets began to accumulate within the dispenser, clogging it. We returned to an FR only schedule to return to baseline behavior. We conducted an additional FRWAIT session with a 2 sec base wait that incremented every 2 sec. It was apparent that the schedule parameters needed adjusting so that fewer pellets were delivered. Therefore, the base wait value was increased from 2 sec to 5 sec. During the next 11 sessions, the mice were tested on an FRWAIT 5sec schedule that increment by 3 sec and the session length was reduced from 30 to 20 min. This generated a stable baseline. However, the increment value still generated too many pellets, and the mice became satiated prior to the end of the session. Thus, the 14 final baseline sessions were run under a 20 min FRWAIT 5sec schedule which incremented every 5 sec.

Pharmacokinetics of MPD

142 C57BL/6J mice (71 male/71 female) were used to assess MPD across time and dose. For the time course, mice were exposed to either 4 or 8mg/kg MPD via a single i.p. injection and sacrificed 10, 30, 90 or 120min later. These doses were chosen based on our previous data showing their effective reversal of impulsive-like behavior in mice. In the dose response group, mice were exposed to either 4 or 8mg/kg MPD, then killed 30min later (this timeframe corresponds to the time during which mice were exposed during behavior test sessions).

All mice were sedated using $C0_2$, and blood was collected by cardiac puncture with a 1cc disposable syringe affixed with a 26 gauge needle. Blood was immediately transferred to Vaccutainer tubes coated with Potassium Oxolate and Sodium Fluoride to prevent clotting and preserve the MPD, then spun 3500rpm for 15min. Resulting plasma was then transferred to 2mL polypropylene tubes and stored at -80º C until analysis. The mice were then decapitated and frontal cortex and striatum were removed.

Plasma (100uL) was thawed to room temperature (RT) and mixed by brief centrifugation. Internal standard (40µL phenacetin in methanol) was added to 100µL of plasma and vortexed. An 0.9mL volume of 3% aqueous acetic acid in water (v/v) was added by vortex mixing. To brain tissue, 50µL of 3% acetic acid was added and homogenized. Tissue homogenate was spun down at 14,000g at 4°C for 15min then transferred to clean 1.5mL centrifuge tubes. To the resultant pellet, an additional 400µL of 3% acetic acid was added then vortexed. Internal standard was added to each sample then vortexed. The resultant plasma and tissue samples were then applied to an Oasis HLB cartridge (30mg, 1mL, Waters Inc., Milford, MA, USA). Prior to use, the solid phase extraction (SPE) cartridges were equilibrated with 1mL of methanol and 1mL of 2% aqueous acetic acid. After the diluted plasma sample was applied, the SPE cartridge was washed with 0.5mL of 5% methanol/2% acetic acid, then 0.5mL of water. The methanol/5% acetic acid followed by 0.5mL of methanol and evaporated to dryness using a heated centrifugal evaporator (Speed-Vac, Thermo Scientific ISS 110). Dried samples were reconstituted in HPLC vials with 200µL of mobile phase (0.1% formic acid in water:0.1% formic acid in methanol).

Samples were added to a Thermo Finnnigan LCQ Deca (San Jose, CA) with LC pump Spectra System P4000, auto sampler Spectra System AS300 via LC with a Gemini HPLC C18 column (100 x 4.6mm, 5um particle size (Phenomenex, Torrence, CA). Chromatography was performed with isocratic elution using 50% methanol containing 0.1% formic acid and 50% 0.1% aqueous formic acid, v/v. The flow rate for all analyses was 0.3mL/min and the entire effluent was introduced into the electrospray ionization probe. Aliquots of 25µL were analyzed using a fixed volume loop. Instrument conditions were optimized for the detection of MPD using standard solution (1 µg/ml) introduced by infusion at a flow rate of 3 µl/min together with HPLC eluent at flow rate 0.3 ml/min. The instrument was auto-tuned before analysis of each sample set with values for capillary voltage, tube lens and sheath gas flow changed when necessary during auto-tuning. In order to maintain effective ionization spray, sheath gas flow was 1.425 L/min while the spray voltage was maintained at 5kV. The offset voltage on the ion transfer capillary tube was set to 35V in the positive electrospray ionization mode. The tube lens offset was set to 40V. The heated capillary was set at 260 °C. These ESI parameters were determined to maximize ion fragmentation of the MPD molecules within the spray cone.

The MS responses were determined in 100uL aliquots of plasma spiked with varying amounts of methylphenidate and ritalinic acid (0-20,000pg) and a constant amount of phenacetin (40,000pg) and the response ratio was computed. Quantitation was done using Selective Reaction Monitoring (SRM). The MS^2 spectra were obtained using the settings detailed in Table 3.

Quantitation was based on integrated peak area ratios MPD/IS and Ritalinic Acid/IS. Integration was performed using Xcalibur software, ICIS peak integration with 15 point smoothing, the minimum peak height for detection a signal to noise (S/N) ratio of 3. Calibration curves were generated by plotting the peak areas against the amount of analytes. The curve was fit using a linear regression algorithm using 1/x as a weighting factor.

Statistical Analyses

The following FI performance measures were determined for each subject: (1) rate of responding or rate (total number of responses divided by the total session time; (2) PRP (the time between delivery of a food pellet and the emission of the next response; (3) RR (the total rate of responding minus the PRP; and (4) IRT (the time elapsed between responses). To determine the extent to which developmental DM exposure altered FI performance, each of these dependent measures were calculated across blocks of 5 sessions, resulting in 7 blocks of 5 sessions each. The means of the resultant 7 blocks were analyzed by repeated measures analysis of variance (RMANOVA) with DM exposure and gender as a between-group factors and session blocks as a within-group factor. When RMANOVA revealed statistically significant results, Bonferroni post-hoc analyses were performed to further elaborate group differences. To determine the impact of D1- and D2-antagonist treatment, two-way ANOVAs were conducted with DM as a between-group factor and antagonist dose as a within-group factor. The impact of acute eticlopride and SCH 23390 exposure on DM-induced FI alterations was compared to each respective saline control value, which was determined by averaging the three

intervening saline days between each dose of both antagonists. When two-way ANOVA yielded a statistically significant main effect ($p < 0.05$), Bonferonni's correction was applied using Prism 5.0, GraphPad Software, Inc. The Bonferonni correction lowered the significance value to .05 divided by the number of comparisons (or dose of antagonist). All analyses were carried out separately for each FI performance measure.

FR response rates were determined by dividing the total time spent in the FR component and the total number of responses. The mean longest time to wait for a "free" pellet (WAIT) was calculated as the mean longest time a subject would wait between "free" pellet deliveries. Responses per reinforcer (RESPSR) were derived by dividing the total number of responses by the total number of reinforcers earned in both components and was used as a measure of efficiency. Fourteen baseline sessions were conducted and analyzed by repeated measures analysis of variance (RMANOVA), with treatment and gender as between-group factors and session as a within-group factor. The effect of MPD challenge on DM-induced changes in FRWAIT performance was analyzed by two-way analysis of variance (ANOVA). When ANOVA yielded statistically significant effects and the data met normal distribution and equal variance assumptions, Dunnett's multiple comparison tests ($p < 0.05$) were used to compare all treatment groups to the control group (Prism5.0, GraphPad Software, Inc.). For MPD challenge analyses, the saline data from the preceding session was used for all comparisons. Analysis of each dependent variable was conducted separately.

Autoradiography data were analyzed by two-tailed, unpaired t-tests. Receptor binding data were analyzed by two-way ANOVA with Bonferonni's correction applied to all significant main effects using Prism 5.0, GraphPad Software, Inc. Pharmacokinetic

data were obtained using Excel 2003 (Microsoft Corporation). Briefly, data were input as ng of MP and RA per ml or mg (plasma and tissue respectively). Time following MP administration of plasma and tissue sample collection was also input to obtain areas under the concentration vs. time curves (AUCs). Half-lives were calculated by regression analysis of concentration-time data with the plasma sample estimated to represent the beginning of the terminal elimination phase.

CHAPTER 3:

DEVELOPMENTAL DELTAMETHRIN EXPOSURE MODULATES FIXED-INTERVAL RESPONSE PATTERNING: ROLE OF DOPAMINE RECEPTORS

INTRODUCTION

Preliminary observations from this laboratory have demonstrated that mice exposed during development to low levels of the pyrethroid pesticide DM exhibit signs similar to those observed in children with ADHD. These include elevated DAT levels, hyperactivity that is ameliorated by methylphenidate treatment, and behavioral deficits in crude tests of working memory and sustained attention. Given that deficits in working memory are another common characteristic of ADHD (Doyle, 2006), we examined Y-maze performance where successful completion of a trial occurs when the mouse explores each arm of the maze once without reentering a previously entered arm. This exploration pattern in which the mouse explores one arm, then the next and finally the last unexplored arm is considered a measure of working memory. Arm reentry is considered a crude measure of attention. We found mice exposed perinatally to DM had significantly reduced alternation behavior and increased same arm entries (data not shown). The Y-maze does not permit comprehensive analysis of attention and lacks the ability to examine impulsivity. Considering that impulsivity is often considered the most disruptive symptom of ADHD, we sought to determine whether DM exposure results in impulsive-like behavior. Impulsivity has been operationally defined as a preference for an immediate, small reward over a larger, but delayed reward. Such a preference has

been reported in ADHD children (Sagvolden et al., 1998). Two studies by Darcheville et al., (1992, 1993) found that fixed-interval (FI) response rates reliably predicted performance of normal children on a self-control (impulsivity) paradigm in which a choice between a smaller but immediate reward (impulsive choice) or a larger but delayed reward (self-controlled choice) was presented. Specifically, children that emitted higher FI response rates made significantly more impulsive choices than those with lower rates, leading the authors to conclude that FI rates were predictive of impulsive responses (Darcheville et al., 1992, 1993). FI rates may be predictive of impulsivity and eventual ADHD diagnosis. Indeed, Sagvolden et al. (1998) found a similar relationship between FI rates in children diagnosed with ADHD and impulsivity (Sagvolden et al., 1998). Specifically, the children diagnosed as ADHD exhibited increased response rates during the FI component of the schedule, shorter interresponse times (IRT: time which elapses between successive responses), decreased post-reinforcement pause time (PRP: time that elapses between the delivery of a reinforcer and the next response), and increased run rates (RR: rate of responding once the interval begins). The relationship between FI performance and impulsivity has also been shown in the Spontaneously Hypertensive Rat, a commonly used animal model of ADHD (Berger et al., 1998). Therefore, we chose to use a FI paradigm to determine whether developmental DM exposure produces impulsive-like behavior.

There is a substantial body of evidence that the dopaminergic innervations of the NA is critical in the regulation of reinforcement processes [for reviews see (Berridge et al., 1998; Cardinal et al., 2001; Pierce et al., 2006; Salamone et al., 2005; Wise, 2004)]. More specifically, it has been demonstrated that rodent timing ability can be

systematically altered by manipulation of DA (Cory-Slechta et al., 1997; Maricq et al., 1983; Maricq et al., 1981). DA agonists have been reported to result in overestimation of the passage of time, whereas DA antagonists result in underestimation of the page of time (Church, 1989; Frederick et al., 1996). Furthermore, preliminary data for our laboratory demonstrates that administration of the D1- and D2-like receptor antagonists (SCH23390 and eticlopride respectively), significantly reduces the locomotor activity in DM-exposed male offspring to control levels (data not shown). To further examine the responsiveness of postsynaptic receptors, we determined the locomotor response of animals administered the general DA agonist apomorphine. Administration of apomorphine resulted in significantly greater locomotor activity (64%) in the male but not female offspring compared to controls (data not shown). Since the D1-specific antagonist SCH23390 produced a greater effect, compared to the D2-receptor antagonist (eticlopride), we administered the full D1 agonist SKF82958 to explore whether the D1 receptor response was altered by developmental DM exposure. Locomotor activity was increased following SKF82958 (0.5mg/kg) treatment by 137% in control mice. However, the DM treated mice exhibited a much greater response than control mice in that locomotor activity increased by 208% (data not shown). These data suggest that alteration of the DA system during development by DM results in enhanced responsiveness of D1 receptors. However, these data were preliminary, the number of subjects per group was small (n=3) and the role of D2 receptors on these effects was not determined.

Alteration in DA receptor function may also play a role in the expression of impulsive-like behavior. For example, lead exposure has been demonstrated to produce impulsive-like behavior in rats and decreases in DA D1 D2 and DAT binding in the

nucleus accumbens but not in the dorsal striatum was found in rats exposed to lead (Pokora et al., 1996). Brockel et al (1999) examined whether acute administration of both D1 (SKF82958) and D2 (quinpirole) agonists and antagonists (SCH 23390 and eticlopride, respectively) altered baseline performance of lead-exposed rats on a fixed-ratio waiting for reward (FRWAIT) paradigm. This study also found that the D2 agonist quinpirole reversed the impulsive-like behavior produced by lead. In addition, D2 compounds exerted a greater magnitude of effects than did D1 compounds.

Further, environmental factors that affect the mesolimbic DA system may lead to impulsive-like behaviors. Here, we wanted to examine the impact of developmental DM exposure on DA function, DA receptor binding and its consequent effect on behavior. Pregnant mice were exposed to either 0, 1 or 3mg/kg DM throughout gestation and lactation. In addition, we wanted to explore the potential role of dopamine receptors (D1 and D2) in DM-induced modulation of FI performance.

RESULTS

Our pilot data depicted below in figures 3-6 demonstrate that mice developmentally exposed to DM throughout gestation and lactation exhibit response patterns similar to the ADHD children studied by Sagvolden's group (1998). Offspring of pregnant mice exposed to either 0 or 3mg/kg DM throughout gestation and lactation were tested in operant chambers on a FI60 sec schedule in which reinforcement was available every 60 sec over a 45 min test period. Following a three day training period, mice underwent seven days of testing on the same schedule. As depicted, male offspring of mice exposed to DM had higher FI response rates (Fig.'s 3 and 4A), longer PRP (Fig. 5A) and shorter IRT's (Fig. 6A). Female offspring were not affected (Fig.'s 3-6B). Based on the association of FI response patterns with impulsive-like behavior observed in ADHD children and in the SHR model of ADHD, these data suggest that mice developmentally exposed to the pyrethroid pesticide, DM, exhibit impulsive-like behavior. Here, we are proposing to expand these preliminary findings and characterize the impulsive-like behavior along with determining the ability of the commonly used ADHD therapeutic, methylphenidate, to ameliorate these effects.

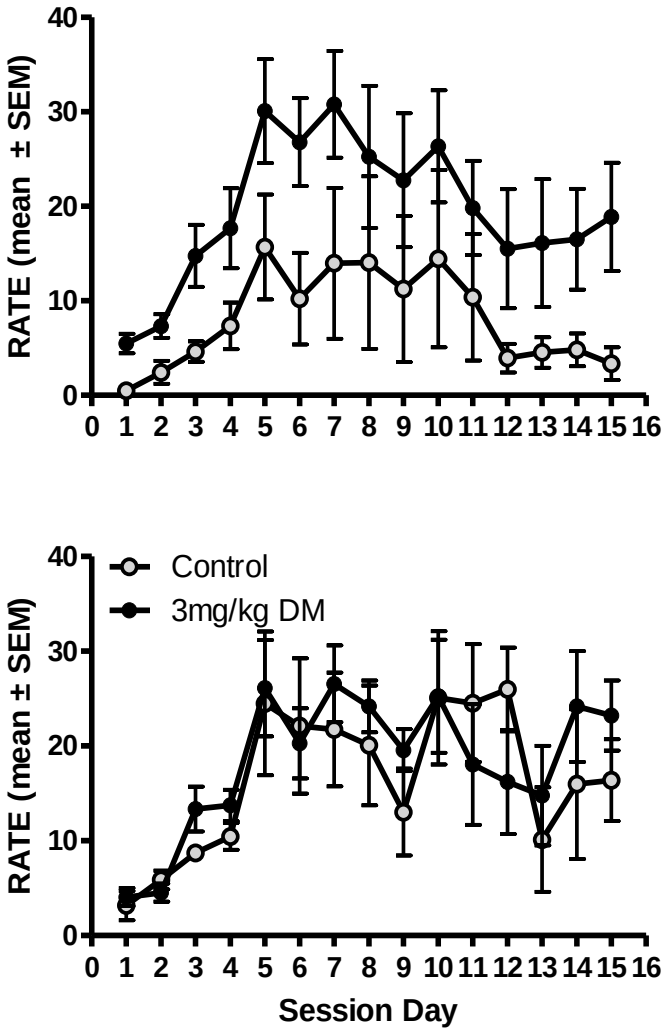

Figure 3. Pilot data: Rate of responding in mice exposed to either 0 or 3mg/kg DM on a FI60s schedule of reinforcement. Top and bottom panels represent males (n=3 control; n=6 DM) and females (n=3 control; n=5 DM) respectively. Data represent mean + SEM and were analyzed by RMANOVA. Litter is the unit of measure (n=number of litters represented).

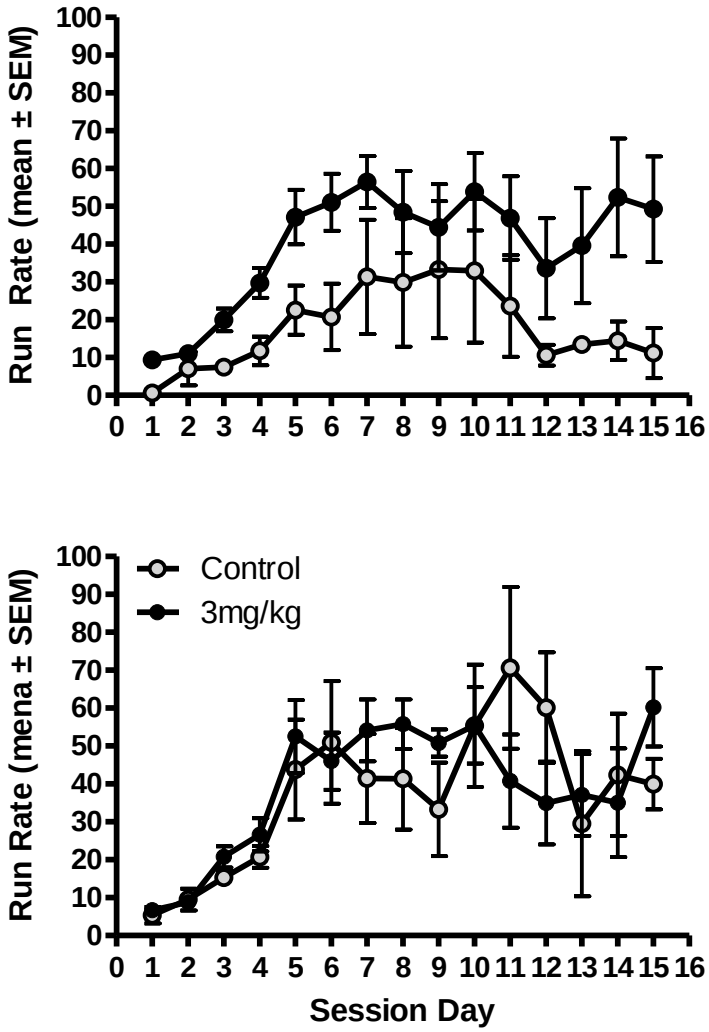

Figure 4. Pilot data: Run rate in mice exposed to either 0 or 3mg/kg DM on a FI60s schedule of reinforcement Top and bottom panels represent males (n=3 control; n=6 DM) and females (n=3 control; n=5 DM) respectively. Data represent mean + SEM and were analyzed by RMANOVA. Litter is the unit of measure (n=number of litters represented).

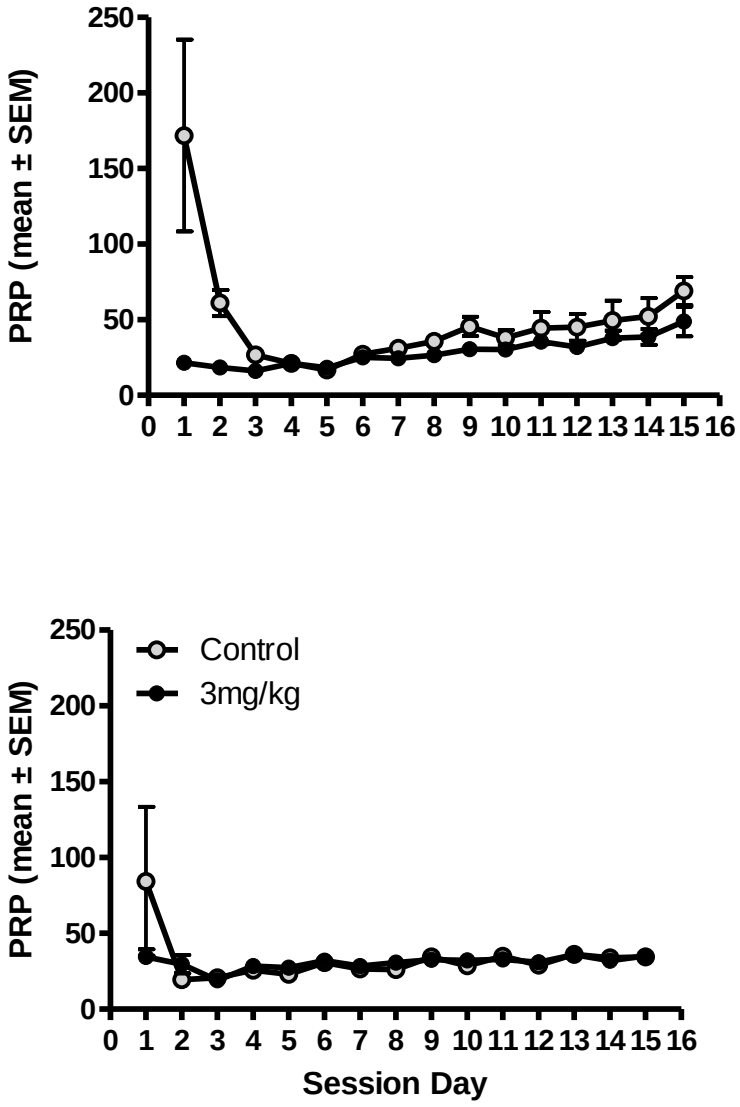

Figure 5. Pilot data: Post-reinforcement pause in mice exposed to either 0 or 3mg/kg DM on a FI60s schedule of reinforcement. Top and bottom panels represent males (n=3 control; n=6 DM) and females (n=3 control; n=5 DM) respectively. Data represent mean + SEM and were analyzed by RMANOVA. Litter is the unit of measure (n=number of litters represented).

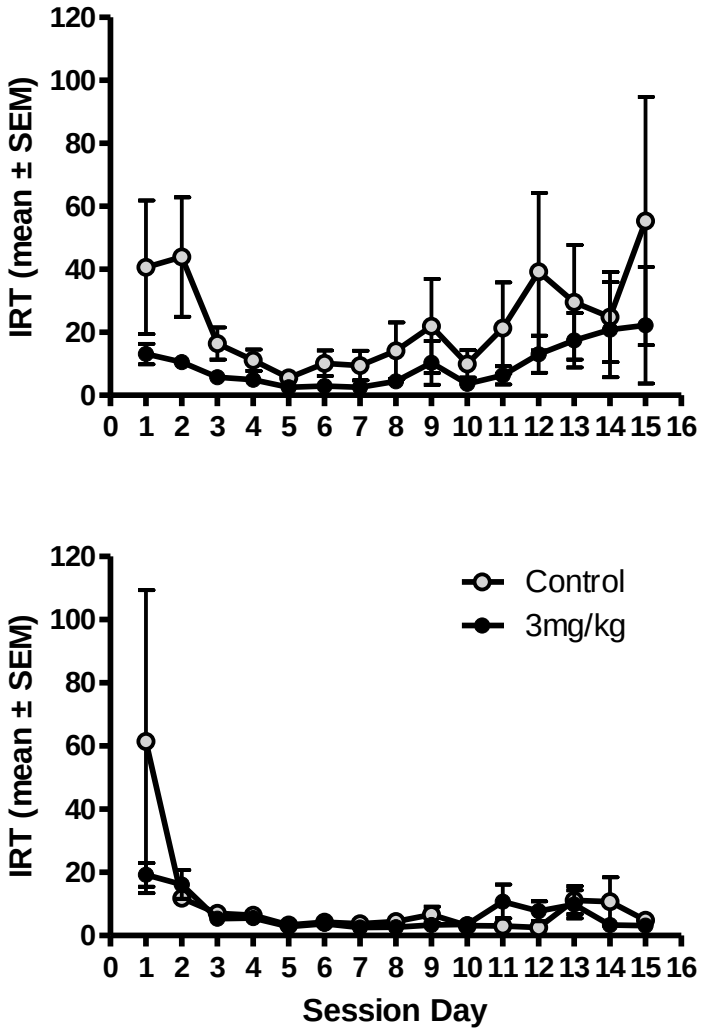

Figure 6. Pilot data: Interresponse time in mice exposed to either 0 or 3mg/kg DM on a FI60s schedule of reinforcement. Top and bottom panels represent males (n=3 control; n=6 DM) and females (n=3 control; n=5 DM) respectively. Data represent mean + SEM and were analyzed by RMANOVA. Litter is the unit of measure (n=number of litters represented).

DM-induced modulation of FI performance

Response rates gradually increased across baseline sessions, with DM-induced increases emerging within the 1st session block in both genders (Fig.'s 7A and B, males and females respectively). DM exposure was associated with higher response rates in both males and females. RMANOVA revealed a significant main effect of DM ($F[2, 41]=3.995$, $p=.0260$) and gender ($F[1,41]=21.278$, $p<.0001$). There was also a DM by gender interaction ($F[2,41]=6.639$, $p=.0032$). Bonferroni post-hoc analysis revealed that all three male treatment groups significantly differed from each other. However, in females, the low dose DM group differed from both controls and high dose DM but the control and high DM groups did not differ from each other. Males exposed to DM emitted on average (across all sessions) 108 and 292% more responses than controls and low dose females emitted 30 and 41% more responses per minute than both their control and high dose counterparts, respectively. Figures 8A and B depicts robust increases in RR in DM-treated males and female low dose DM mice as well. RMANOVA confirmed an overall effect of gender (gender: $F[2,41]=11.221$, $p=.0017$) but not treatment or DM by gender interaction (DM: $F[1,41]=1.795$, $p=.1789$; DM by gender: $F[2,41]=2.153$, $p=.1291$). Bonferroni post-hoc analyses revealed a dose-dependent increase in RR. In males, all three treatment groups significantly differed from each other. In females however, low dose DM differed from both control and high dose DM but control and high dose DM did not differ from each other as determined by Bonferroni post-hoc analysis. Robust increases in RR were observed in males 99 and 257% (1 and 3mg/kg DM respectively). Female RR increased slightly by 25 and 29% (1 and 3mg/kg DM respectively).

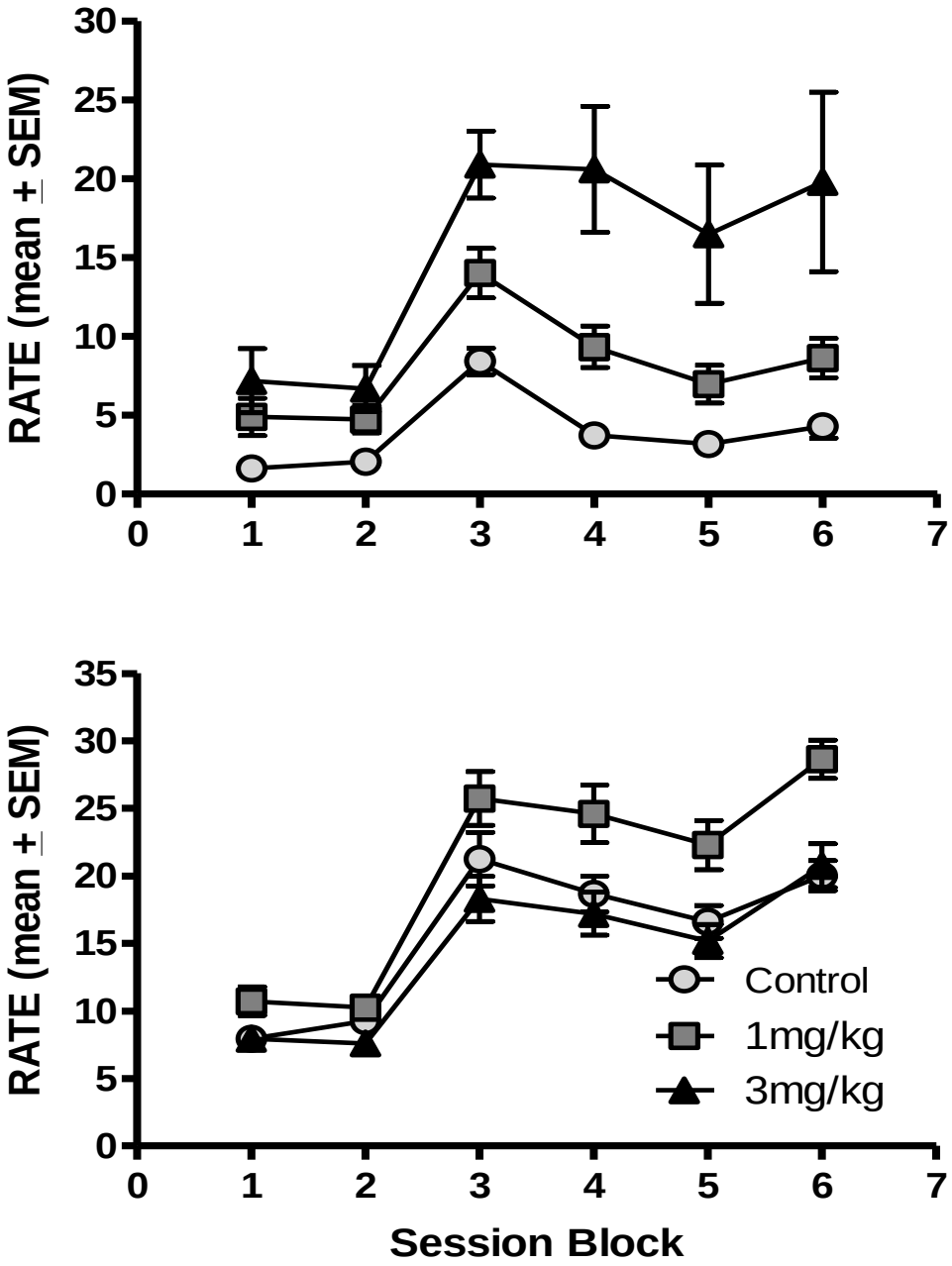

Figure 7. Rate of responding in mice exposed to either 0, 1 or 3mg/kg deltamethrin on a FI60s schedule of reinforcement Males (top panel) and females (bottom panel) exposed to 0, 1, or 3mg/kg DM. Data represent mean + SEM (n=6-7) and were analyzed by RMANOVA. Data are plotted across seven blocks of five sessions each.

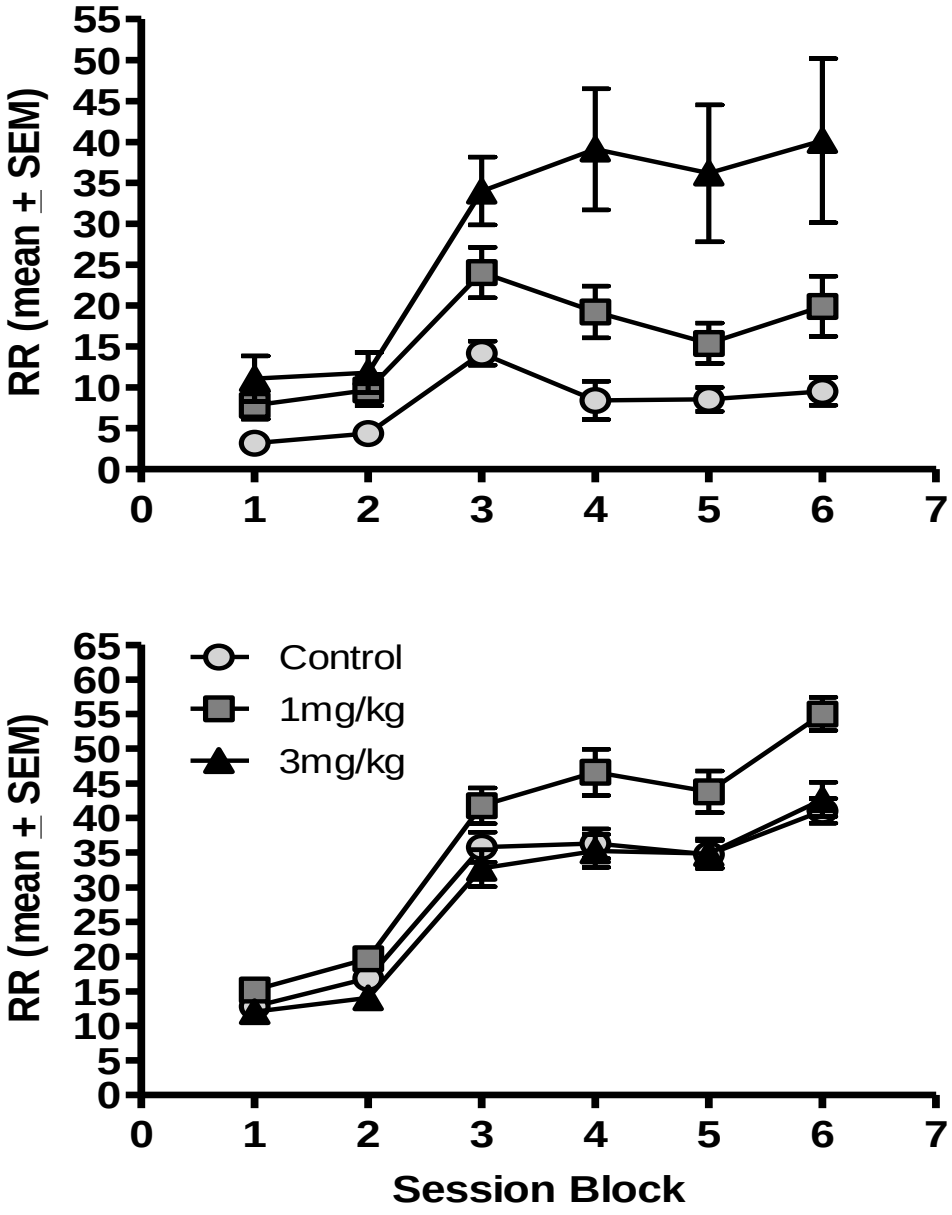

Figure 8. Run rate in mice exposed to either 0 or 3mg/kg deltamethrin on a FI60s schedule of reinforcement Males (top panel) and females (bottom panel) exposed to 0, 1, or 3mg/kg DM. Data represent mean + SEM (n=6-7) and were analyzed by RMANOVA. Data are plotted across seven blocks of five sessions each.

DM exposure also resulted in significantly shorter PRP times in both genders (Fig.'s 9A and B). A RMANOVA revealed an overall effect of treatment and gender (DM: $F[2,41]=3.873$, $p=.0288$; gender: $F[1,41]=17.745$, $p=.0001$). Post-hoc analysis confirmed a significant dose-dependent decrease in pause length between controls and the two DM groups (the low and high dose DM groups did not differ) in the males. In females, there was no significant difference among the treatment groups as determined by post hoc analysis. While the 1 and 3mg/kg DM groups both had significantly shorter PRP times than controls, they did not differ from each other. When averaged across all the sessions PRP significantly decreased by 23 and 29% in males (1 and 3mg/kg DM respectively) but not females 11 and 2% (1 and 3mg/kg DM respectively) compared to controls. Finally, a decrease in IRT was apparent in males (Fig. 10A) but not females (Fig. 10B) as early as the 1st session block. A RMANOVA revealed a significant effect of gender ($F[1,41]=9.486$, $p=.0037$) but not treatment ($F[2,41]=2.604$, $p=.0861$). Still, 1 and 3mg/kg DM decreased IRT, on average, by 69 and 66% in males and 57 and 1% in females compared to controls. Post-hoc analysis revealed that both 1 and 3mg/kg DM groups significantly differed from controls but not each other in males. In females, post-hoc analysis showed that both controls and 1mg/kg DM differed from 3mg/kg DM (but not each other).

Performance stability during injection acclimation

In order to evaluate dopamine receptor involvement in DM-induced FI performance changes, we administered D1 and D2 receptor antagonists SCH23390 and eticlopride. For five days prior to antagonist testing, the mice were acclimated to the injection process by administration of saline 15min prior to behavioral testing. The resultant mean values of rate, RR, PRP and IRT illustrate that the injection itself did not impact FI rates or PRP in either control or DM-exposed males (Table 1). A RMANOVA of the raw data showed an effect of DM-treatment on rate ($F[1,56]=9.907$, $p=.007$), RR ($F[1,56]=10.546$, $p=..006$), and IRT ($F[1,56]=22.55$, $p=.0003$) but not PRP ($F[1,56]=2.22$, $p=.158$). To determine whether FI performance changed across days, a two-way ANOVA (day by treatment) of the means was performed. There was a significant effect of treatment but not day on rate (TRT: $F[4,70]=45.65$, $p=.0001$; DAY: $F[4,70]=636.$, $p=.638$); RR (TRT: $F[4,70]=52.50$, $p=.0001$; DAY: $F[4,70]=.267$, $p=.898$) as well as PRP (TRT: $F[4,70]=7.477$, $p=.0079$; DAY: $F4,70]=1.871$, $p=.125$). While rate and PRP were, there was both a significant effect of treatment and day on IRT during saline injection acclimation (TRT: $F[4,70]=72.13$, $p=.0001$; DAY: $F[4,70]=3.67$, $p=.009$). However, there was no interaction of treatment and day on IRT (TRT by DAY: $F[4,70]=2.332$, $p=.0642$). As depicted in table one, the IRT values decreased across days in control but not DM-treated males.

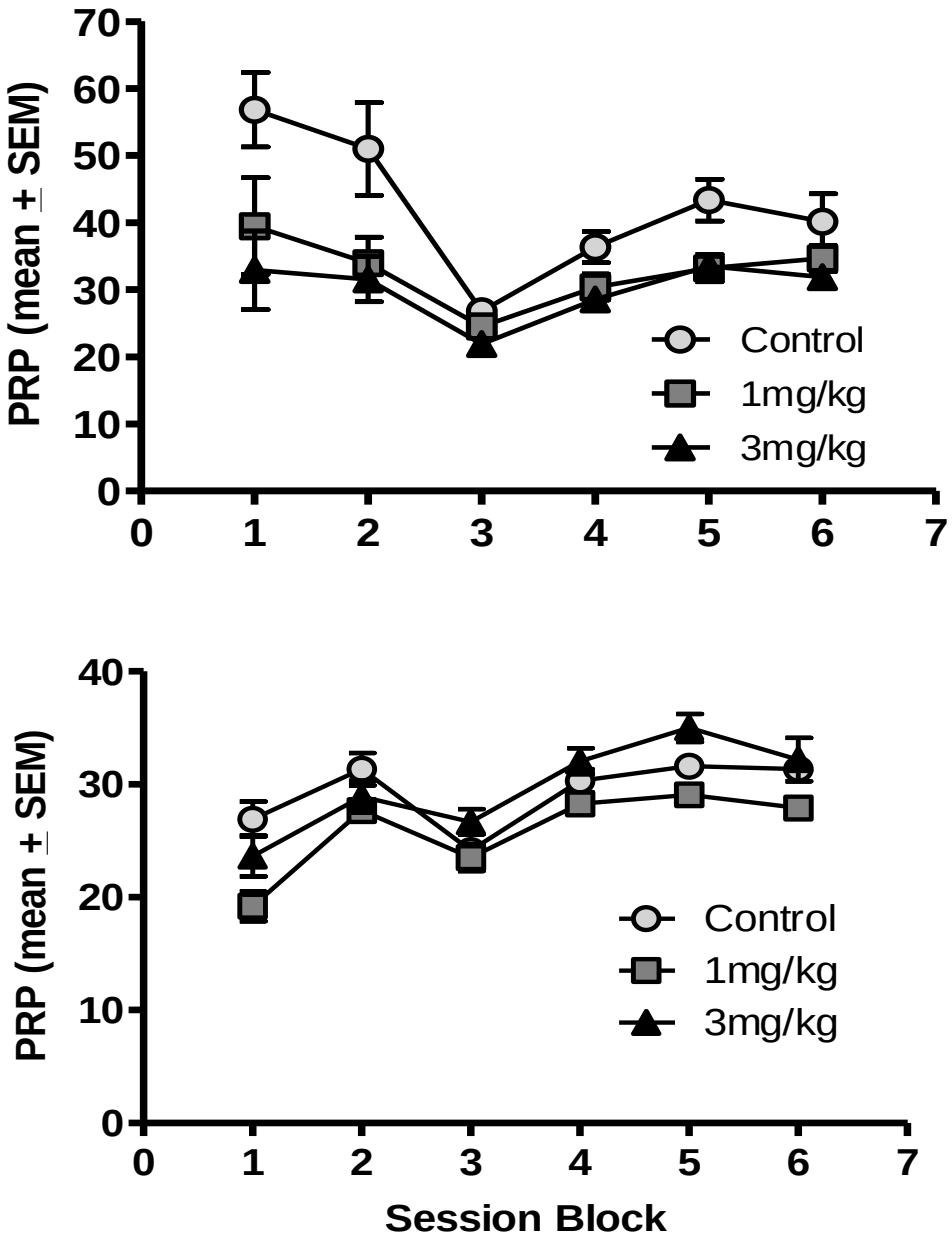

Figure 9. Post reinforcement pause in mice exposed to either 0 or 3mg/kg deltamethrin on a FI60s schedule of reinforcement Males (top panel) and females (bottom panel) exposed to 0, 1, or 3mg/kg DM. Data represent mean + SEM (n=6-7) and were analyzed by RMANOVA. Data are plotted across seven blocks of five sessions each.

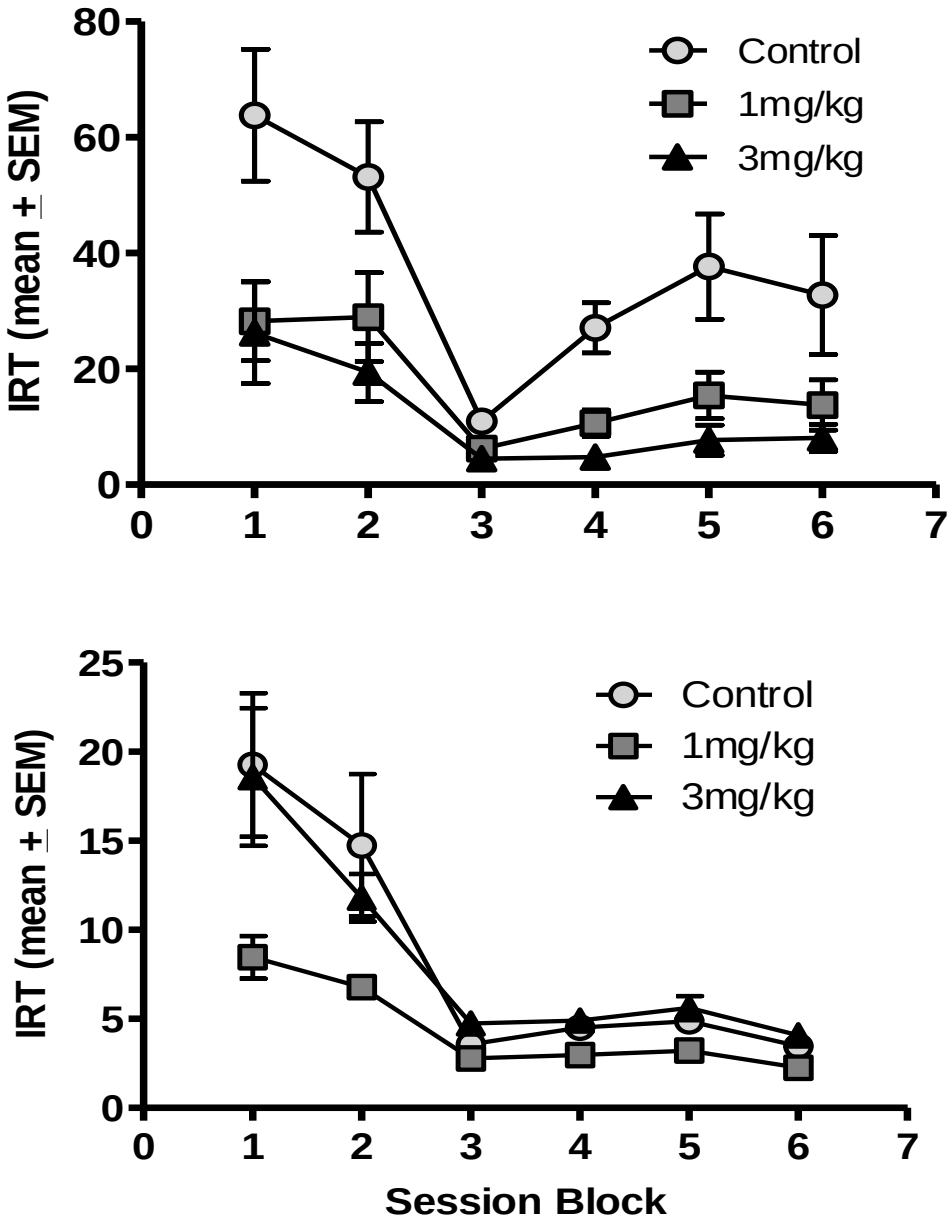

Figure 10. Interresponse time in mice exposed to either 0 or 3mg/kg deltamethrin on a FI60s schedule of reinforcement Males (top panel) and females (bottom panel) exposed to 0, 1, or 3mg/kg DM. Data represent mean + SEM (n=6-7) and were analyzed by RMANOVA. Data are plotted across seven blocks of five sessions each.

Effects of D1 antagonist SCH23390

SCH23390 dose-dependently decreased rate and RR as well as increased PRP and IRT in males. Figures 11 (rate and RR) and 12 (PRP and IRT) depict effects of SCH23390 in males. The 1mg/kg DM group no longer differed significantly from the control group prior to antagonist testing; therefore that group is not depicted. A two-way ANOVA confirmed an overall significant effect of DM on rate in males ($F[1,53]=7.214$, $p=.0096$) that SCH23390 dose-dependently, but not signficantly reduced ($F[3,53]=2.616$, $p=.06$) (Fig. 10). There was no interaction of DM exposure and SCH23390 ($F[3,53]=.487$, $p=.692$). Following .025mg/kg SCH23390, mean rate in DM-exposed males decreased to control levels. Specifically, the mean rates in control and DM males were 16.51 and 36.356, respectively, prior to SCH23390. Likewise, a two-way ANOVA confirmed an overall effect DM on RR in males ($F[1,53]=10.10$, $p=.0025$) that SCH23390 dose-dependently decreased. However, the RR of control and 3mg/kg DM males still differed significantly following SCH23390 ($F[3,53]=3.235$, $p=.0294$). There was no interaction of DM exposure and SCH23390 treatment on RR ($F[3,53]=.1425$, $p=.934$). SCH23390 treatment dose-dependently increased PRP and IRT in both control and DM-exposed males (Fig. 12). A two-way ANOVA showed that SCH23390 produced an overall significant increase in PRP ($F[3,53]=6.015$, $p=.0013$) that was independent of DM exposure (DM by SCH23390 interaction: $F[3,53]=.1267$; $p=.944$). In fact, there was no difference in PRP between control or DM-exposed males prior to ($F[1,53]=.556$, $p=.459$) or following SCH23390 (DM by SCH23390 interaction: $F[3,53]=.1267$; $p=.944$). There was also no effect of DM or SCH23390 on IRT in males (DM: $F[1,53]=1.015$; $p=3.189$; SCH: $F[3,53]=2.167$, $p=.104$).

The effects of SCH23390 on female performance are depicted in figures 13 (rate and RR) and 14 (PRP and IRT). It was determined during testing that .00625 and .025mg/kg SCH would be the lowest and highest dose tested. This was based on the observation that .025mg/kg SCH resulted in a 60% decrease in rate and a 546% increase in IRT in the controls. Several of the females were observed to be engaging in behaviors incompatible with lever pressing such as running, spinning and attempting to jump onto the houselight. Males exposed to the same dose had a 36% decrease and 252% increase in control rate and IRT respectively. It should be noted that, in general, the female mice were more sensitive than the males to the injection process and became increasingly difficult to handle throughout the remainder of the experiment, suggesting a possible gender difference in the stress response to the injection. SCH23390 produced a significant dose-dependent reduction in both rate and RR in control and DM-exposed females (RATE: $F[1,53]=5.282$, $p=.0029$; RR: $F[1,53]=4.384$; $p=.0079$). There was no overall significant effect of DM on rate or RR however (RATE: $F[1,53]=1.886$, $p=.1775$; RR: $F[1,53]=.4628$, $p=.4993$). There was also no interaction (RATE: $F[3,53]=.2899$; $p=.8325$; RR: $F[3,53]=.047$; $p=.9863$). SCH23390 also increased both PRP and IRT in control and DM-exposed females (PRP: $F[1,53]=5.833$; $p=.0016$; IRT: $F[1,53]=7.309$; $p=.003$). The effects of SCH23390 on PRP (DM by SCH23390 interaction: $F[3,53]=.098$; $p=.9607$) and IRT (DM by SCH23390 interaction: $F[3,53]=.099$; p=9601) were independent of DM exposure however. As depicted in figure 13, control and DM-exposed female PRP and IRT did not differ prior to the antagonist challenges (PRP: $F[1,53]=.98$; $p=.3267$; IRT: $F[1,53]=.039$; $p=.844$).

Saline Days Preceeding Antagonist Testing								
DAY	RATE		RR		PRP		IRT	
	Control	DM	Control	DM	Control	DM	Control	DM
1	9.13±.1.91	34.45±9.98	18.52±2.79	59.41±15.82	32.69±3.16	23.14±1.88	9.33±1.79	2.58±.51
2	11.18±1.56	34.38±7.20	20.71±2.37	66.39±13.08	26.23±2.35	25.56±1.89	6.78±1.16	2.18±.31
3	9.91±1.44	36.57±6.45	21.46±2.36	66.86±11.84	31.82±2.46	27.43±1.59	7.47±1.00	2.04±.33
4	16.33±2.74	43.15±8.03	29.50±3.11	77.52±12.23	26.12±2.95	23.42±1.21	4.38±.532	1.69±.24
5	13.89±1.52	32.46±6.82	29.04±1.87	60.78±11.64	30.79±2.99	28.48±1.05	4.96±.551	2.32±.33

Table 1. Male FI Rate, RR, PRP and IRT values during the five days prior to D1 and D2 antagonist testing. Values represent mean ± SEM and demonstrate consistent differences in male mice exposed developmentally to 0 (n=8) or 3mg/kg DM (n=8).

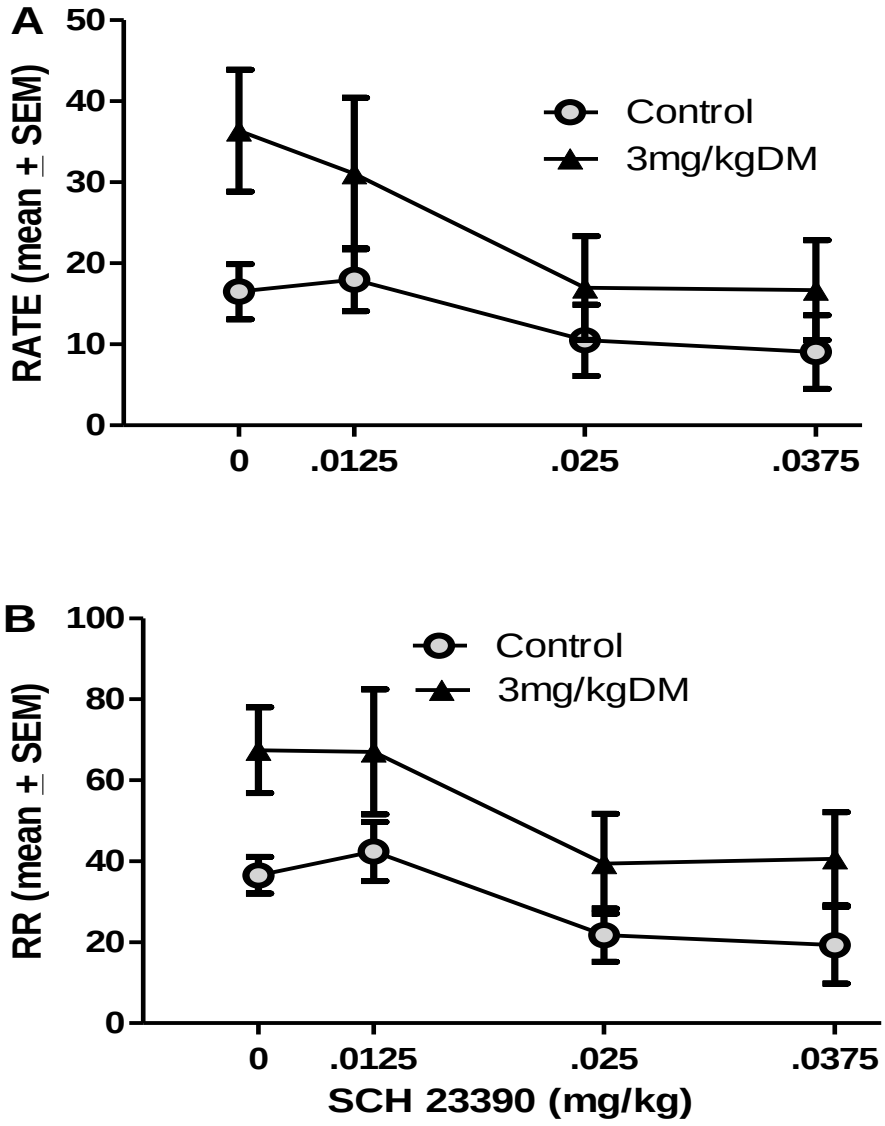

Figure 11. Effects of D1 receptor antagonist SCH23390 on RATE (A) and RR (B) on a FI60s schedule of reinforcement in males exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=7-8) and were analyzed by two-way ANOVA. Mice were challenged with .0125, .025 and .0375mg/kg SCH 23390. SCH23390 was given via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (+ SEM) of the three intervening saline days between SCH23390 doses.

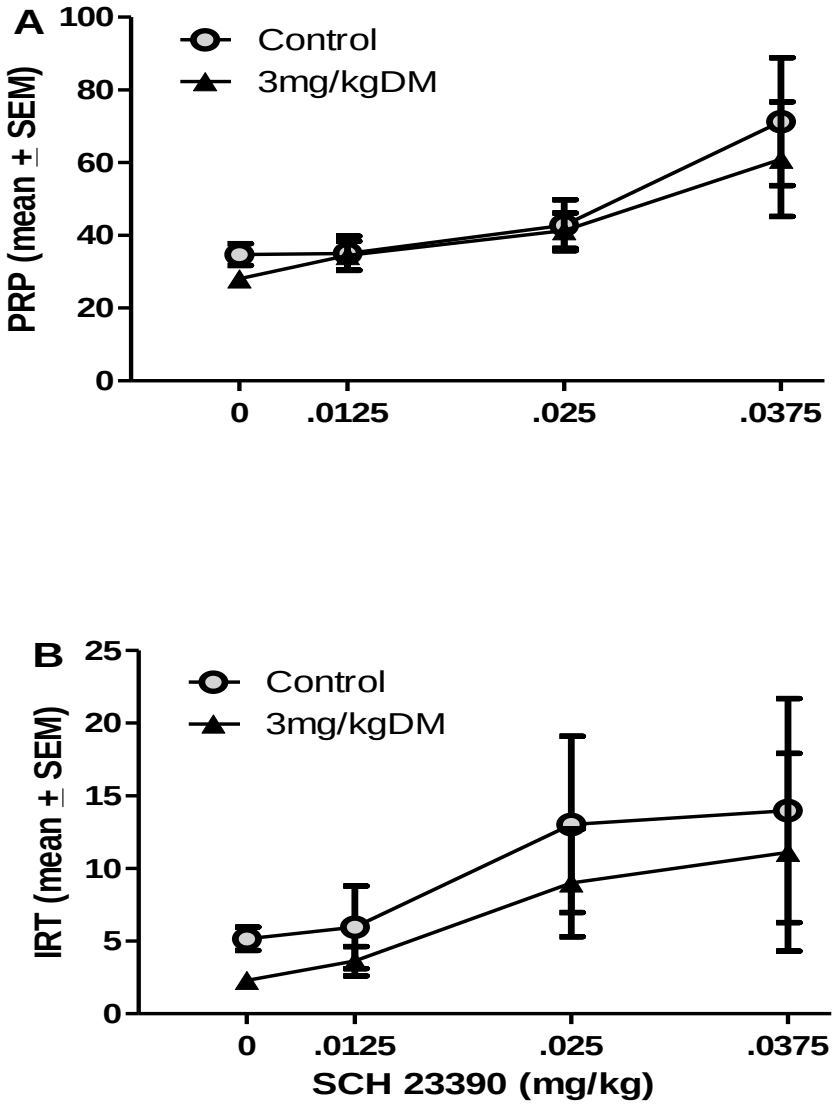

Figure 12. Effects of D1 receptor antagonist SCH23390 on PRP (A) and IRT (B) on a FI60s schedule of reinforcement in males exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (PRP: n=7-8; IRT: n=6-8) and were analyzed by two-way ANOVA. Mice were challenged with .0125, .025 and .0375mg/kg SCH 23390. SCH23390 was given via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between SCH23390 doses.

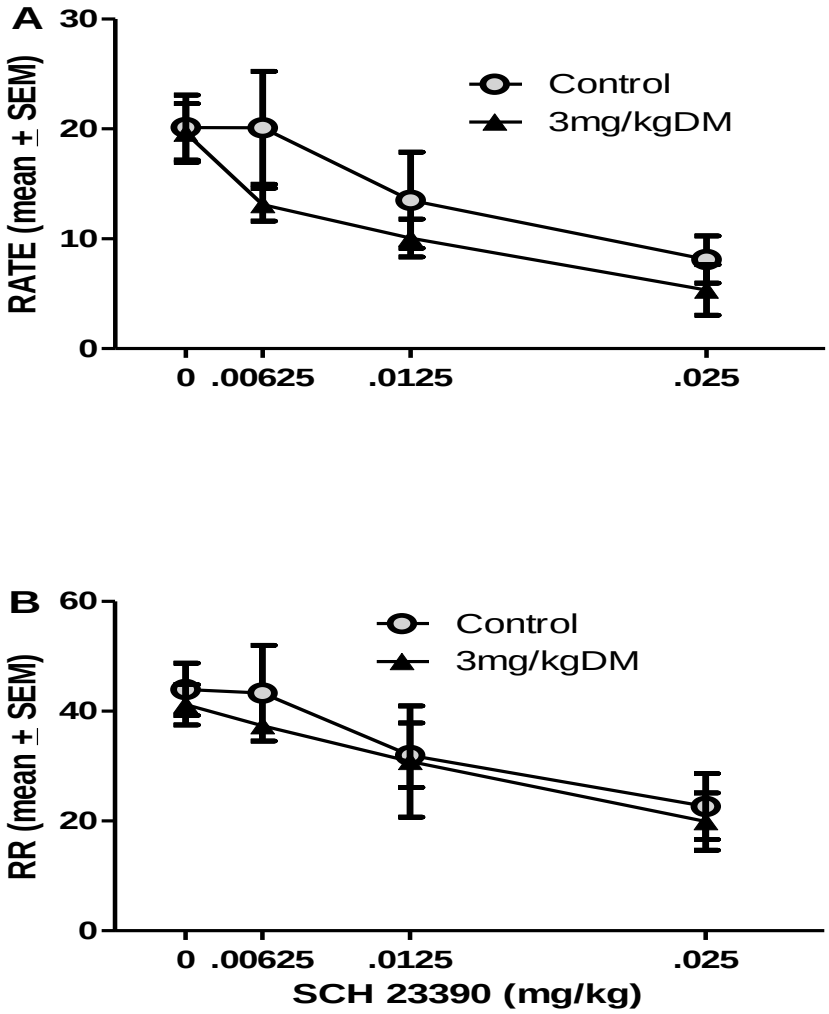

Figure 13. Effects of D1 receptor antagonist SCH23390 on RATE (A) and RR (B) on a FI60s schedule of reinforcement in females exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-8) and were analyzed by two-way ANOVA. Mice were challenged with .00625,.0125 and .025mg/kg SCH23390. SCH23390 was given via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between SCH23390 doses.

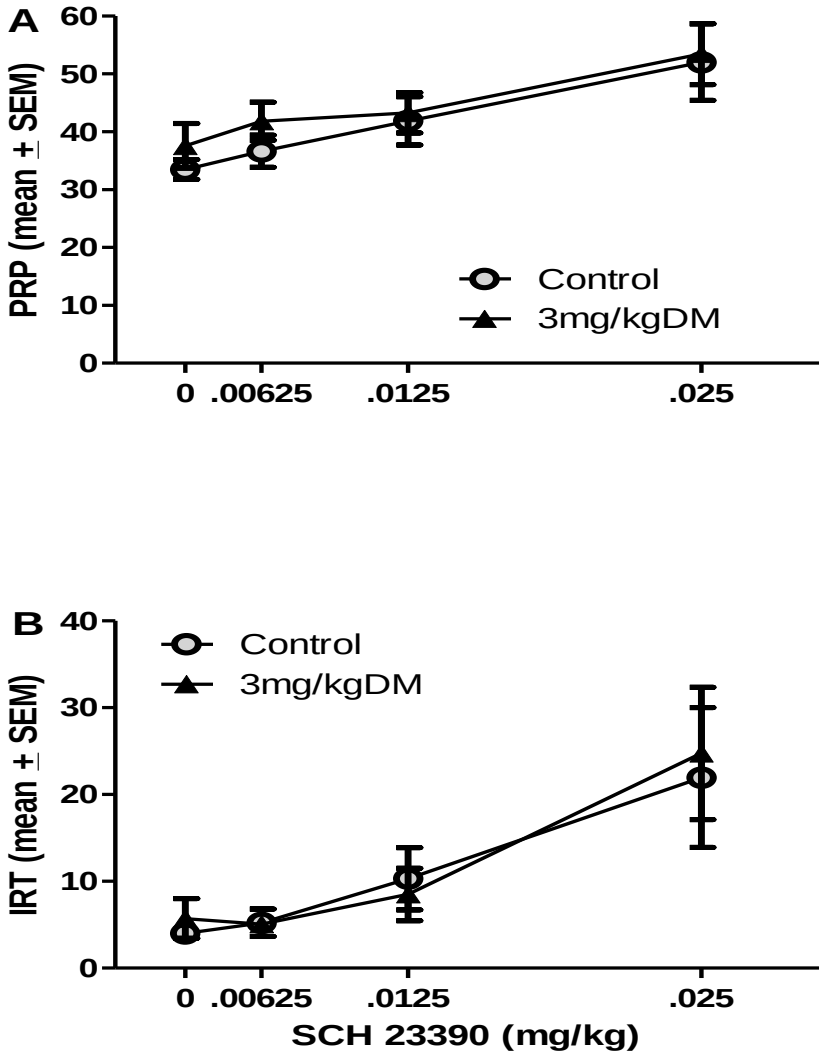

Figure 14. Effects of D1 receptor antagonist SCH23390 on PRP (A) and IRT (B) on a FI60s schedule of reinforcement in females exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-7) and were analyzed by two-way ANOVA. Mice were challenged with .0125, .025 and .0375mg/kg SCH 23390. SCH23390 was given via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between SCH23390 doses.

Effects of D2 antagonist Eticlopride

Figures 15 (rate and RR) and 16 (PRP and IRT) depict reversal of DM-induced alterations by the higher doses of eticlopride (ETI) in males exposed perinatally to 3mg/kg DM. Administration of low dose ETI (.025mg/kg) robustly increased rate and RR as well as decreased both PRP and IRT compared to control values. In contrast, higher doses of ETI (.05 and .1mg/kg) decreased rate and RR as well as increased both PRP and IRT. As depicted in figure 15, DM-exposed males emitted significantly more responses per minute on average relative to controls throughout ETI testing ($F[1,56]=22.98$, $p<.0001$). Bonferroni post hoc analysis revealed that control and DM-exposed males given saline differed significantly ($p<.05$) and that .05 and .1mg/kg ETI reduced DM-induced rate increases to control levels ($p>.05$). While there was a significant difference in PRP between control or DM-exposed males prior to ETI treatment ($F[1,56]=9.921$, $p=.0035$) and ETI produced an overall significant increase in PRP ($F[3,56]=4.338$, $p=.0081$); the effect of ETI was independent of DM exposure (DM by ETI interaction: $F[3,56]=.5810$; $p=.63$). There was no effect of ETI on IRT in males ($F[1,56]=2.175$; $p=1.011$).

As noted previously, the difference between DM-treated and control females diminished during establishment of the D1 and D2 antagonist dose response. Indeed, female baseline rate no longer differed significantly during SCH23390 or ETI testing ($p>.05$). Still, ETI produced the same inverted u-shaped dose response as in males; low dose (.025mg/kg ETI) increased rate and RR (Fig.17) and decreased PRP and IRT (Fig.18). High doses (.05 and .1mg/kg ETI) decreased rate and RR and increased PRP and IRT, although these changes were independent of DM exposure.

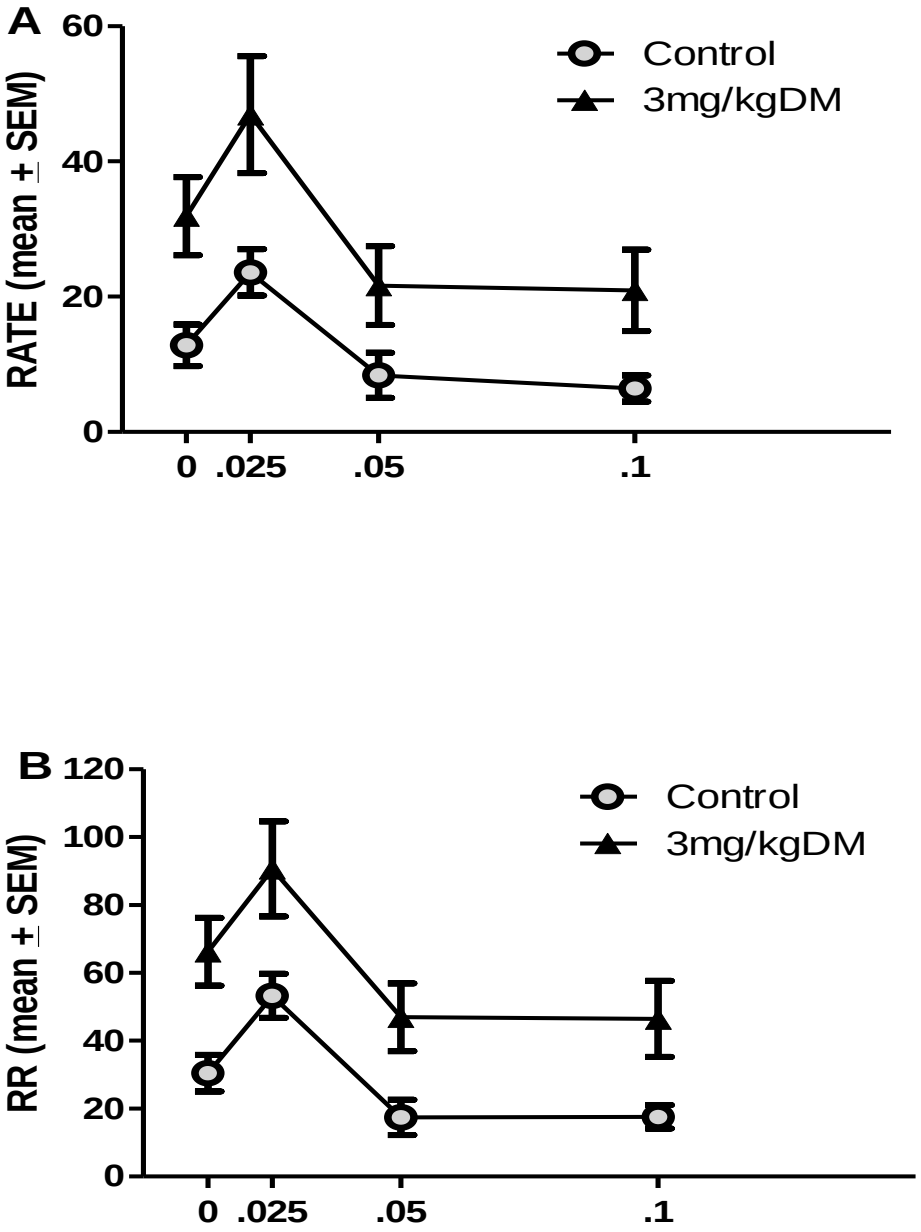

Figure 15. Effects of D2 receptor antagonist eticlopride on RATE (A) and RR (B) on a FI60s schedule of reinforcement in males exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-7) and were analyzed by one-way ANOVA. Mice were challenged with .025, .05 and .1mg/kg eticlopride (ETI) via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between ETI doses.

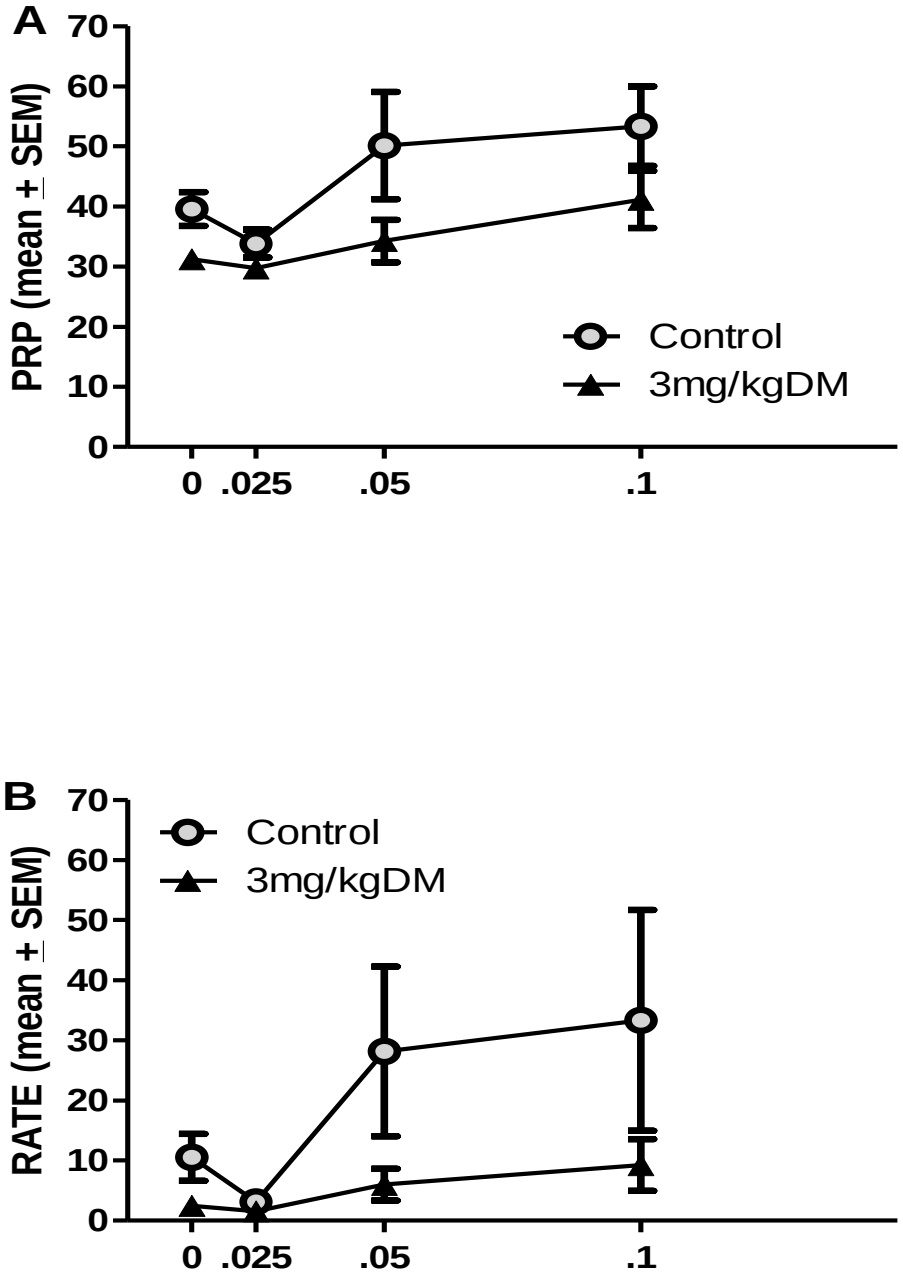

Figure 16. Effects of D2 receptor antagonist eticlopride on PRP (A) and IRT (B) on a FI60s schedule of reinforcement in males exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-7) and were analyzed by one-way ANOVA. Mice were challenged with .025, .05 and .1mg/kg eticlopride (ETI) via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between ETI doses.

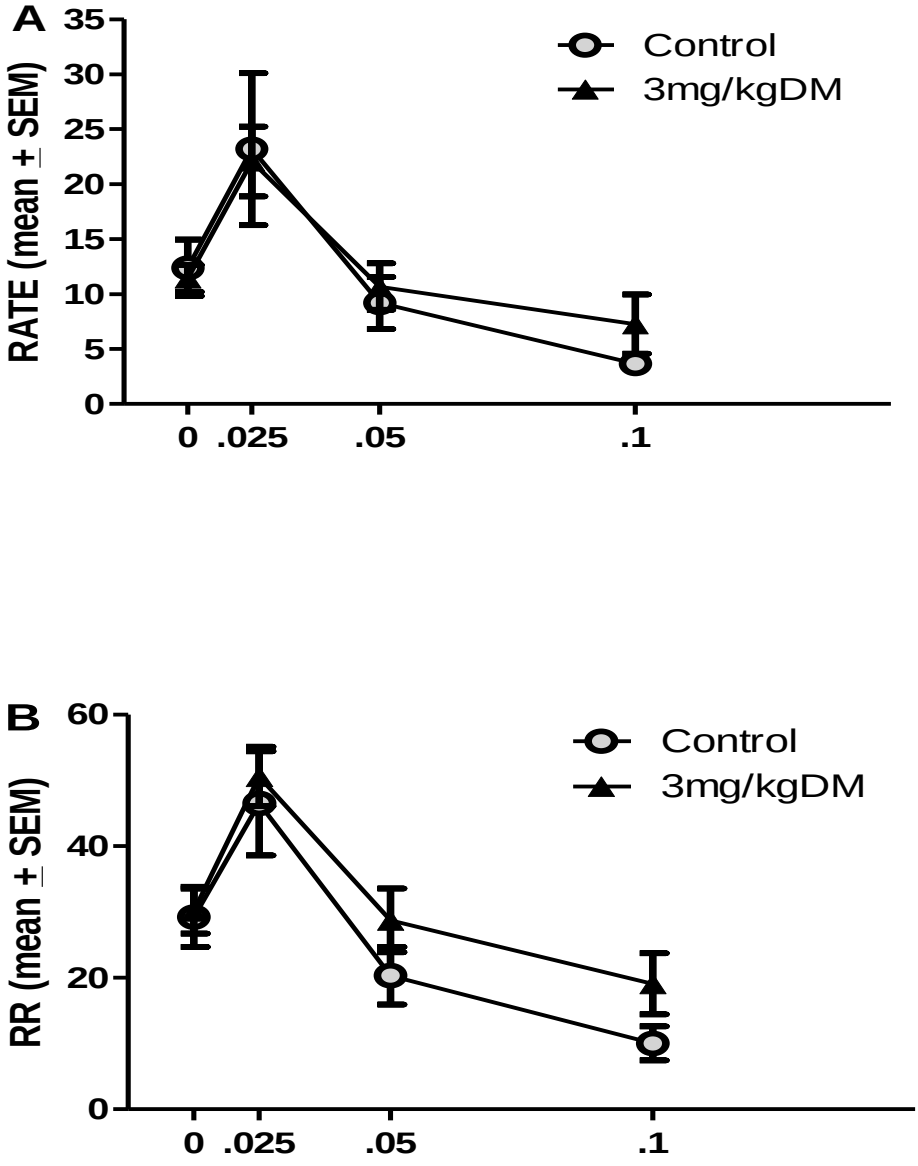

Figure 17. Effects of D2 receptor antagonist eticlopride on rate (A) and run rate (B) on a FI60s schedule of reinforcement in females exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-7) and were analyzed by two-way ANOVA. Mice were challenged with .025, .05 and .1mg/kg eticlopride (ETI) via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between ETI doses.

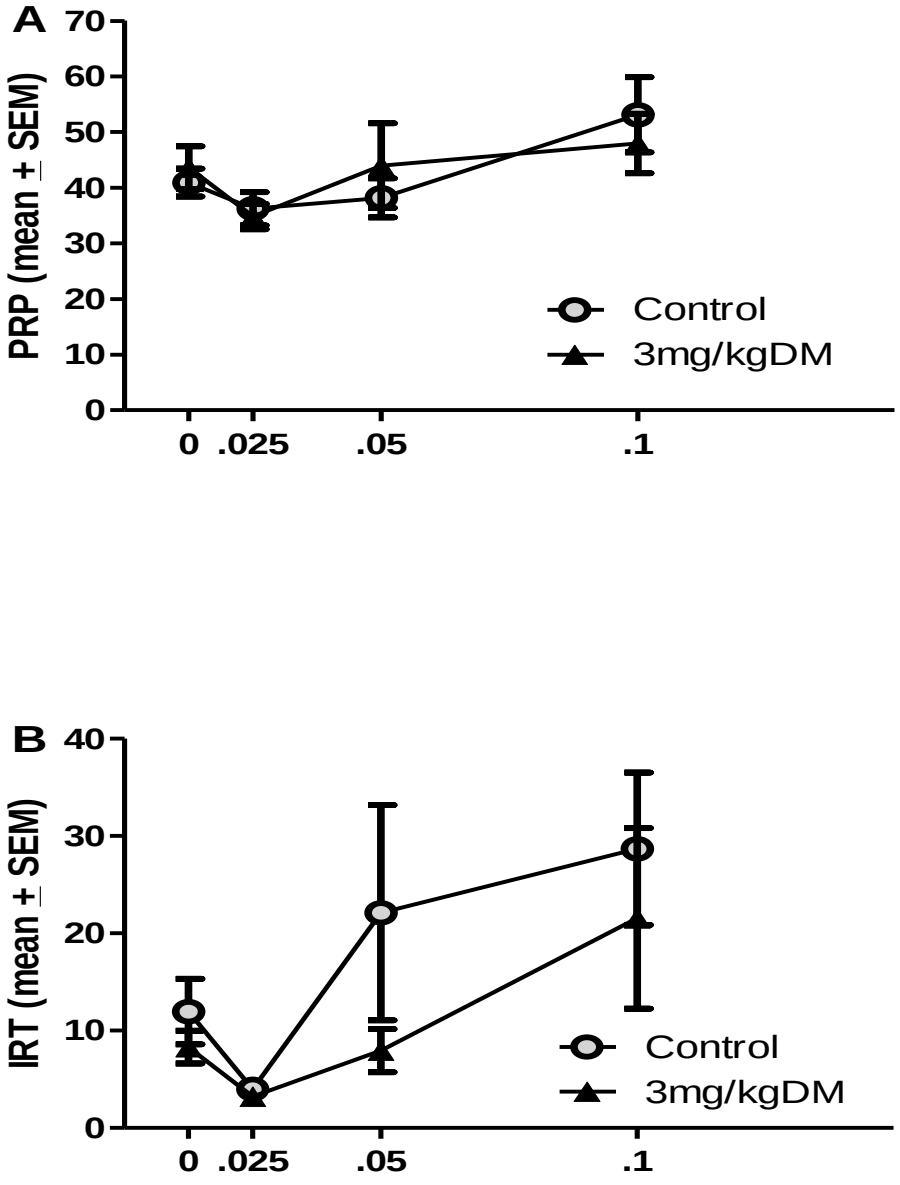

Figure 18. Effects of D2 receptor antagonist eticlopride on PRP (A) and IRT (B) on a FI60s schedule of reinforcement in females exposed to 0 or 3mg/kg DM during development. Data represent mean ± SEM (n=6-7) and were analyzed by one-way ANOVA. Mice were challenged with .025, .05 and .1mg/kg eticlopride (ETI) via i.p. injection 15min prior to behavioral testing. Saline values represent the mean (± SEM) of the three intervening saline days between ETI doses.

Autoradiographical Analyses

Figure 19 depicts autoradiographical measurements in the STR of D1 and D2 DA receptor binding in males only. As shown graphically (Fig. 19A), there was a robust increase in D1 binding. Binding values for DM-treated mice(575.316 $\pm$ 44.667) were 58% higher than those in the control group (365.065 $\pm$ 51.338; (t[10]=3.09; p=.0114). D1 autoradiographs of mice perinatally exposed to corn oil vehicle and DM (Fig.s 20A and B respectively) illustrate that D1 binding appears to be increased in the STR of DM-exposed mice, particularly in the accumbens (nucleus accumbens core and shell, NAc and NAs respectively). As shown in figure 19B, there was a slight increase in D2 binding in the DM-exposed mice (mean control and DM binding values were 173.038 $\pm$ 9.433 and 201.379 $\pm$ 12.722 respectively). However, an unpaired t-test revealed no significant treated related difference (t[12]=1.789; p=.099).

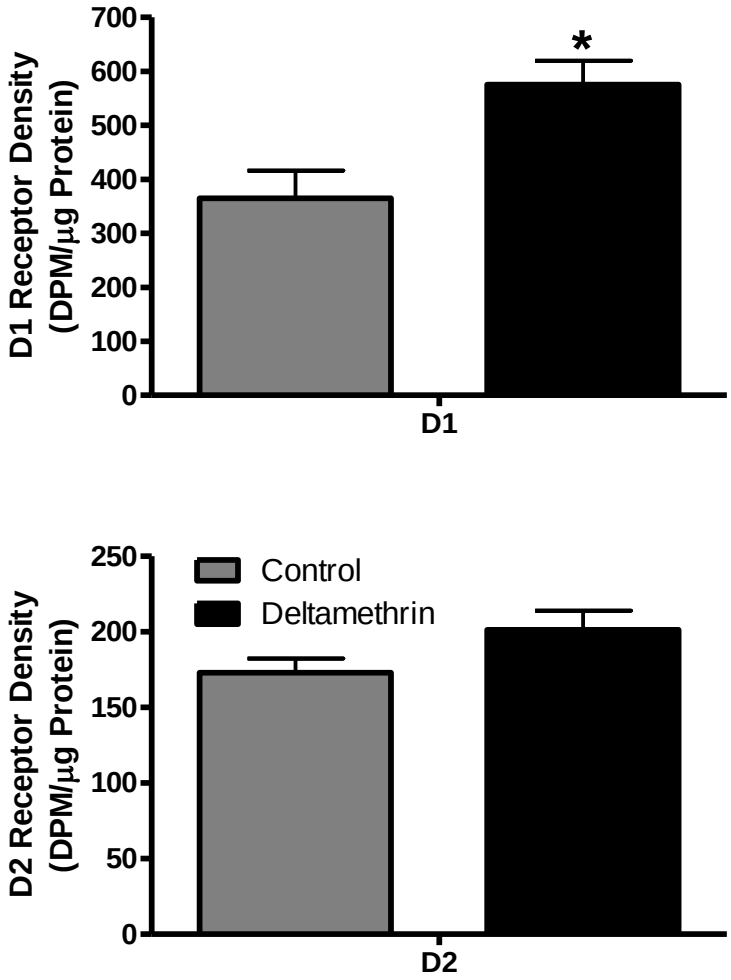

Figure 19. Mean D1 and D2 receptor binding values in male striatum. Group mean ± SEM of D1 and D2 DA receptor binding in the striatum of male mice exposed prenatally to either 0 or 3mg/kg DM. Mean D1 binding values were 365.065 and 575.316 for control and DM mice respectively ($p=.0114$). Mean D2 binding values were 173.038 and 201.722 for control and DM mice respectively ($p>.05$).

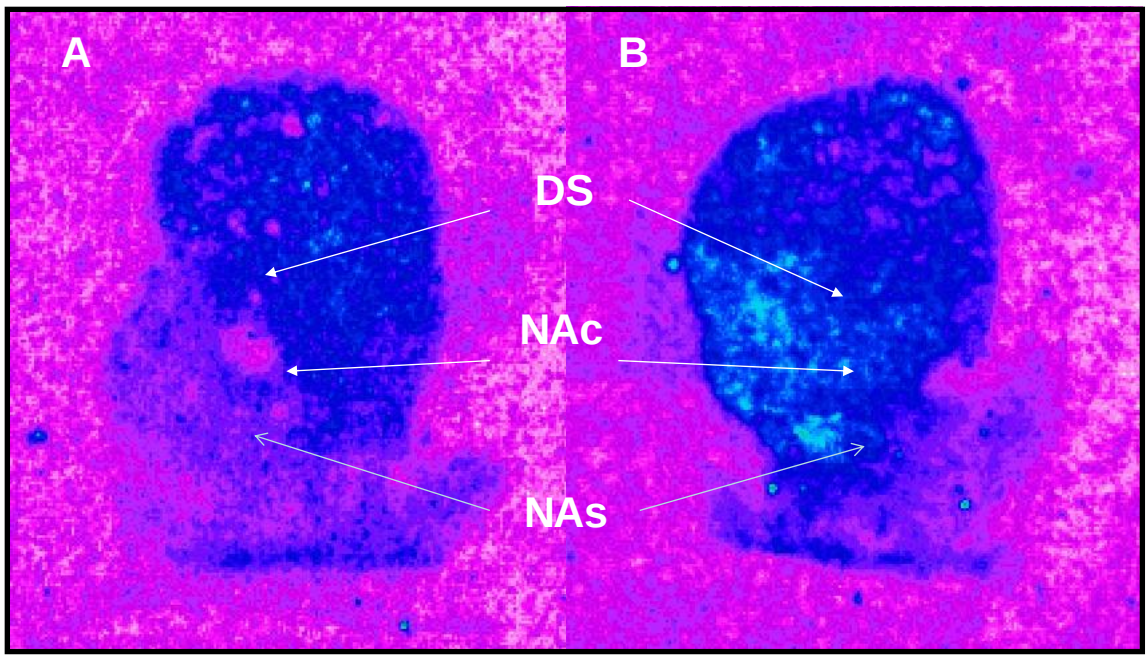

Figure 20. Representative autoradiograms measuring D1 receptors in males. Control (A) and DM-treated (B) mice. DS: dorsal striatum; NAc: nucleus accumbens core; NAs: nucleus accumbens shell.

Homogenate Binding

D1- and D2-like DA receptor binding in STR tissue homogenate of male and female mice exposed to either 0 or 3mg/kg DM is plotted in figure 21. Comparable to binding determined by autoradiography, there was a robust increase in D1 but not D2 binding. The increase in D1 density was found in males only. Indeed, mean D1 binding values were 40% higher in DM-exposed than in control males (470.2 $\pm$ 20.675 and 655 $\pm$ 16.994). Meanwhile, average D2 binding values in males did not differ (control: 193.6 $\pm$ 8.681; DM: 218.2 $\pm$ 8.249). There was no DM-related difference in either D1 (control: 385.8 $\pm$ 17.65; DM: 392.4 $\pm$ 12.086) or D2 (control: 257.4 $\pm$ 9.647; DM: 264.4 $\pm$ 18.84) binding in females. This was confirmed by a two-way ANOVA which revealed a main effect of treatment ($F[1,16]=31.21$, $p<.0001$) and gender ($F[1,16]=102.6$, $p<.0001$) as well as a treatment by gender interaction ($F[1,16]=27.05$; $p<.0001$) on D1 binding. In contrast, there was a main effect of gender ($F[1,16]=20.46$, $p=.0003$) but not DM ($F[1,16]=1.688$, $p=.212$) on D2 binding. A two-way ANOVA of D1 and D2 binding in control animals revealed that D1 and D2 binding significantly differed between genders ($F[3,32]=31.82$, $p<.0001$). Post-hoc analyses of control D1 and D2 binding revealed that compared to males, females had decreased D1 and increased D2.

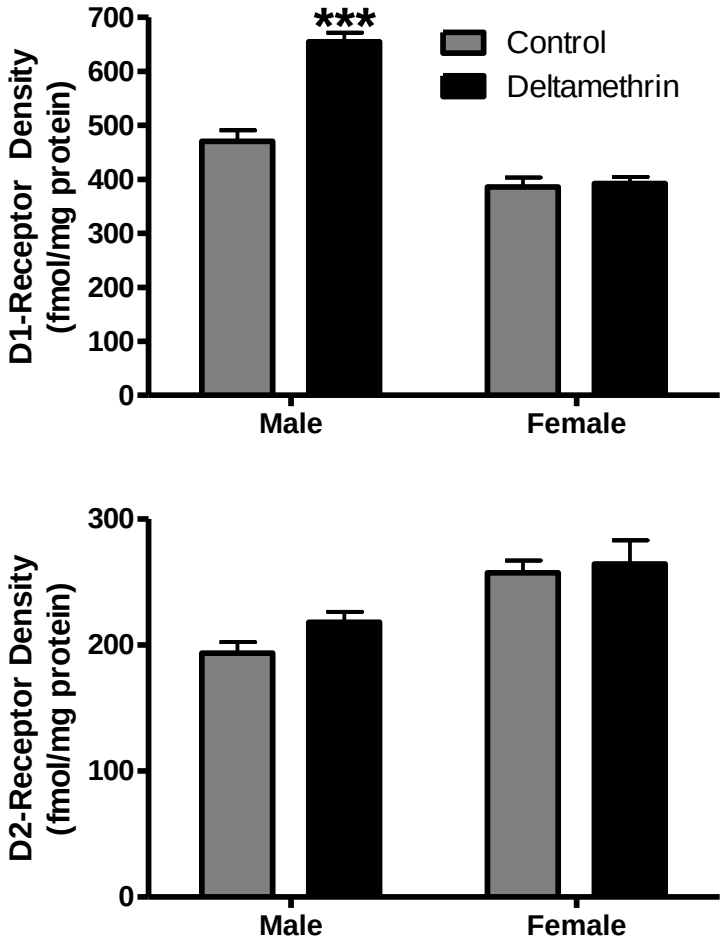

Figure 21. Striatal dopamine D1 and D2 receptor density in DM-exposed mice. D1 (top panel) and D2 (bottom panel) receptor density in male (left) and female (right) mice exposed to either 0 or 3mg/kg DM during development. Data are plotted as mean $\pm$ SEM (n=5).

DISCUSSION

Impulsivity has been operationally defined as preference for an immediate, small reward over a larger, but delayed reward. In normal children, Darcheville et al. (1992, 1993) demonstrated that increased rate of responding under a FI schedule of reinforcement is a predictor of impulsive response patterns. Similar results have also been reported in ADHD children (Sagvolden et al., 1998).We implemented the same FI schedule to explore whether perinatal DM exposure results in impulsive-like behavior as evidenced by increased response rates, shortened PRP and IRT. Such response patterning does not accelerate or magnify reinforcement; thus, responses emitted prior to the completion of the interval are inefficient and have been ascribed to impulsiveness. Here we demonstrate that DM exposure in pregnant mice produces impulsive-like behavior in the offspring. Specifically, perinatal DM exposure dose-dependently increased rate of responding in both genders. Averaged across all sessions, DM (3mg/kg) produced robust increases in RATE (males: 292%; females: 41%) and RR (males: 257%; females: 29%). The emergence of DM-dependent increases was evident by the third and fourth (males and females respectively) session block (which consisted of 5 sessions each). Furthermore, 3mg/kg DM decreased PRP by 29 and 2% and IRT by 66 and 1% in males and females respectively. Exposure of 1mg/kg DM also produced decreases in PRP and IRT. Administration of both ETI and SCH23390 effectively returned many of these impulsive-like behaviors to control levels. Autoradiographic and homogenate binding data suggest a prominent role for D1 receptors. These data are consistent with the profile of effects observed in ADHD patients (Darcheville et al., 1992, 1993).

The dopaminergic system has been implicated in the manifestation of impulsive-like behaviors (Cardinal et al., 2001). Impulsivity is the most disruptive symptom in ADHD which often manifests as dangerous behavior in both children (such as darting into the street without first looking for traffic or climbing then jumping from high places like a roof or tree) and teens (drinking and driving or drug use) (Malone et al., 1993; Rappaport et al., 1986; Schweitzer et al., 1995; Sonuga-Barke et al., 1992; Sonuga-Barke et al.). Selective lesions to the nucleus accumbens core induce persistent impulsive choice in rats (Cardinal et al., 2001). Prior to lesioning, the rats were given a choice between a small, immediate and large, delayed reward. There was no difference in choice between the sham or lesion groups. Then, the rats were lesioned in the nucleus accumbens core, the anterior cingulated cortex and medial prefrontal cortex. Lesions in the nucleus accumbens core produced a robust and persistent deficit in the rats' ability to choose the delayed reinforcer (Cardinal et al., 2001).

Data from the Richardson laboratory demonstrate that DM exposure during development increases DAT mRNA and protein levels in mice which may produce a hypodopaminergic state. This in turn may result in altered D1 and D2 receptor responsiveness. It has been demonstrated that the nuclear transcription factors Nurr1 and Pitx3 control DAT levels during development (Martinat et al., 2006; Smidt et al., 2004; Smits et al., 2003). Further, increased Nurr1 levels have been linked to increased neuronal activity (Brosenitsch et al., 2001; Volpicelli et al., 2007) and DM has been shown to increase neuronal activity and enhance dopamine release (Mubarak et al., 2006; Narahashi, 1996). DM significantly increases Nurr1 and Pitx3 mRNA preferentially in males. It is suggested that developmental DM increases DAT through the coordinate up-

regulation of Nurr1 and Pitx3 expression, resulting in a hypodopaminergic state. In turn, the hypodopaminergic state alters D1 sensitivity and produces the ADHD-like profile effects in mice such as increased FI response patterns. Such response patterning is highly inefficient as it does not accelerate reinforcement availability and has been ascribed to impulsivity (Brockel et al., 1998, 1999; Rice, 1992). To determine the role of the DA system in DM-associated FI changes, we examined whether antagonism of D1 and D2 receptors would ameliorate DM-induced FI changes. Acute administration of the D1 antagonist, SCH 23390, reduced DM-induced RATE and RR increases while it concomitantly lengthened PRP and IRT in both genders. Notably, control and DM groups receiving saline no longer differed significantly for PRP (both genders) and IRT (males only). Therefore, comparing the impact of SCH 23390 on these two dependent measures is inappropriate. Nevertheless, in males, SCH 23390 did increase PRP and IRT. In fact, SCH 23390 administration increased PRP and IRT beyond control levels. Each drug challenge was followed by two drug free days, a no injection day then saline injection day during which rate of responding returned to baseline.

Similarly, ETI reduced DM-induced RATE (males only) and RR (both genders) increases. In addition, increases in PRP (both genders) and IRT (males only) were decreased following ETI exposure. It is important to note that female mice became increasingly difficult to handle and inject as antagonist testing progressed, negatively impacting performance on the baseline FI and complicating DM-related comparisons. This was particularly apparent during ETI testing which followed SCH 23390 in the drug test sequence and may suggest the emergence of behavioral sensitization. However, this was not directly examined and needs to be explored further. By the time we began the

ETI dose response there was no longer a significant difference in RATE or IRT between female saline and DM groups. In general, all of the female mice became progressively more difficult to handle for injections and place in as well as remove from the operant chambers. They were more active during home cage observation and extremely hard to capture and hold for injections, many bit every time they were handled. It may be that stress hormones released during handling and injection just prior to testing during these drug challenges brought these two baselines together. Control and DM-exposed females may simply habituate at a different rate. However, given the home cage observations and difficulty handling, future studies should include collection of blood for hormone levels and regression analyses conducted to compare baseline values and hormone levels to rule out stress in alteration of this baseline. The collection of blood in and of itself is stressful and if done after the session may reduce this confounding variable. Finally, DM exposure did not modify the effects of SCH 23390 or ETI, which uniformly exerted performance changes across all perinatal exposure groups. While SCH 23390 was more effective, both antagonists generally ameliorated the DM-induced behavior changes by decreasing RATE, RR and increasing PRP and IRT. Previously our lab showed administration of SCH 23390 and ETI both reduce DM-induced hyperlocomotion, although SCH 23390 was slightly more effective (data not shown). Furthermore, administration of the full D1 agonist SKF82958, (0.5mg/kg) increased locomotor activity by 137% in control mice. DM-treated mice exhibited a much greater response than control mice in that locomotor activity increased by 208% (data not shown). Contrastingly, administration of quinpirole, a D2 autoreceptor agonist reduced hyperactivity in males but not females to control levels (data not shown). Collectively,

these data suggest a hyper-responsive state at the post-synaptic level due to either enhanced responsiveness or levels of D1 receptors.

Further bolstering the notion that D1 receptors play a critical role in DM-induced FI changes, robust increases in striatal D1 receptor density in both autoradiography and homogenate binding were found. There were no significant alterations observed in DM-exposed females. D2 receptor density was unchanged in both genders. Previous autoradiographic data has shown that D1 receptors are present in higher density than D2 receptors in all brain structures except VTA and pituitary gland (Aiso et al., 1987; Boyson et al., 1986; Dawson et al., 1988; Huang et al., 1992; Levey et al., 1993; Richfield et al., 1989; Yung et al., 1995). The highest density of D2 receptors are found in the NAc, olfactory tubercle, olfactory bulb (glomelular layer) and caudate putamen (Bouthenet et al., 1985; Boyson et al., 1986; Charuchinda et al., 1987; Dawson et al., 1986; Jastrow et al., 1984; Levey et al., 1993; Palacios et al., 1981; Richfield et al., 1989). This is consistent with what we found in our autoradiographic and homogenate binding data: control mice had higher D1 density than D2. While D1 receptors in ADHD patients have not been directly measured, two studies have reported a potential association between D1 dopamine receptor polymorphisms with ADHD (Bobb et al., 2005; Misener et al., 2004). Yet another study demonstrated an association between the D1 receptor interacting protein, calcyon, with ADHD (Laurin et al., 2005). Modest alterations in receptor density can have a dramatic impact on dopaminergic tone by modifying uptake, release, or synthesis.

It is well established that dopamine can act on two types of receptor; D1 and D2 (Cooper et al., 2003), each distinguished by whether they stimulate or inhibit the enzyme

adenylyl cyclase (Fig. 22). D1-like receptors activate adenylyl cyclase and stimulate cyclic adenosine monophosphate (cAMP) via stimulatory G protein. This results in increased cAMP formation and the activation of cAMP-dependent protein kinase A (PKA) which in turns results in increased phosphorylation of DARPP-32 (dopamine and cAMP-regulated phosphoprotein). Phospho-DARPP-32 inhibits protein phosphatase-1 which in turn increases the phosphorylation state of numerous phosphoproteins involved in the regulation of important physiological processes. In contrast, D2-like receptors decrease DARPP-32 phosphorylation via two pathways. One mechanism involves inhibition of adenylyl cyclase, thereby decreasing cAMP and PKA activity (depicted in Fig. 22). Another mechanism involves increased intracelular calcium and calcineurin activation which in turn increases dephosphorylation of phospho-DARPP-32 (not depicted in Fig. 22). Increased phosphorylation of phospho-DARPP-32 is associated with decreased activity of the Na+/K+-ATPase, GABA receptors and Na+ channels and with increased activity of NMDA and AMPA glutamate receptors, L-, N-, and P-type calcium channels and CREB (Greengard et al., 1999). The disparate actions of these two receptor subtypes may facilitate understanding of DM-induced changes in FI behavior. D1-like receptors have been implicated in learning (Beninger et al., 1989; Beninger et al., 1998; Beninger et al., 1995). Evidence of D1 receptor involvement in reward-based learning has been observed by several investigators (Fowler et al., 1994; Hoffman et al., 1988, 1989; Hunt et al., 1994). Moreover, D2 agonists and antagonists have been shown to produce perseveration and extrapyramidal side effects (Fowler et al., 1994; Senyuz et al., 1993) respectively. Beninger and Miller (1998) suggest that D1 receptors mediate reward value and are involved in reward based learning, while D2 receptors are more

strongly linked to impaired motor performance. Increased D1 but not D2 receptor levels and homogenate binding in males in the present experiments suggest that reward value may have been modified in DM-exposed mice, resulting in a shift in the FI baseline. One possible mechanism for the DM-induced shift in baseline may relate to dopamine that is released during reward, followed by D1 receptor stimulation and cAMP/PKA activation. Stimuli presented just prior to reward may activate glutamatergic synapses. Therefore, as learning on the FI schedule progresses, dopamine acting on D1-like receptors changes in the striatum which form the basis of FI performance.

Modification of dopamine receptor function may also play a role in the expression of impulsive-like behavior. For instance, lead exposure has been demonstrated to produce impulsive-like behavior in rats and decreases in DA D1, D2 and DAT binding in the nucleus accumbens but not in the dorsal striatum (Pokora et al., 1996). Brockel et al (1999) examined whether acute administration of both D1 (SKF82958) and D2 (quinpirole) agonists and antagonists (SCH 23390 and eticlopride, respectively) altered baseline performance of lead-exposed rats on a fixed-ratio waiting for reward (FRWAIT) paradigm. Further, they reported that the D2 agonist quinpirole reversed the impulsive-like behavior produced by lead. In addition, D2 compounds exerted greater magnitude effects than did D1 compounds. Spontaneously Hypertensive Rats (SHR), which are often used as an animal model of ADHD. During the selection process of the SHR strain for familial hypertension (Okamoto, 1969), it was discovered that the rats were hyperactive. The strain has been utilized since as a model of ADHD since they exhibit increased motor activity in an open field (Hendley et al., 1985; Sagvolden et al., 1993; Tilson et al., 1977) and in mazes (Aspide et al., 1996) and show alterations in FI

performance (Kantak et al., 2008; Sagvolden et al., 2005). It has also been demonstrated that this strain has a higher density of D1-like receptors in the dorsal striatum, as well as in the nucleus accumbens core and shell (Carey et al., 1998). Interestingly, methylphenidate treatment returned D1-like receptor density to control levels in SHR rats (Carey et al., 1998). The increase in D1 but not D2 receptor density in the present experiments is consistent with these findings.

Our data suggest D1 receptors are intricately involved in mediating DM's effect on behavior. However, additional evaluation of D1 and D2 receptor agonist and antagonist administration on DM-induced behavioral changes is warranted, with careful attention to the arrangement of drug test order. Moreover, systematic co-administration of dopamine receptor agonists and antagonists may shed light on possible D1 and D2 interactions. Finally, during antagonist testing, the robust DM-induced changes in baseline FI performance began to wane. Any future replication should ensure maintenance of baseline performance by having more days in between agonist or antagonist drug challenges. These data implicate developmental DM exposure in alterations of the dopaminergic milieu and consequent impulsive-like behavior in mice

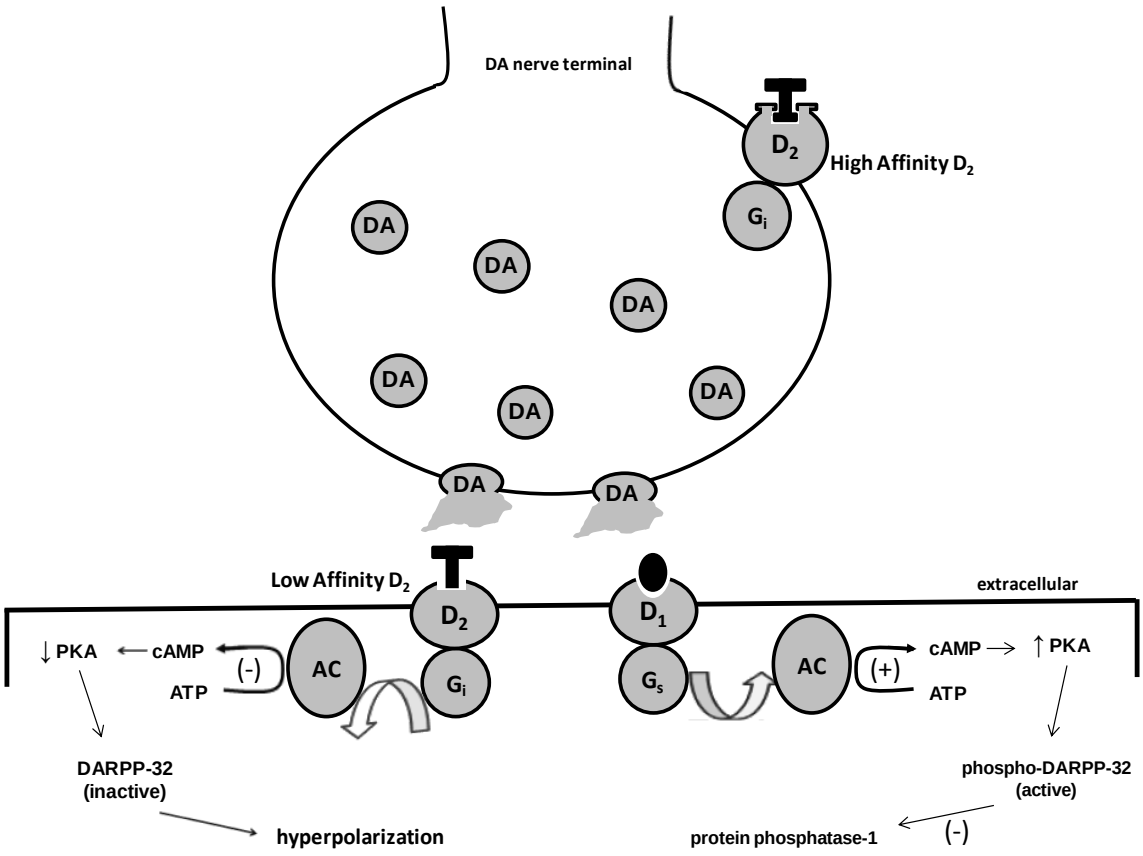

Figure 22. Schematic diagram depicting the different roles of D_1 and D_2 receptors on intracellular adenylate cyclase (AC) activity (modified from (Cooper et al., 2003)

CHAPTER 4:

PERINATAL DELTAMETHRIN EXPOSURE PRODUCES DECREMENTS IN WAITING BEHAVIOR: EFFECTS OF METHYLPHENIDATE

INTRODUCTION

In the previous chapter, we demonstrated that developmental DM exposure produces increases in response rates on a fixed-interval (FI) schedule of reinforcement. This response patterning is highly inefficient as it does not accelerate reinforcement availability and has been ascribed to impulsivity (Brockel et al., 1998, 1999; Rice, 1992). Although studies have reported increased FI responses are associated with impulsivity in humans (Darcheville et al., 1992, 1993; Sagvolden et al., 1998; Sagvolden et al., 1996) impulsivity is operationally defined as preference for an immediate, small reward over a larger, but delayed reward. The behavioral mechanism driving impulsivity has also been ascribed to delay aversion (Sonuga-Barke et al., 1992). The aversion to delay hypothesis states that impulsive behavior in ADHD children represents an adaptive response to a particular situation as opposed to an inability to withhold inappropriate responses (Sonuga-Barke et al., 1992). Therefore, children diagnosed with ADHD are believed to be more sensitive to the passage of time before a reward can be obtained. This, rather than the inability to withhold responding or an attempt to maximize reward, is the reason for choosing immediate small rewards (Sonuga-Barke et al., 1992). While the FI schedule of reinforcement is a time-based schedule, reinforcement density and the time

intervals remain constant across the session. Therefore, increased rate of responding does not produce more immediate or increased reinforcement.

To more specifically address whether DM exposure alters waiting behavior, the ability to inhibit responding and response to delays in reinforcement, we utilized a fixed ratio waiting for reward (FRWAIT) paradigm (as modified from (Bizot et al., 1988) and used previously in lead exposed rats (Brockel et al., 1998, 1999). The task involves completion of the initial FR25, after which "free pellets" are delivered at increasing time intervals (5, 10, 15sec, etc). Intervening responses reset the FR requirement. The outcome measures were FR rate of responding (total number of responses divided by total FR component time), mean longest time to wait for a free pellet (longest time waited before emitted a response, resetting the FR component), responses per reinforcer (the total number of responses divided by the total number of reinforcers). Lead exposure in rats decreased the longest wait time and increased response rates and resetting of the fixed ratio component which in turn resulted in a greater amount of reinforcers being delivered. However, the lead-exposed group emitted doubled the number of responses per reinforcer as did the controls, resulting in an inefficient response pattern. (Brockel et al., 1998, 1999). The present study was designed to determine whether DM exposure in mice alters waiting behavior, the ability to inhibit responding and produces delay aversion. Furthermore, we wanted to determine whether administration of the common ADHD therapeutic methylphenidate (Ritalin®) would reverse DM-induced impulsive-like behavior.

RESULTS

FR Component Performance

The response rates stabilized in both males and females (Fig.'s 22A and B respectively). DM exposure was associated with higher response rates in both males and females as revealed by a significant main effect of DM ($F[1, 22]=5.556$; $p=.0277$) but not gender ($F[1,22]=.169$; $p=.6847$) or DM by gender interaction ($F[1,22]=1.662$; $p=2.108$). Averaged across all of the sessions, male DM treated mice emitted double the number of responses per minute than control mice (males: 100 and 42; females: 85 and 69, DM and control rates respectively). Figures 23A (males) and B (females) depicts robust increases in the number of FR component resets (RESETS) in male DM treated mice. This was confirmed by an overall effect of DM but not gender (DM: $F[1,22]=14.385$; $p=.0010$; gender: $F[1,22]=1.748$; $p=.1997$). In fact, male but not female DM-treated mice reset the FR component twice as often as controls (males: 14 and 5; females: 9 and 6).

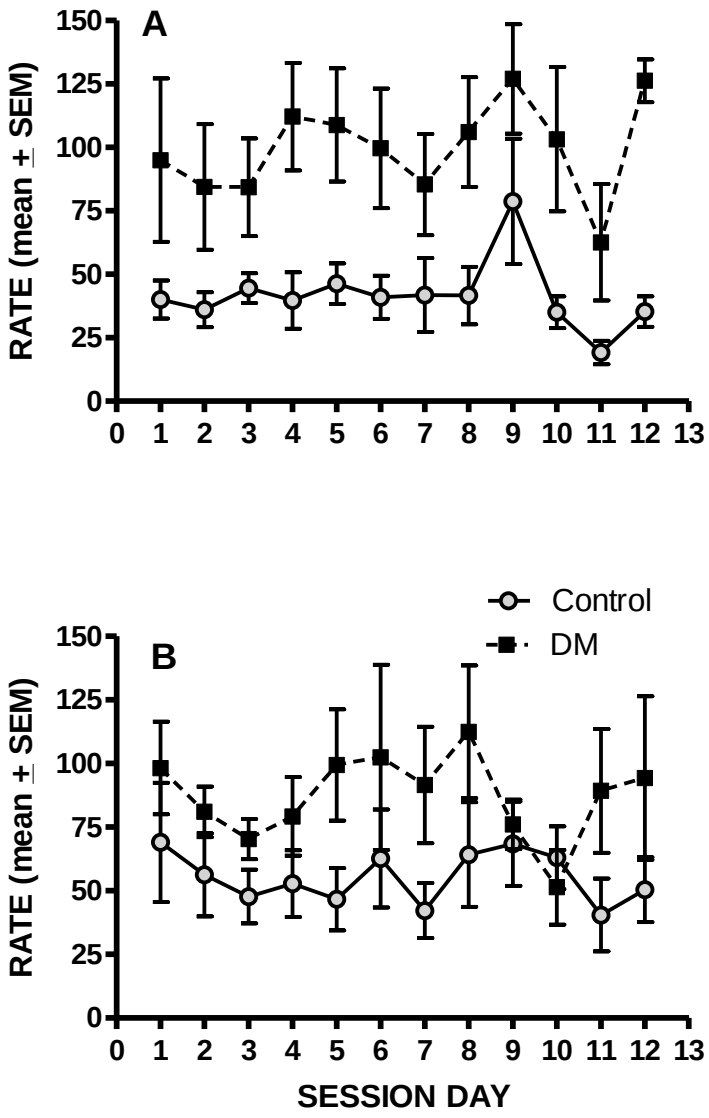

Figure 23. Rate of responding on a FRWAIT schedule in mice exposed perinatally to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and (B) female mice exposed to 0 (grey circles) or 3mg/kg (black squares) DM.

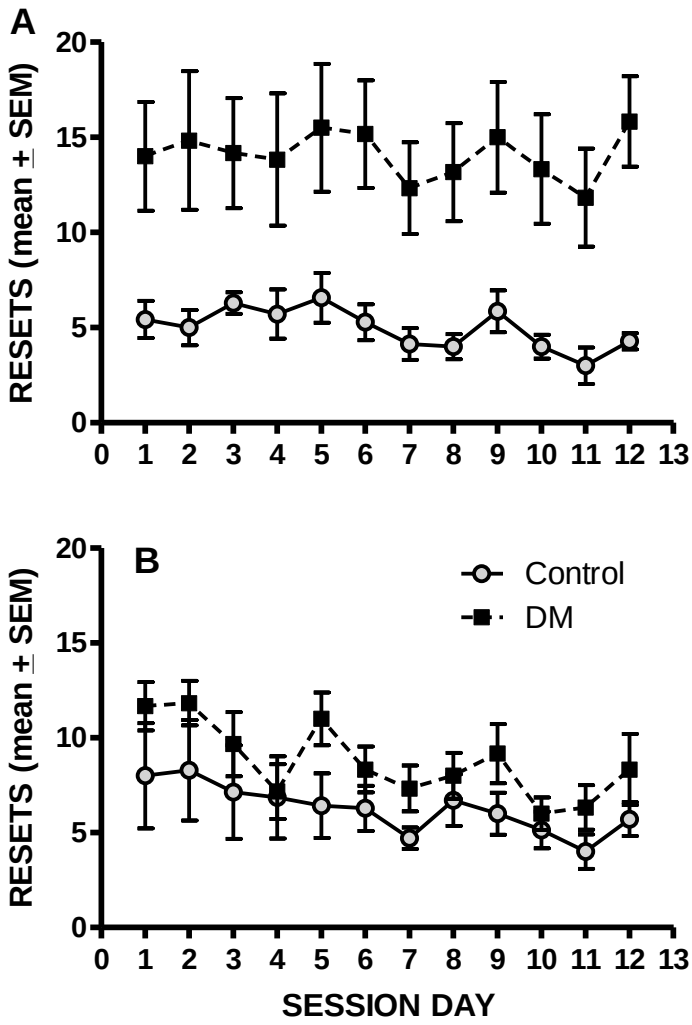

Figure 24. FR resets on a FRWAIT schedule in mice exposed to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and (B) female mice exposed to 0 (grey circles) or 3mg/kg (black squares) DM.

Waiting Behavior

DM exposure resulted in significantly shorter WAIT times in both genders. A RMANOVA revealed a significant main effect of DM ($F[1,22]=14.09$; $p=.0011$), although there was no effect of gender ($F[1,22]=1.075$; $p=.3110$) or gender by DM interaction ($F[1,22]=3.045$, $p=.0949$). Control mice consistently waited longer (18 and 7sec, males and females respectively) before resetting the FR component (Fig.'s 24A and B). Shortened WAIT times and increased RESETS in the DM-exposed group produced a concomitant increase in the number of reinforcers earned for waiting ($F[1,22]=11.542$, $p=.0026$). Specifically, male and female DM mice earned 27 and 13% more "free" reinforcers than their control counterparts respectively (Fig.'s 25A and B).

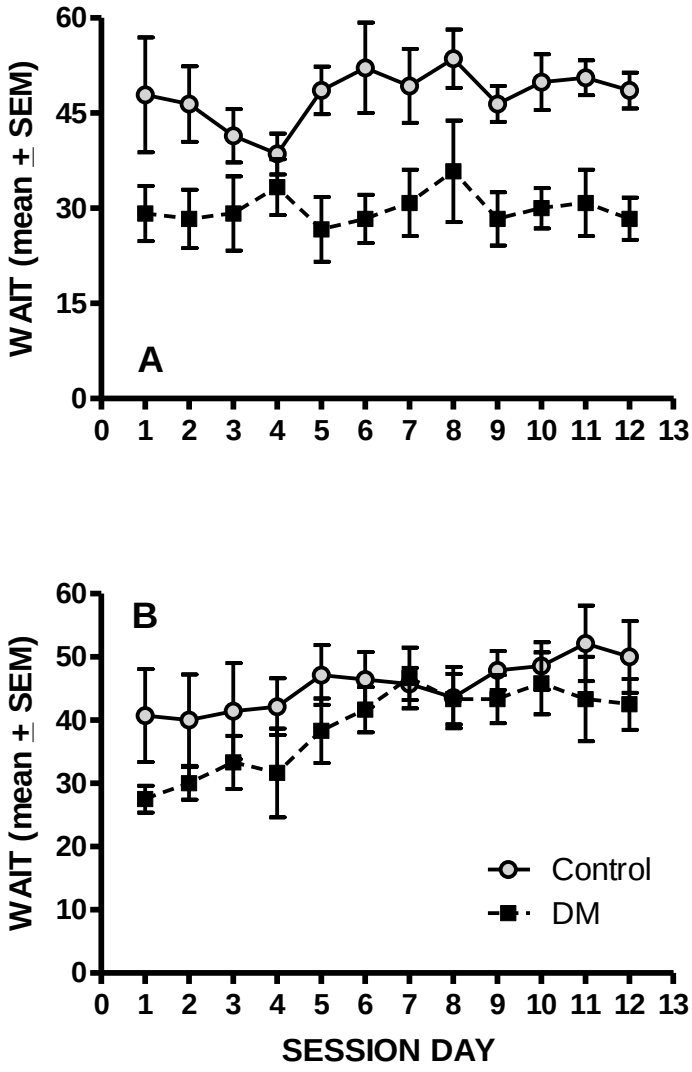

Figure 25. Longest wait time on a FRWAIT schedule in mice exposed perinatally to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and (B) female (bottom panel) mice exposed to 0 (grey circles) or 3mg/kg (black squares) DM.

Response Patterning

Shorter wait times and increased resetting suggest more efficient response patterning in the DM-exposed mice. By resetting the FR component, WAIT time returns to the base value, resulting in more immediate reinforcement availability. Even though DM-exposed mice earned more "free" reinforcers, this was at a higher response cost. In fact, DM-exposed mice emitted 33 and 7% (males and females respectively) more responses per reinforcer than controls, reflecting inefficient responding (Fig.'s 26A and B). This was confirmed statistically (DM: $F[1,22]=7.950$, $p=.0100$). There was no effect of gender ($F[1,22]=.300$, $p=.5897$) or a treatment by gender interaction ($F[1,22]=2.946$, $p=.1001$) however.

A ratio of WAIT over time to complete an FR was calculated to determine the extent to which differences in response rate factor into the DM effect on WAIT. A ratio value of 1 would indicate that prior to resetting the FR component, time between "free" pellets is equivalent to the amount of time needed to complete an FR25 (Brockel et al., 1998). Ratios less than 1 reflect early resetting, in other words, the time to complete an FR is longer than the WAIT time (Brockel et al., 1998). DM mice did not earn reinforcers sooner than controls by resetting the FR than could be achieved by waiting. This was confirmed statistically by a non-significant main effect of DM ($F[1,22]=.005$, $p=.9424$) and gender ($F[1,22]=1.939$, $p=.1777$).

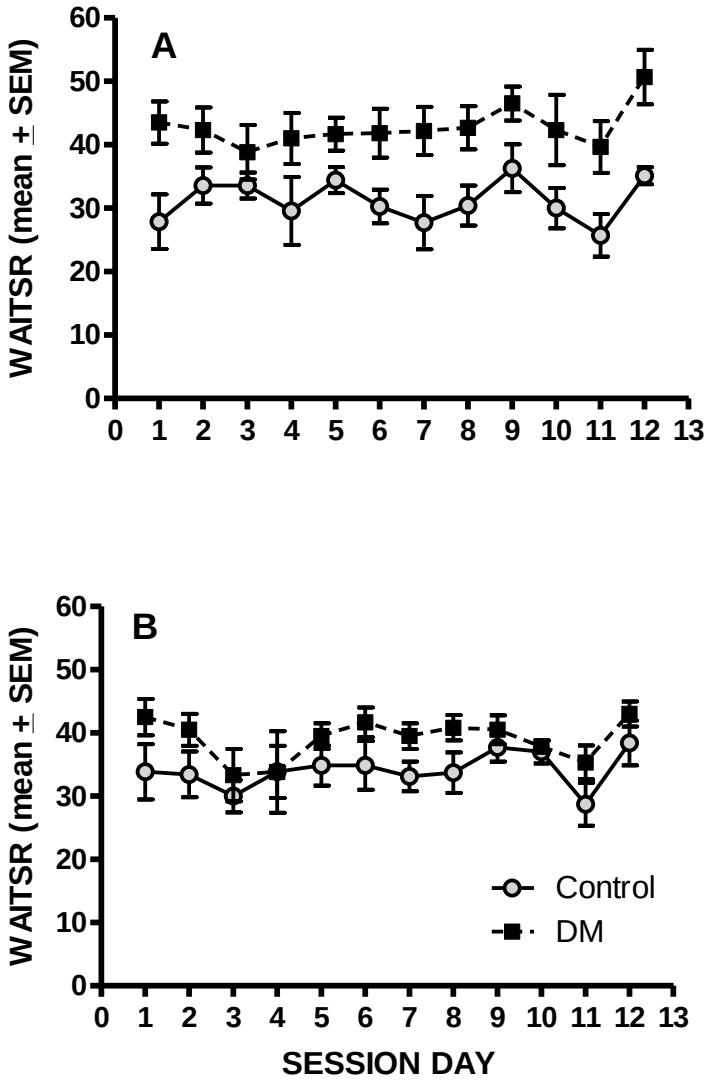

Figure 26. Wait reinforcers earned on a FRWAIT schedule in mice exposed perinatally to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and (B) female mice exposed to 0 (grey circles) or 3mg/kg (black squares) DM.

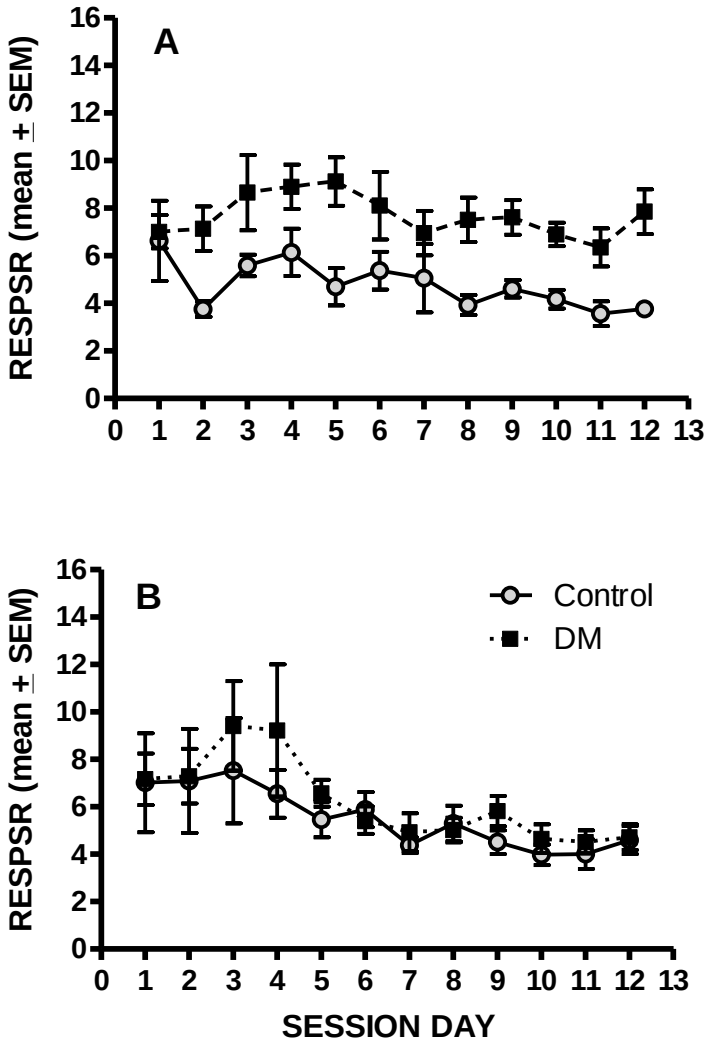

Figure 27. Responses/reinforcer on a fixed-ratio waiting for reward schedule in mice exposed perinatally to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and female mice exposed to 0 (light grey circles) or 3mg/kg (black squares)

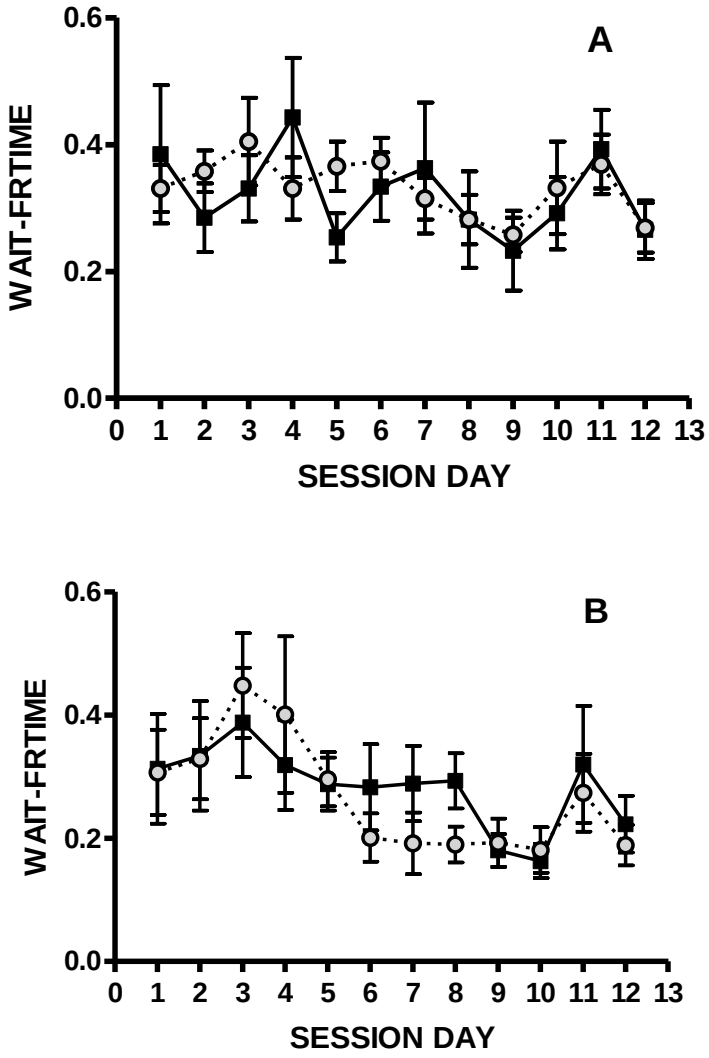

Figure 28. Ratio of wait over time to complete a FR on a fixed-ratio waiting for reward schedule in mice exposed perinatally to 0 (n=7) or 3mg/kg (n=6) DM. (A) Male and (B) female mice exposed to 0 (light grey circles) or 3mg/kg (black squares)

Effects of Methylphenidate

The dose response function of MPD was determined in both genders. Interestingly, pilot testing revealed that male and female mice responded differently to MPD. Specifically, MPD ameliorated DM-induced behavior changes at a lower dose in female than in males. Furthermore, the pre-session injection time at which MPD returned DM-induced reductions of waiting behavior was longer in females (15 min) than in males (5 min). Therefore, the dose response functions were conducted differently in male and female mice. Male mice were given .5, 1, 2, 4, 6 and 8mg/kg MPD 15min (Fig.'s 28-32, top panels) and 4, 6 or 8mg/kg MPD 5min (Fig.'s 28-32, middle panels) prior to behavioral testing. Females were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing (Fig.'s 28-32, bottom panels). Each MPD day was separated by two days, one injection free day followed by a saline control day. The pilot testing and dose response functions revealed the effective doses of MPD in DM-exposed male (8mg/kg, 5min pre-session injection) and female (4mg/kg, 15min pre-session injection) mice.

Figures 33 and 34 (males and females respectively) illustrate that MPD treatment reduced DM-induced rate increases in both genders. A two-way ANOVA revealed an overall significant effect of MPD treatment (given 15 and 5min prior to testing) on RATE in males (15min: $F[1,84]=154.6$, $p<.0001$; 5min: $F[1,48]=27.30$, $p<.0001$). A two-way ANOVA did not reveal an overall significant effect of MPD treatment in females ($F[1,60]=2.770$, $p=.1013$). Post hoc analysis however, showed that control and DM-exposed mice of both genders given saline did not differ significantly ($p>.05$).

MPD treatment impacted RESETS in both males and females (Fig.'s 33 and 34B respectively) as well. A two-way ANOVA revealed a significant overall effect of DM in both males (15min: $F[1,84]=103.8$, $p<.0001$; 5min: $F[1,48]=93.05$, $p<.0001$) and females ($F[1,60]=15.29$, $p=.0002$). While MPD treatment reduced the number of resets in both genders, post hoc analysis revealed a significant reversal in females, and only after 1, 2 and 4mg/kg MPD; it also revealed that DM and control females did not differ significantly following saline injection. In fact, post-hoc analysis showed that female resets differed between DM and controls only following .5mg/kg MPD. Male wait length increased in duration following MPD; however not significantly. A two-way ANOVA revealed an overall effect of DM on waiting behavior in males (15min: $F[1,84]=84.76$; $p<.0001$; 5min: $F[1,48]=24.12$, $p<.0001$) but post-hoc analysis showed that MPD treatment did not significantly increase wait length at any dose or pre-session injection time. Wait time in females exposed to DM did not differ significantly from controls following saline ($F[1,60]=3.154$, $p=.0808$) or following any dose of MPD. Two-way ANOVA showed a significant overall effect of DM in males on the number of responses emitted per reinforcer (15min: $F[1,84]=237.7$, $p<.0001$; 5min: $F[1,48]=78.23$, $p<.0001$). Post-hoc analysis revealed that MPD did not reduce the number of responses emitted per reinforcer at any dose or pre-session injection time. The number of responses females emitted per reinforcer did not differ significantly between DM and controls before or after MPD treatment.

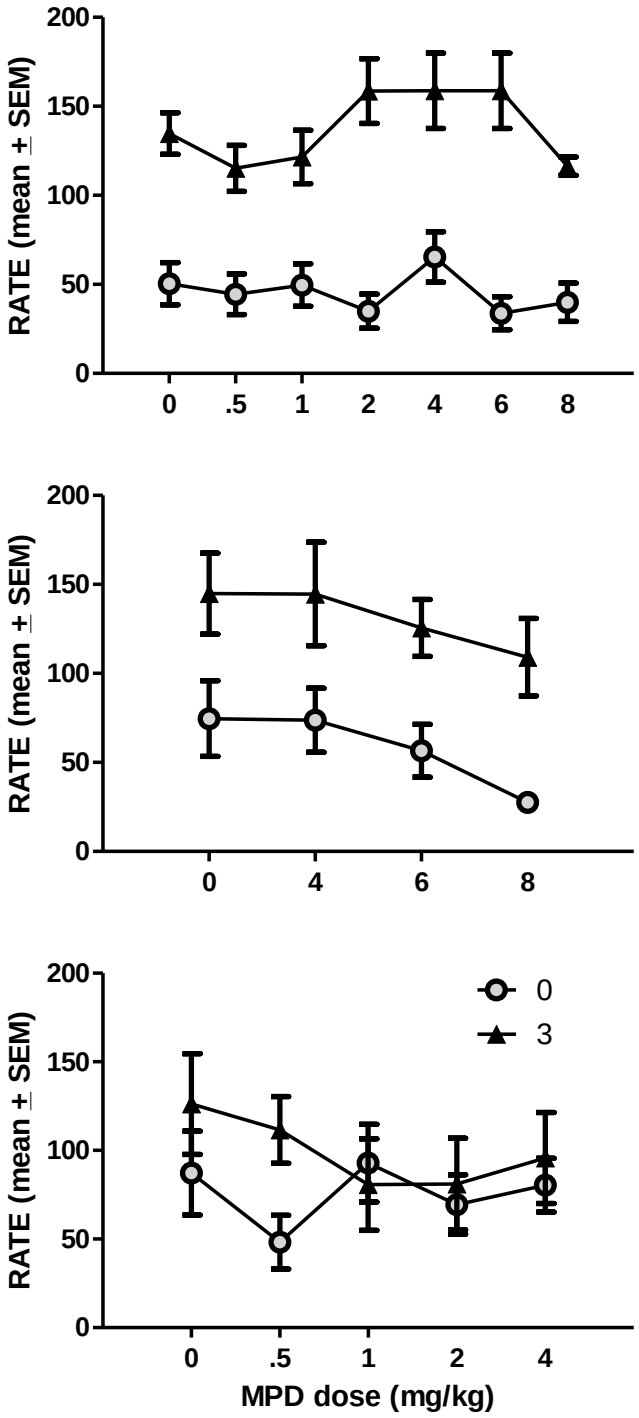

Figure 29. Dose response effect of MPD on rate of responding under a FRWAIT schedule. Rate plotted as mean ± SEM. Males given MPD via oral gavage 15 and 5min prior (top and middle panels respectively) to behavioral testing. Females (bottom panel) were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing. n=7 for all groups.

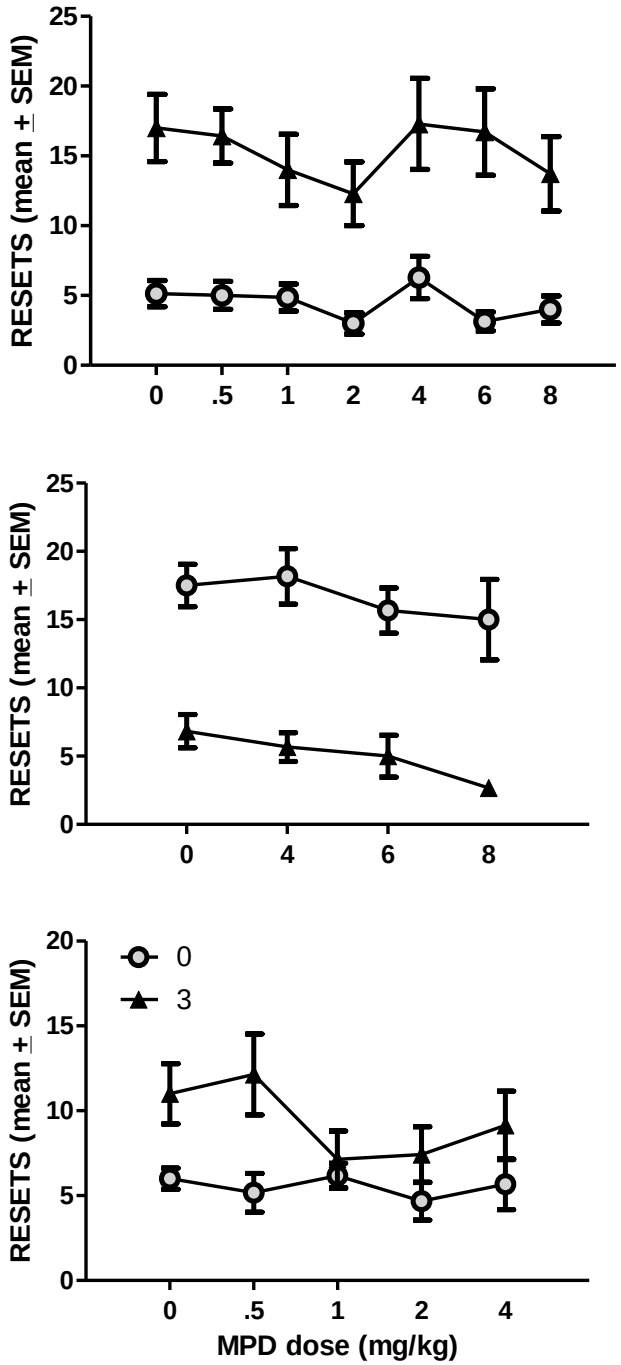

Figure 30. Dose response effect of MPD on resets under a FRWAIT schedule. Rate plotted as mean ± SEM. Males given MPD via oral gavage 15 and 5min prior (top and middle panels respectively) to behavioral testing. Females (bottom panel) were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing. n=7 for all groups

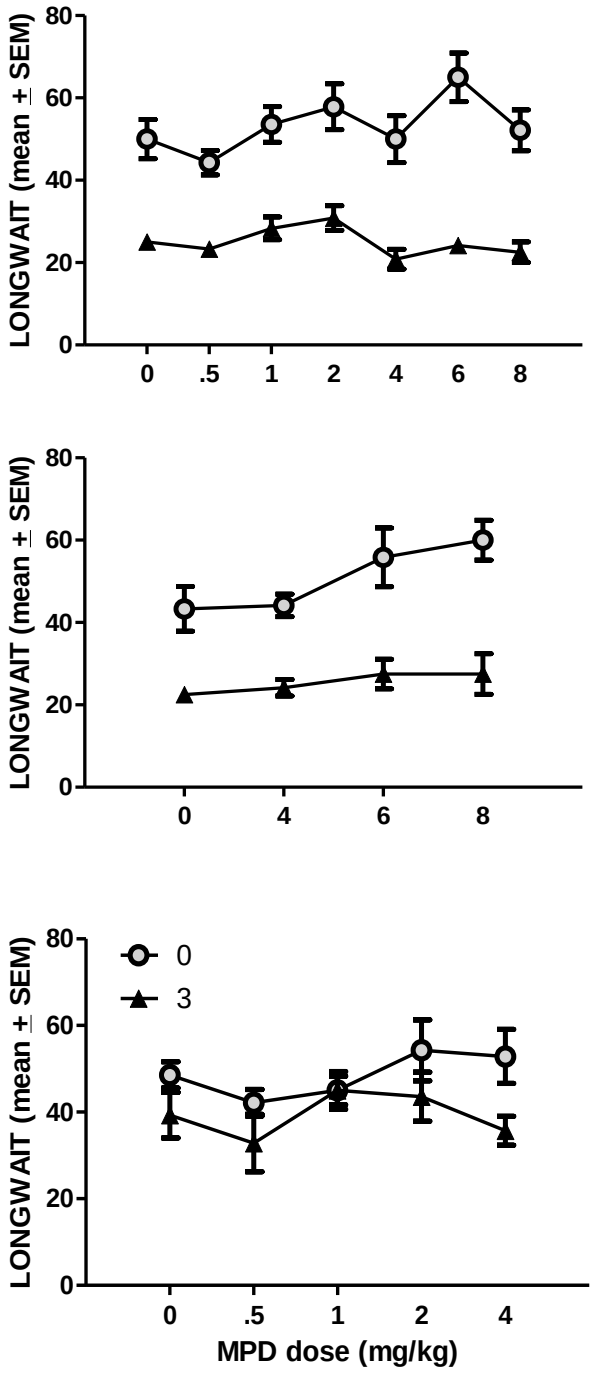

Figure 31. Dose response effect of MPD on longest wait time under a FRWAIT schedule. Rate plotted as mean ± SEM. Males given MPD via oral gavage 15 and 5min prior (top and middle panels respectively) to behavioral testing. Females (bottom panel) were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing. n=7 for all groups.

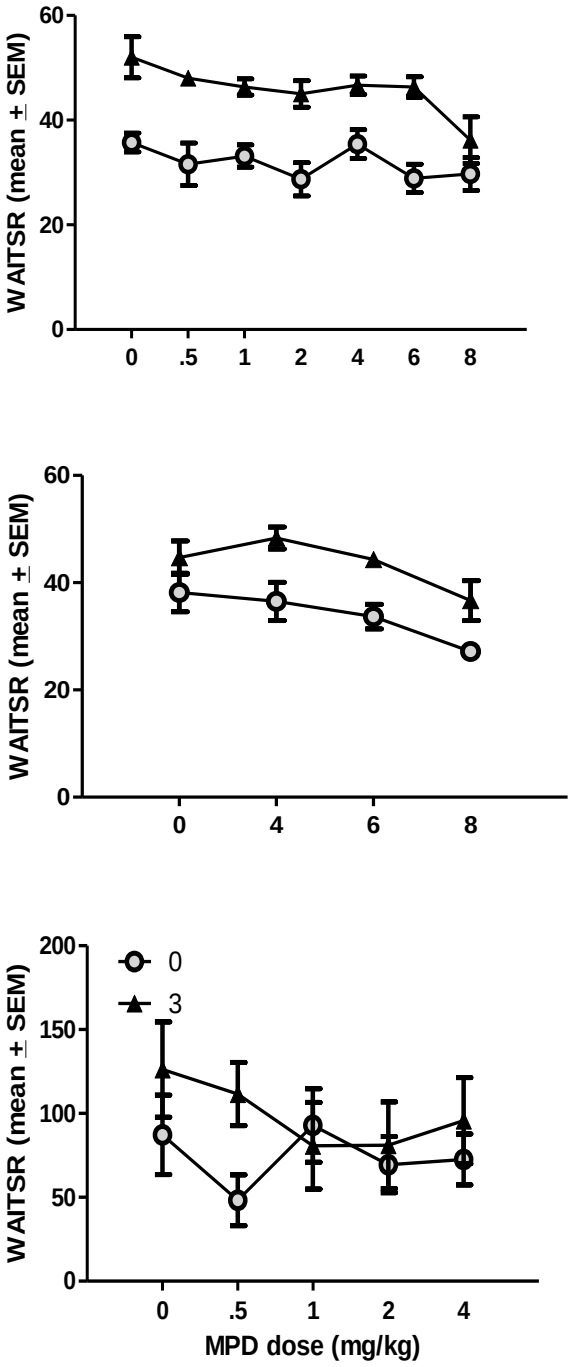

Figure 32. Dose response effect of MPD on number of WAITSR's earned under a FRWAIT schedule plotted as mean ± SEM. Males given MPD via oral gavage 15 and 5min prior (top and middle panels respectively) to behavioral testing. Females (bottom panel) were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing. n=7 for all groups

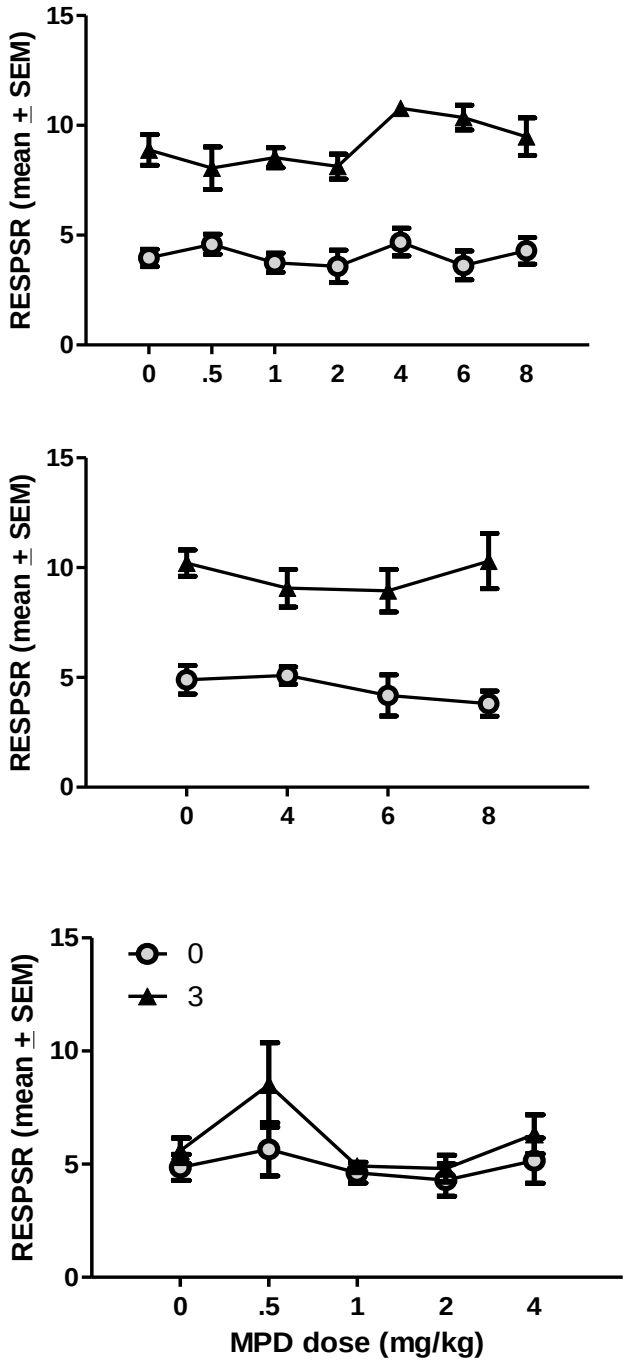

Figure 33. Dose response effect of MPD on responses per reinforcer under a FRWAIT schedule. Rate plotted as mean ± SEM. Males given MPD via oral gavage 15 and 5min prior (top and middle panels respectively) to behavioral testing. Females (bottom panel) were given .5, 1, 2, and 4mg/kg MPD via oral gavage 15min prior to behavioral testing. n=7 for all groups

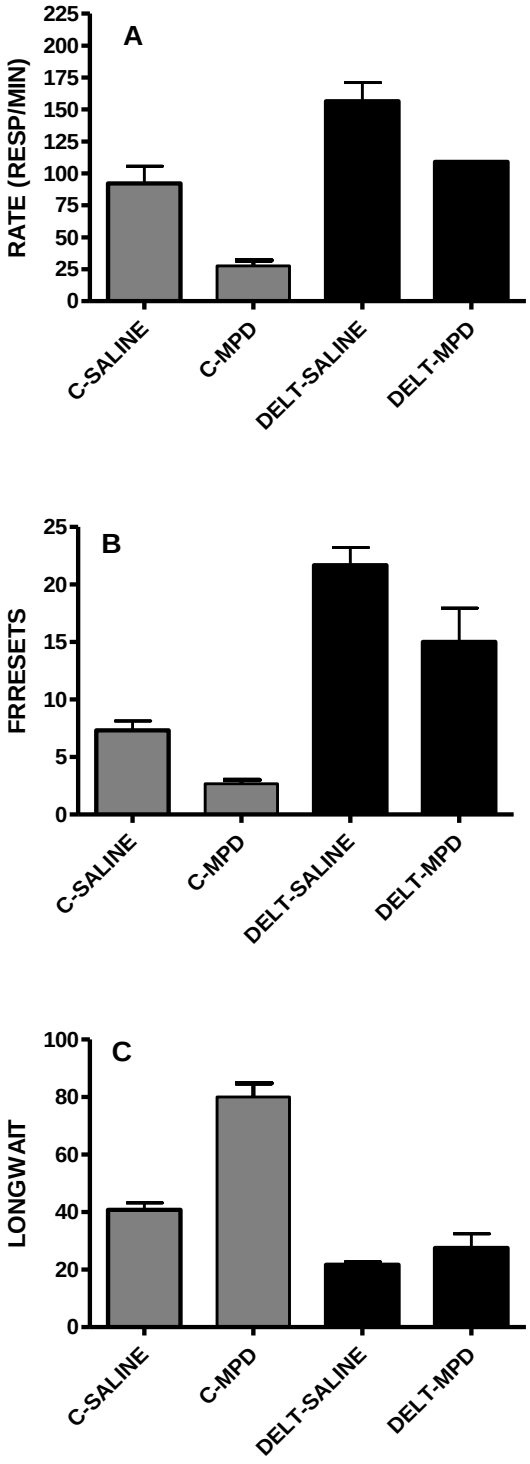

Figure 34. Effects of 8mg/kg MPD on FRWAIT performance in male mice. Mean rate, resets, and longwait in males (A, B and C respectively) following 8mg/kg MPD 5min prior to the behavioral test session. n=7 for all groups

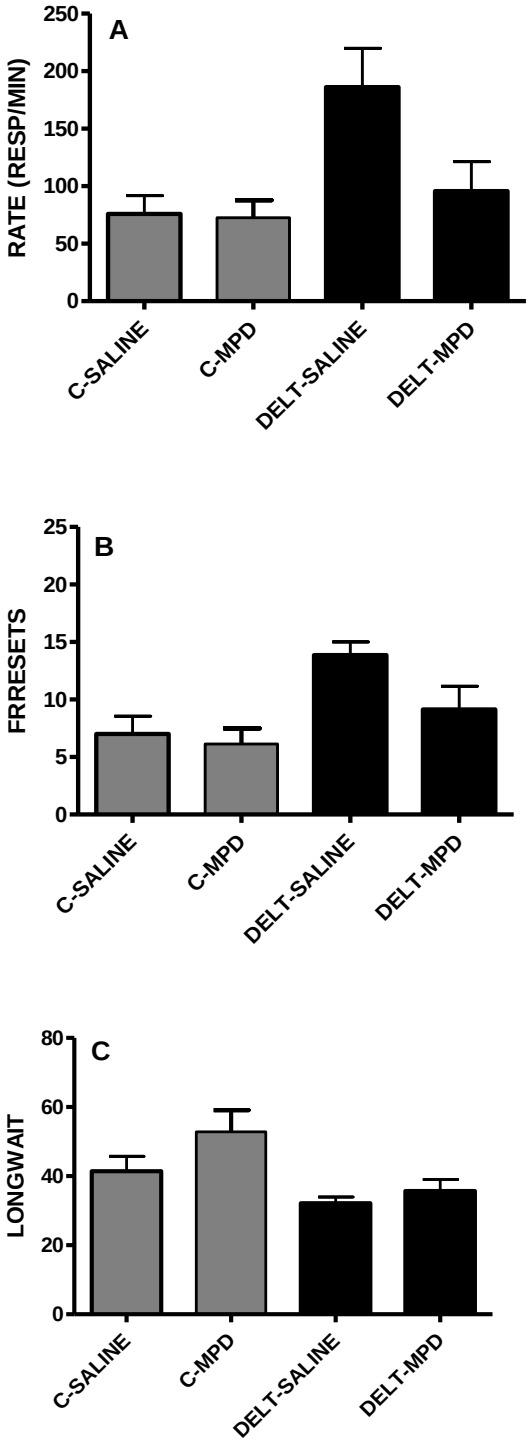

Figure 35. Effects of 8mg/kg MPD on FRWAIT performance in female mice. Mean rate, resets and longwait (A, B and C respectively) following 8mg/kg MPD 5min prior to behavioral test session. n=7 for all groups

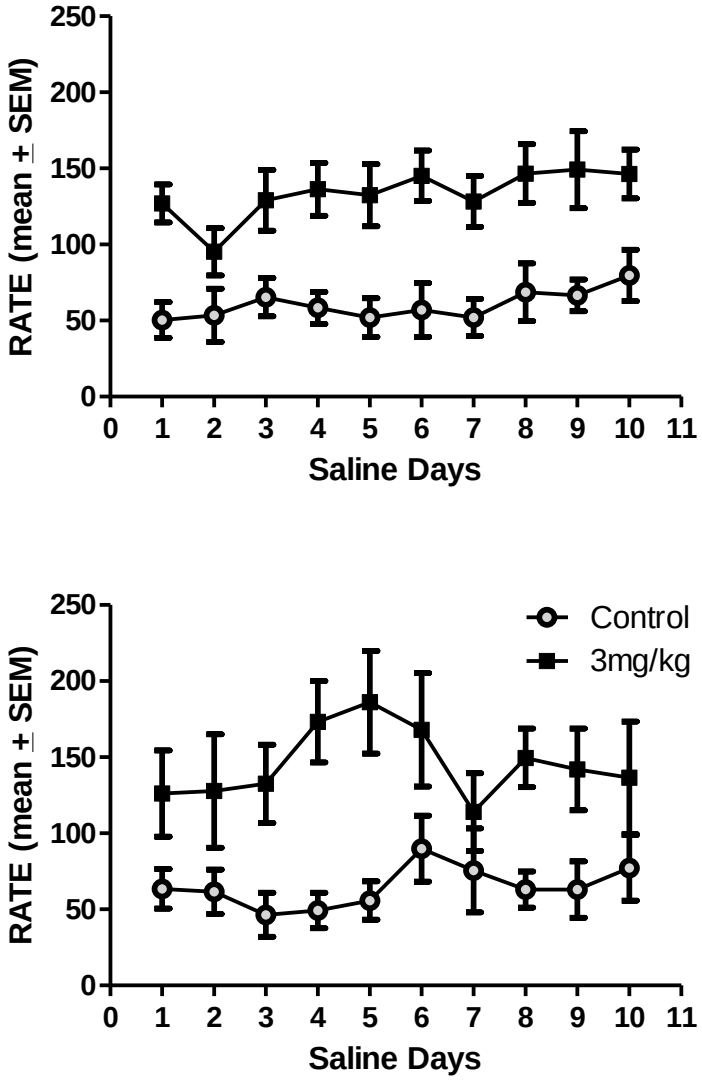

Figure 36. FRWAIT rate of responding across the intervening saline days during MPD testing. Males and females (top and bottom panels respectively) were administered 15min prior to behavioral testing. n=7 for all groups

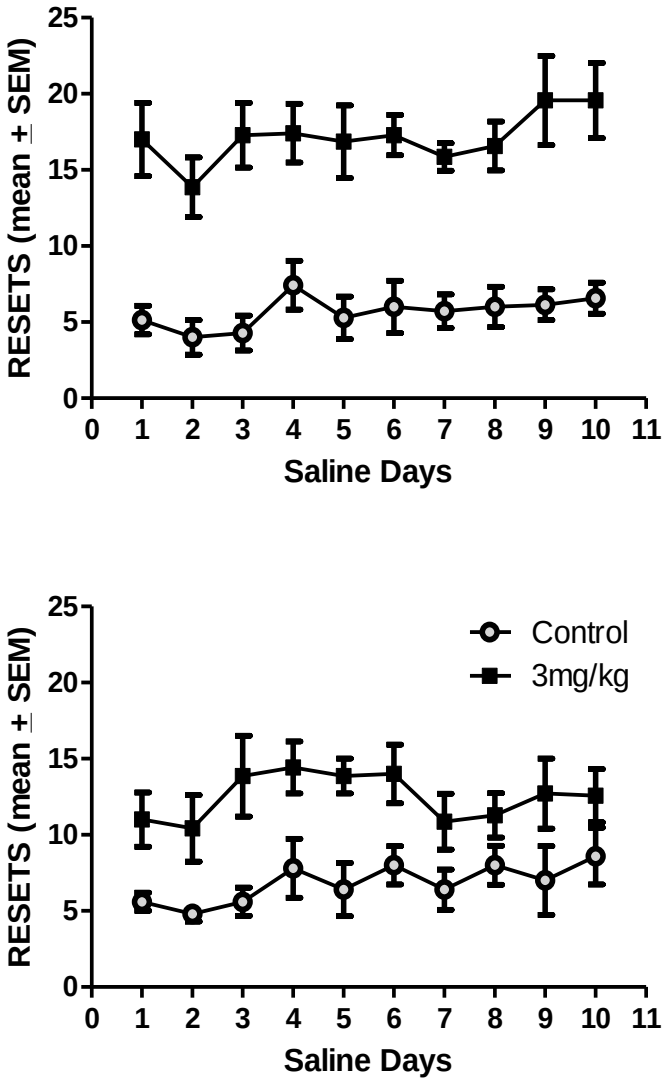

Figure 37. FRWAIT resets across the intervening saline days during MPD testing. Males and females (top and bottom panels respectively) were administered 15min prior to behavioral testing. n=7 for all groups

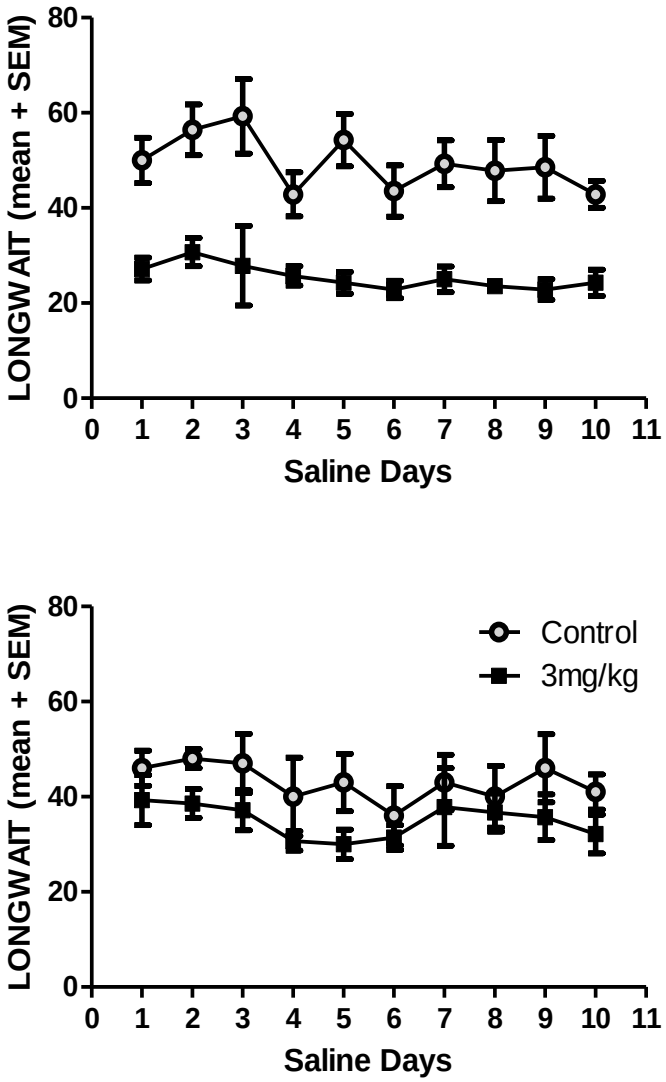

Figure 38. FRWAIT longest wait time across the intervening saline days during MPD testing. Males and females (top and bottom panels respectively) were administered 15min prior to behavioral testing. n=7 for all groups

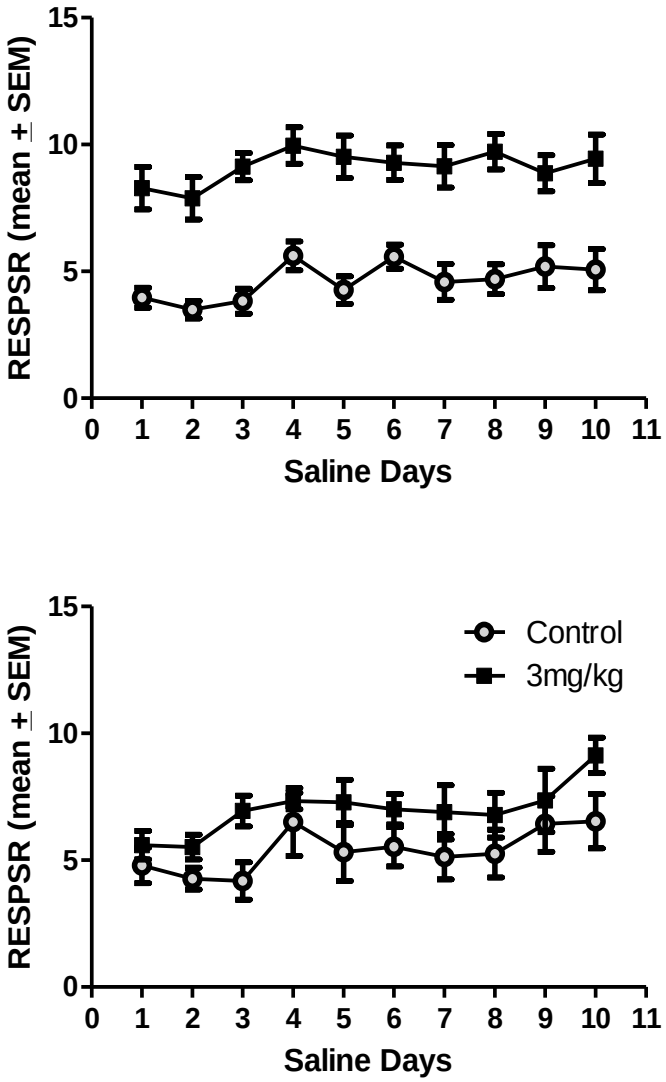

Figure 39. FRWAIT responses per reinforcer across the intervening saline days during MPD testing. Males and females (top and bottom panels respectively) were administered 15min prior to behavioral testing. n=7 for all groups

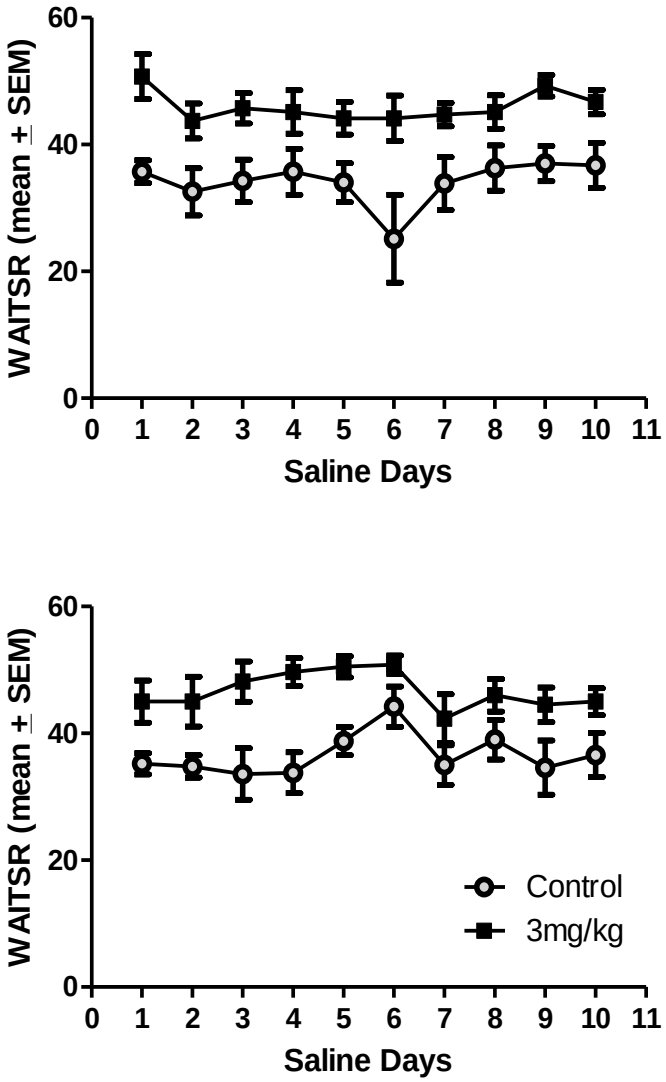

Figure 40. FRWAIT responses earned for waiting across the intervening saline days during MPD testing. Males and females (top and bottom panels respectively) were administered 15min prior to behavioral testing. n=7 for all groups

TABLE 2
SUMMARY OF DUNNETT'S MULTIPLE COMPARISON TESTS
OF SALINE AND METHYLPHENIDATE TREATMENT
IN DM-EXPOSED VS CONTROL MICE

	RATE		FRRESETS		LONGWAIT		WAITSR		RESPSR	
	M	F	M	F	M	F	M	F	M	F
CON-SAL VS DM-SAL	**	**	*	**	*	***	***	*	*	*
CON-SAL VS DM-MPD	***	***	**	***	**	***	***	***	*	*

* = < 0.01, ** = < 0.05, *** = > 0.05. Administration of 4 or 8mg/kg MPD (15 and 5min prior to session) reversed DM-Induced increases on RATE in females and males respectively. In addition, MPD returned FRRESETS and WAITSR to control levels in females.

Table 2. Summary of Dunnett's multiple comparison tests of saline and MPD treatment in DM and control mice.

DISCUSSION

This study demonstrated that developmental exposure to DM alters the pattern of responding on a multiple FRWAIT schedule of reinforcement. This schedule has been utilized to address issues of reinforcement delay, impulsivity and inability to inhibit responding as behavioral mechanisms of lead-induced learning impairments in rats (Brockel et al., 1998, 1999). Here, we wanted to determine whether perinatal DM exposure results in impulsive-like behavior, as evidenced by deficits in waiting behavior and the capacity to inhibit responding or to refrain from responding during delays in reinforcement. Furthermore, we examined the ability of oral MPD to ameliorate these deficits. DM exposure significantly increased response rates (males: 167%; females: 101%) and FR resets (males: 167%; females: 103%). As a consequence of early resetting, the DM group earned a greater number of wait reinforcers (males: 38%; females: 28%). In other words, early resetting kept the wait duration from incrementing to longer intervals which is evident by significantly reduced wait time in both genders exposed to DM (males: 35%; females: 21%). While this strategy seems optimal in that it maximizes the number of reinforcers delivered, in fact it is highly inefficient. As depicted in figures 26A and B, DM mice, particularly the males, emitted more responses per reinforcer (53% and 20% respectively), thereby exerting unnecessary effort. Interestingly, male rats exposed to lead performing on a FRWAIT schedule of reinforcement generated similar response patterns (Brockel et al., 1998, 1999). That is they had higher response rates, number of resets, wait reinforcers and responses per reinforcer. Moreover, the longest mean wait time for the lead-exposed rats was much

shorter than their control counterparts. Several hypotheses within the lead literature have been proposed to explain such response patterns which may apply to our findings.

Rice (Rice, 1992, 1992) posits that lead-induced alterations in responding are due to an inability to inhibit responding. An inability to inhibit responding is suggestive of an active behavioral process and might be construed as an adaptive response that functions to make time pass more quickly or alter the perception of the passage of time (Cory-Slechta, 2003). It may be that DM-exposed mice exhibit such inabilities, given the increased rate of responding and number of resets. This behavior may also be maintained by the increased reinforcement density inherent in the altered response pattern. Higher reinforcement density is achieved in two ways. First, increased response rates result in faster reinforcement delivery, the sooner the FR requirement is completed; the faster reinforcement is obtained. Second, the higher number of FR component resets results in more frequent opportunity to obtain reinforcement. Our current data are also consistent with the notion that DM exposure is (Rice, 1992) correlated with the inability to manage delays. Indeed, the behavioral mechanism driving impulsivity has been ascribed to delay aversion (Sonuga-Barke et al., 1992). In other words, DM-exposed mice are delay aversive, and the altered response pattern is an adaptive response to the delay as opposed to an inability to withhold inappropriate responses (Sonuga-Barke et al., 1992). This, rather than the inability to withhold responding or an attempt to maximize reward, is the reason for choosing immediate small rewards (Sonuga-Barke et al., 1992). Moreover, aversion to delays may serve as a discriminative stimulus for inappropriate or perseverative responding. Alternatively, it could be asserted that DM mice would exhibit longer wait times if they were exposed to a procedure in which they were forced to

experience a long delay, during which more "free" pellets would be delivered. In turn, the rate of responding for FR pellets would decrease. However, while mean wait values did increase following MPD administration, they immediately returned to pre-MPD levels afterward, making this assertion unlikely. In fact, MPD-induced decrements in waiting behavior and response patterns was consistent across intervening saline injection days (FIG's 36-38). In other words, exposure to free pellets in and of itself does not directly modify wait times in DM-exposed mice. It is possible that DM-induced decreases in wait are directly related to rate and therefore, a decrease in rate should increase wait times. However, such a relationship was not apparent in this experiment. In fact, MPD significantly decreased rate but did not produce a concomitant increase wait, suggesting that these two components of performance are dissociated. Finally, it could be argued that DM-induced rate increases might reflect a perseverative pattern of responding or repetitive motor responses and not an inability to inhibit responding (Hilson et al., 1997). Since this was not directly addressed in the current experiment, it cannot be ruled out. In order to more accurately determine whether repetitive response patterns are being generated, we could measure responding during time outs, delays or extinction trials. Persistent repetitive responding during these periods is suggestive of perseveration. Such response patterns likely influence other behavioral functions, particularly learning, by engendering behaviors incompatible with those required by the paradigm (Cory-Slechta, 2003).

In ADHD patients, MPD is effective in treating the symptoms of hyperactivity, impulsivity and attention deficits. This lab has established that intraperitoneal (i.p.) dosing of .1mg/kg of MPD is sufficient to abolish deltamethrin-induced increases in

locomotor activity in mice. However, i.p. dosing does not correlate with the clinical ADHD population which is given methylphenidate orally. There are differences in the kinetics of methylphenidate as a function of route of administration (oral vs i.p.) (Volkow, 2003). Indeed, relative to i.p. administration, oral administration results in lower bioavailability (Gerasimov et al., 2000). Therefore, to more closely represent the dosing procedure used in the human population, MPD was administered via oral gavage. Figures 33 (males) and 34 (females) show MPD effectively increased DM-induced reductions of waiting behavior. Specifically, administration of 8 or 4mg/kg MPD (5 and 15min prior to session) significantly reduced of DM-induced increases on rate in females and males respectively (Table 2). In addition, MPD returned resets and number of reinforcers earned for waiting to control levels in females. It is important to note that MPD given to control mice robustly reduced rate, resets and reinforcers earned for waiting. However, the comparison of interest was that of the DM-exposed mice before and after MPD exposure in relation to the controls. Accordingly, like the clinical ADH D population, MPD successfully ameliorated DM-induced impulsive-like behavior.

Several studies in animals and humans (Crawford et al., 1998; Vaidya et al., 1998) suggest that the striatum, a major component of the reward system and a region critical to executive function and selection of motor response (Grillner et al., 2005; Monchi et al., 2006), is affected by MPD. Indeed, MPD has been shown to alter activity of striatal neurons during stimulus-controlled tasks involving motor inhibition (Wang et al., 2004). Specifically, it has been shown that, stimulants, such as MPD, block the reuptake of dopamine and norepinephrine into presynaptic neurons and increase their release into the extraneuronal space, thereby acting as an indirect catecholaminergic

agonist (Wilens, 2009). Interestingly, the distribution of MPD binding in the brain parallels that of DAT. Distribution of DAT is highest in the caudate putamen. While moderate levels are also present in the nucleus accumbens, substantia nigra, ventral tegmental area, only marginal levels of DAT exist in the PFC (Madras et al., 1998; Madras et al., 1998). In humans, MPD has been demonstrated to both occupy and block DAT (Dougherty et al., 1999; Sonders et al., 1997; van Dyck et al., 2002; Vles et al., 2003), amplifying DA signaling. Furthermore, a reduction in radioligand binding to DAT following MPD has been shown in adults and children diagnosed with ADHD (Dresel et al., 2000; Krause et al., 2003; Vles et al., 2003).

Data suggest that the effects of MPD on impulsive-like behavior in mice exposed to DM may be mediated in part by alterations in the striatum and the motor cortex. The ability of MPD to reverse DM-induced impulsivity may be related to the normalization of striatal and cortical dopamine activity necessary for response inhibition. A model for the action of MPD on the dopaminergic system in DM-exposed mice is depicted in figure 39. which illustrates a hypodopaminergic state characterized by low levels of DA along with high levels of DAT and inhibited presynaptic D2 receptors. MPD, acting as an indirect DA agonist, increases the accumulation of DA in the extracellular space by blockade of DAT. It is posited that increased extracellular DA concentrations occurs not only by blocking DAT, which recaptures less DA back up into the cell, but also by disinhibition of D2 autoreceptors on the presynaptic terminal and activation of D1 receptors on the postsynaptic terminal. This is manifest behaviorally as decreased rate of responding and resetting of the FR component as well as increasing waiting times.

Future studies using the model of developmental DM exposure should incorporate other therapeutics known to decrease impulsive behavior, including guanfacine (alpha-adrenergic receptor agonist), atomoxetine (selective norepinephrine reuptake inhibitor) or other DA agonists, such as Adderall which is a mixture of amphetamine salts. Guanfacine (brand name Tenex and the extended release Intuniv) works by improving attention and working memory via modulation of post-synaptic α_{A2}-adrenergic receptors in the prefrontal cortex. Using a delayed-match to sample behavioral task in which a delay separates the presentation of a stimulus and two or more comparison stimuli. A response on the correct, matching, comparison stimulus results in reinforcement. Typically, a decrement in accuracy occurs as the delay increases. Developmental DM exposure may decrease accuracy on this task compared to controls. Further, guanfacine administration may return the performance of DM-exposed animals to control levels which would then suggest a role of the noradrenergic system.

CHAPTER 5:

METHYLPHENIDATE AND ITS METABOLITE RITALINIC ACID IN PLASMA AND BRAIN TISSUE OF MICE DEVELOPMENTALLY EXPOSED TO DELTAMETHRIN: ANALYSIS BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

INTRODUCTION

Data in the previous chapter revealed that MPD ameliorated the DM-induced alteration of waiting behavior in both male and female mice. However, we found that the dose at which MPD reduced impulsive-like behavior differed by gender. In fact, 4mg/kg MPD administered 15min prior to testing was sufficient to ameliorate rate increases in DM-exposed females but not males. Contrastly, the male MPD dose effect curve was shifted to the right, and rate increases were reduced to control levels only after 8mg/kg MPD was administered 5min prior to testing. This implicates a gender differences in metabolism. To explore these differences, the pharmacokinetic profile of MPD was determined. Further, given that MPD reduced impulsive-like behavior, we sought to determine brain tissue levels during a timeframe which corresponded to the behavioral effects.

MPD is rapidly absorbed with plasma concentrations peaking 1 to 3 hrs after oral administration in humans (Faraj et al., 1974). It is readily and extensively metabolized via first-pass metabolism (Fig. 40). Specifically, it is metabolized by deesterification via carboxylesterase CES1A1 to methyl (alpha)-phenyl-2-piperidineacetate hydrochloride

(RA) (Wargin et al., 1983), the primary metabolite. Indeed, plasma concentrations of RA are 30-60 fold higher than that of MPD (Ding et al., 2004). The pharmacokinetic parameters of MPD have been evaluated previously in both children and adults (Chan et al., 1980; Chan et al., 1983; DeVane et al., 2000; Hungund et al., 1979; Markowitz et al., 2000; Markowitz et al., 2003; Modi et al., 2000; Shaywitz et al., 1982) yet little data in mice exists. Recently, MPD disposition in plasma and whole brain was characterized in male C57/BL6 mice (Williard et al., 2007). Notably, MPD was coadministered with ethanol and was given via i.p. not orally. Methodological differences such as exposure (i.p. vs oral and MPD alone or coadministered), and target tissue (whole brain vs region) complicate direct comparison to humans. This lab has established that i.p. dosing of 1mg/kg and of MPD is sufficient to abolish DM-induced increases in locomotor activity in mice. However, i.p. dosing does not correlate with the clinical ADHD population which is given MPD orally. There are differences in the kinetics of MPD as a function of route of administration (oral vs i.p.) (Volkow, 2003). Relative to i.p. administration, oral administration results in lower bioavailability and increased metabolism to RA, a compound with negligible stimulant properties (Gerasimov et al., 2000). The levels of MPD delivered i.p. on rat nucleus accumbens dopamine (assessed by in vivo microdialysis) and on locomotor activity revealed levels are not only higher but reach the brain more quickly than with oral administration. MPD (5 and 10mg/kg) delivered via i.p. was twice as potent as oral MPD with regard to increasing extracellular dopamine levels and increased locomotion (Gerasimov et al., 2000). In the present research, we demonstrate that orally administered MPD reverses DM-induced impulsivity. Although, the dose and time of MPD administration which reduced impulsive-like behavior differed

by gender (4mg/kg MPD administered 15min prior to testing was sufficient to ameliorate rate increases in DM-exposed females but not males). The male MPD dose effect curve was shifted to the right, implicating gender differences in metabolism. Still, the route of administration utilized in the current experiment is clinically relevant and its pharmacokinetic profile needs to be established in mice.

The pharmacokinetic profile of MPD has been examined in blood and brain microdialysates from anaesthetized rats using a novel technique which utilized three simultaneous probes (Weikop et al., 2004). This technique allowed monitoring of MPD pharmacokinetic profile in both blood (probe 1) and brain (probe 2) and the pharmacodynamic response of released dopamine (probe 3). The extracellular levels of dopamine gradually increased to roughly 450% of basal levels in the striatum and prefrontal cortex (Weikop et al., 2004). In addition, the pharmacokinetic profile of MPD has been established in rats (Aoyama et al., 1996; Aoyama et al., 1997; Bakhtia et al., 2004; Ding et al., 2004). Yet, to date, plasma and tissue concentrations following oral administration of MPD in mice has not been reported in the literature. The concentration of MPD which reaches the plasma and brain to exert it behavioral effects in impulsive-like mice has yet to be determined. Here we evaluated murine blood levels of MPD and its' metabolite RA in control and DM-exposed offspring. The quantification of RA was conducted in order to evaluate the metabolic clearance of MP. We also examined whether there are regional brain differences in MPD and RA concentration and whether these are clinically relevant doses.

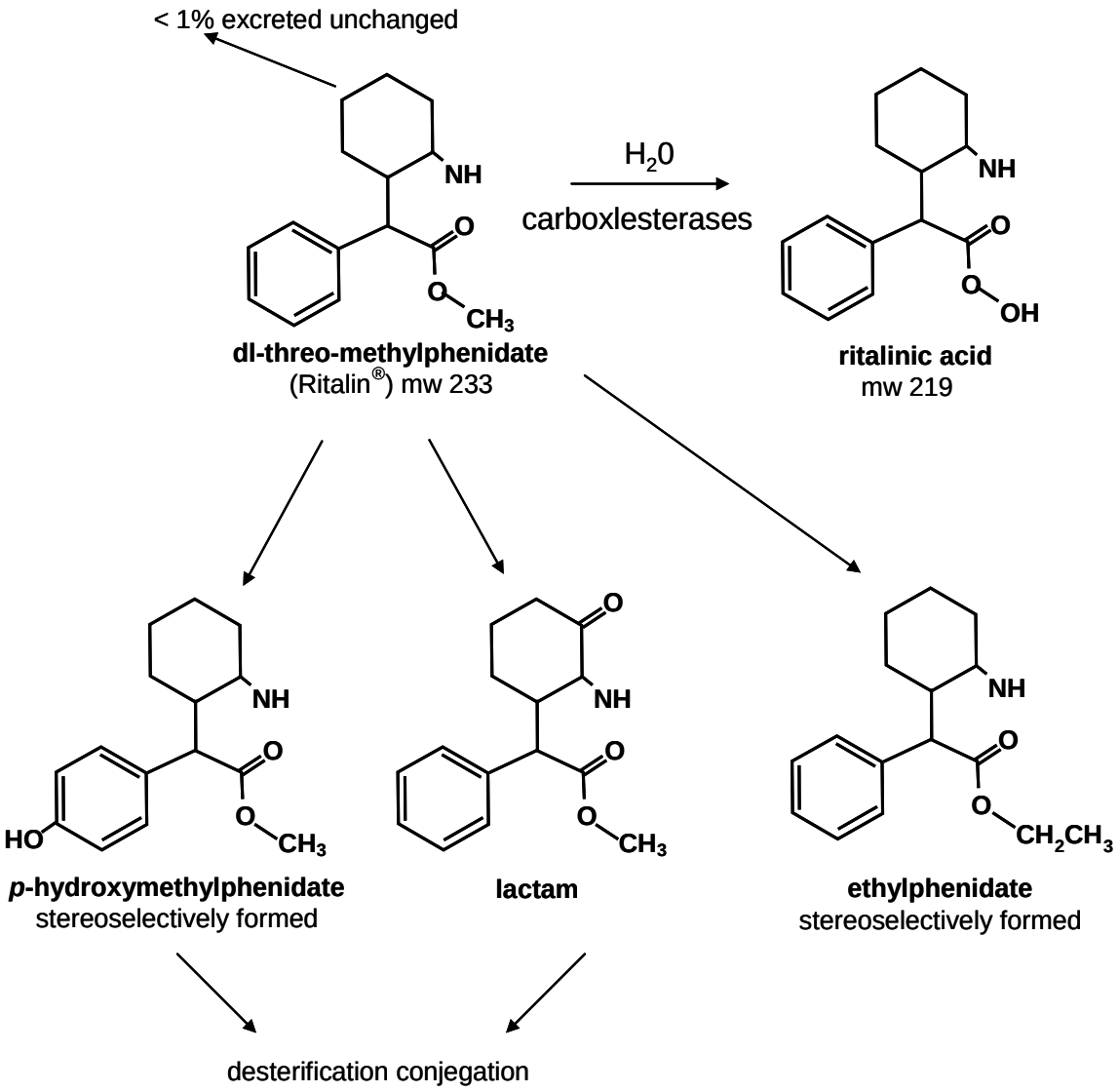

Figure 41. Schematic of MPD metabolism.

RESULTS

The SPE and LC/MS procedures described in the methods section of chapter 2, allowed simultaneous quantification of MPD and RA simultaneously in both plasma and brain tissue of mice. The accuracy of the method was evaluated by spiking blank mouse plasma and tissue homogenate samples with varying amounts of MPD (0-200ng/ml) along with a fixed amount of phenacetin (1ug/ml) (Table 3A and B respectively). Accuracy was determined prior to each sample run. The amount of RA detected in plasma samples of both male (Fig.'s 42 and 44A) and female (Fig.'s 42 and 44B) mice was 10-fold higher than MPD (Fig.'s 41 and 43A and B) at the first two timepoints examined (10 and 30min post-MPD administration). Plasma RA levels were roughly 100-fold higher than MPD at the two later timepoints (90 and 120min) in both genders. The difference between the two increased across time as plasma levels of parent compound decreased and metabolite levels were elevated at the early timepoints and remained elevated at the latest timepoint examined (120min). Indeed, the plasma distribution profiles of MPD show levels decreased across time following 4 and 8mg/kg MPD in males (Fig.'s 41 and 43A) and females (Fig.'s 41 and 43B). The plasma level data were utilized for pharmacokinetic analysis of MPD and RA. Area under the concentration vs. time curves (AUC), half-lives ($t_{1/2}$), and maximum concentration (C_{max}) were determined for plasma levels of MPD and RA in both genders (Tables 6 and 7)

A. Plasma

ng/ml	set1	set2	set3	set4
200	100 - 2%	100 -1%	100 -15%	100 +1%
80	100+ 6%	100 +1%	100 +3%	100 -7%
40	100 - 8%	100 +1%	100 +2%	100 -6%
20	100+ 1%	100 +2%	100%	100+ 8%
8	100 - 2%	100 -2%	100 +7%	100 -1%
4	100+ 5%	100 -2%	100 +4%	100 +5%

B. Tissue

ng/ml	set1	set2	set3
80	100 -1%	100 + 16%	100 + 9%
40	100 + 9%	100 - 7%	100 + 1%
20	100 - 3%	100 - 6%	100 - 3%
8	100 + 3%	100 - 1%	100%
4	100 - 8%	100 - 2%	100 - 8%

Table 3. Accuracy of the PK method determined in plasma and tissue. Accuracy was determined by spiking blank plasma and tissue homogenate samples with varying amounts of MPD (0-200ng/ml) and a fixed amount of phenacetin (1ug/ml).

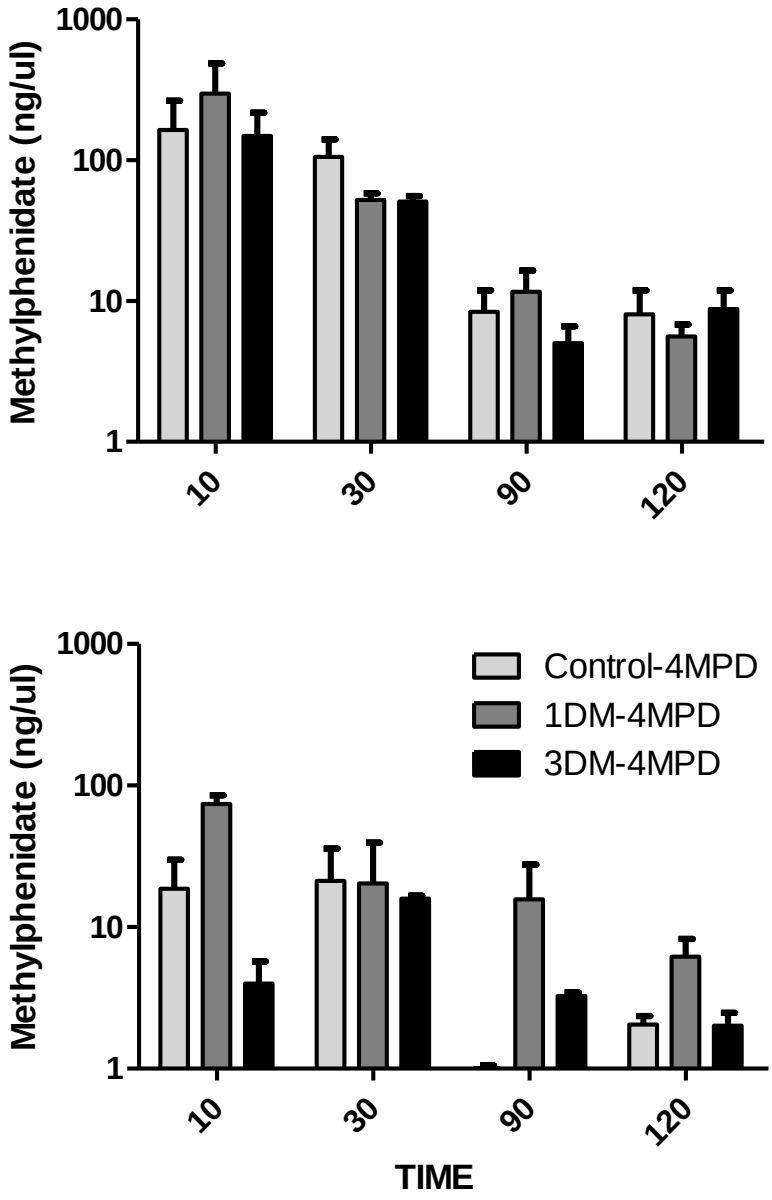

Figure 42. Plasma distribution of MPD in male and female mice following 4mg/kg MPD. Mean plasma MPD AUC values plotted across time in plasma in male (top panel) and female (bottom panel) following oral administration of 4mg/kg MPD. n=3/trt/timepoint/gender.

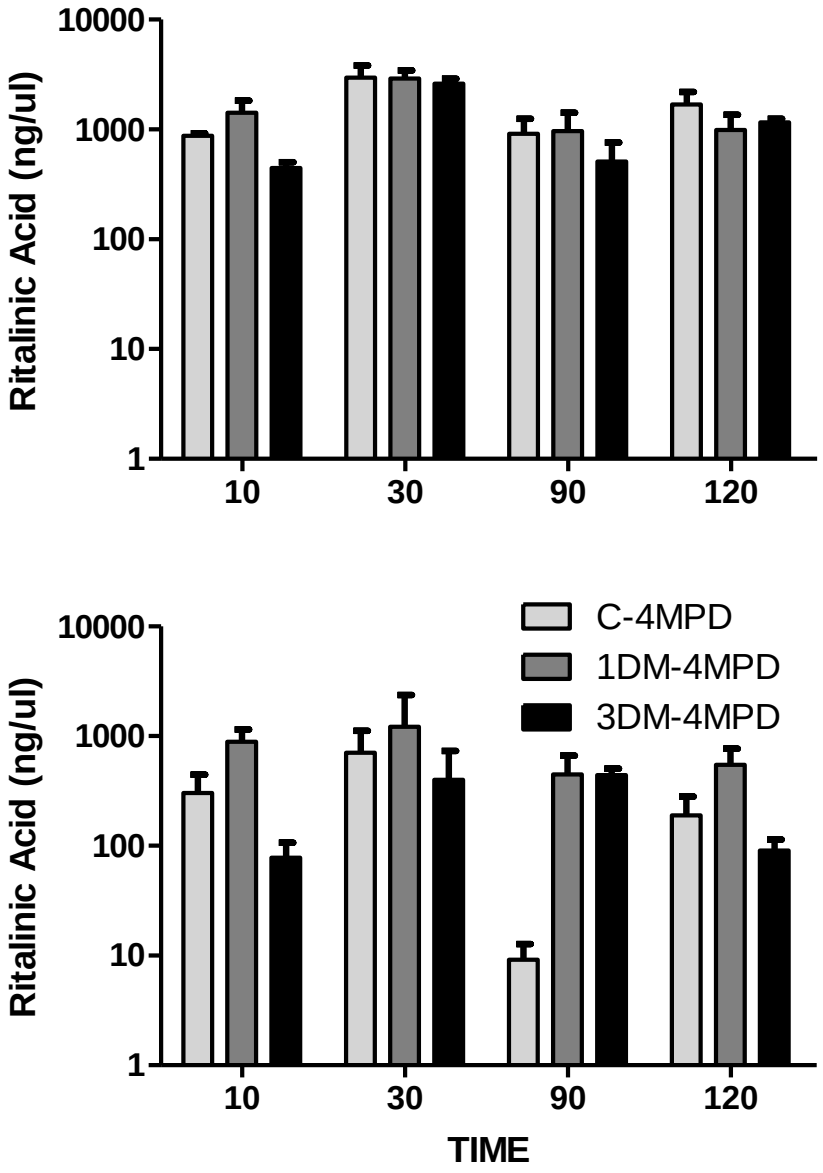

Figure 43. Plasma distribution of RA in male and female mice following 4mg/kg MPD. Mean plasma RA AUC values (ng/ml) plotted across time in plasma in male (top panel) and female (bottom panel) following oral administration of 4mg/kg MPD. n=3/trt/timepoint/gender.

Interestingly, the largest C_{max} value for male MPD and RA was reached in the 1mg/kg and control groups respectively following both low and high dose MPD. In females, the largest C_{max} value for MPD was reached in the 1mg/kg DM group following low dose MPD but in the control group following high dose MPD treatment (Table 7). In addition, $t_{1/2}$ of MPD and RA was shortest in the males exposed developmentally to DM (Table 6). However, in females, the $t_{1/2}$ of MPD was shorter in the controls, while that of RA was shorter in high dose DM following both low and high dose MPD treatment. To further evaluate differences in plasma MPD and RA levels, one-way ANOVA's were carried out.

A one-way ANOVA in males revealed a main effect of DM, MPD and time (DM: $F[2,41]=12.204$, $p<.0001$; MPD: $F[1,41]=40.172$, $p<.0001$, and time: $F[3,41]=38.843$, $p<.0001$] as well as an interaction between DM, MPD and time (DM x MPD x time: $F[6,41]=11.21$, $p<.0001$). Pairwise comparison revealed that the 10min timepoint differed significantly from the three longer timepoints. The difference is largely driven by the robust increase in plasma MPD levels at the 10min timepoint. The impact of MPD treatment in DM exposed males was determined by one-way ANOVA of the low and high dose of MPD separately. A one-way ANOVA of low dose MPD males revealed a main effect of time (time: $F[3,21]=4.775$, $p=.01$) but not DM ($F[2,21]=.286$, $p=.7$) or time x DM [$6,21]=.425$, $p=.85$). However, a one-way ANOVA of high dose MPD males revealed a main effect of time and DM (time: $F[3,20]=30.508$, $p<.0001$; DM: $F[2,20]=11.681$, $p=.0004$) as well as a DM x time interaction (DM x time: $F[6,20]=9.492$, $p<.0001$

In females, a one-way ANOVA confirmed a main effect MPD and time but not DM (MPD: $F[1,44]=10.374$, $p=.0024$, time: $F[3,44]=9.688$, $p<.0001$, and DM:

F[2,44]=1.463, p=.2426). Furthermore, there was a time x MPD and MPD x DM but not a DM x MPD x time interaction (time x MPD: F[3,44]=2.805, p=.05; MPD x DM: F[2,44]=3.506, p=.0387; and DM x MPD x time: F[6,44]=1.875, p=.1066). A one-way ANOVA of the low and high dose of MPD was run separately and revealed a main effect of time (F[3.23]=5.948, p=.0037), DM (F[2,23]=6.519, p=.0057) as well as a time x DM interaction (F[6,23]=3.265, p-.0180) in the low dose MPD group. The impact of the high dose of MPD was less robust, with a main effect of time but not DM or time x DM interaction (time: F[3,21]=5.744, p-.0049; DM: F[2,21]=1.915, p=.1722; and time x DM: F[6,21]=.955, p=.4782).

The $t_{1/2}$ of low dose MPD was dose-dependently decreased in males and increased in females as a result of developmental DM exposure. There were no apparent differences in $t_{1/2}$ of high dose MPD. In addition, there was a robust increase in male peak MPD concentration as a function of DM exposure (8mg/kg group only). No such pattern was observed in females.

RA in both genders (Fig.'s 36 and 38) were elevated at 10min and remained elevated until the last timepoint test (120min). There were no differences observed between the between the mean values for $t_{1/2,}$ C_{max} or AUC for plasma RA in either gender following low or high dose MPD treatment (Tables 4 and 5). In aggregate, these data are suggestive of induction of enzymatic pathways for the elimination of MPD as a function of gender and DM exposure. MPD and RA levels were quantified in brain tissue homogenate derived from the frontal cortex and striatum. As depicted in tables 6 and 7, DM exposure clearly impacted MPD as well as RA AUC values.

Table 4. Pharmacokinetic parameters for MPD and RA in plasma of male mice exposed developmentally to either 0, 1 or 3mg/kg DM. Data shown represent mean ± SEM (n=3).

			4mg/kg MPD-Males			
DM Dose	$t_{1/2}$ MPD (min)	$t_{1/2}$ RA (min)	AUC MPD	AUC RA	C_{max} MPD	C_{max} RA
0	58.58	7896.5	7833	193896	164.54	2969.7
1	43.57	99.65	6500.55	189480.8	298.54	2921.78
3	24.67	-3372.45	3887.84	149498.3	149.16	2614.261
			8mg/kg MPD-Males			
DM Dose	$t_{1/2}$ MPD (min)	$t_{1/2}$ RA (min)	AUC MPD	AUC RA	C_{max} MPD	C_{max} RA
0	25.77	104.28	21374.13	459449	433.7	6472.57
1	16.23	313.49	41342.57	412665	1931.26	6174.32
3	24.67	-3372.45	3887.84	149498	149.164	2614.261

Table 4. Pharmacokinetic parameters of MPD and RA in male mouse plasma (data represent ng/ml).

Table 5. Pharmacokinetic parameters for MPD and RA in plasma of female mice exposed developmentally to either 0, 1 or 3mg/kg DM. Data shown represent mean ± SEM (n=3).

4mg/kg MPD-Females						
DM Dose	$t_{1/2}$ MPD (min)	$t_{1/2}$ RA (min)	AUC MPD	AUC RA	C_{max} MPD	C_{max} RA
0	25.79	36.31	1109.39	34420.48	21.168	703.51
1	38.08	95.61	2354.635	85943.68	73.83	1216.44
3	60.82	-1756.62	854.51	37919.18	15.93	440.17
8mg/kg MPD-Females						
DM Dose	$t_{1/2}$ MPD (min)	$t_{1/2}$ RA (min)	AUC MPD	AUC RA	C_{max} MPD	C_{max} RA
0	23.57	56.53	6150.68	187465	168.53	2589.57
1	33.10	108.9	3100.54	129818	58.30	2020.23
3	28.81	55.66	2999.69	106313	89.67	1589.71

Table 5. Pharmacokinetic parameters of MPD and RA in female mouse plasma (data represent ng/ml).

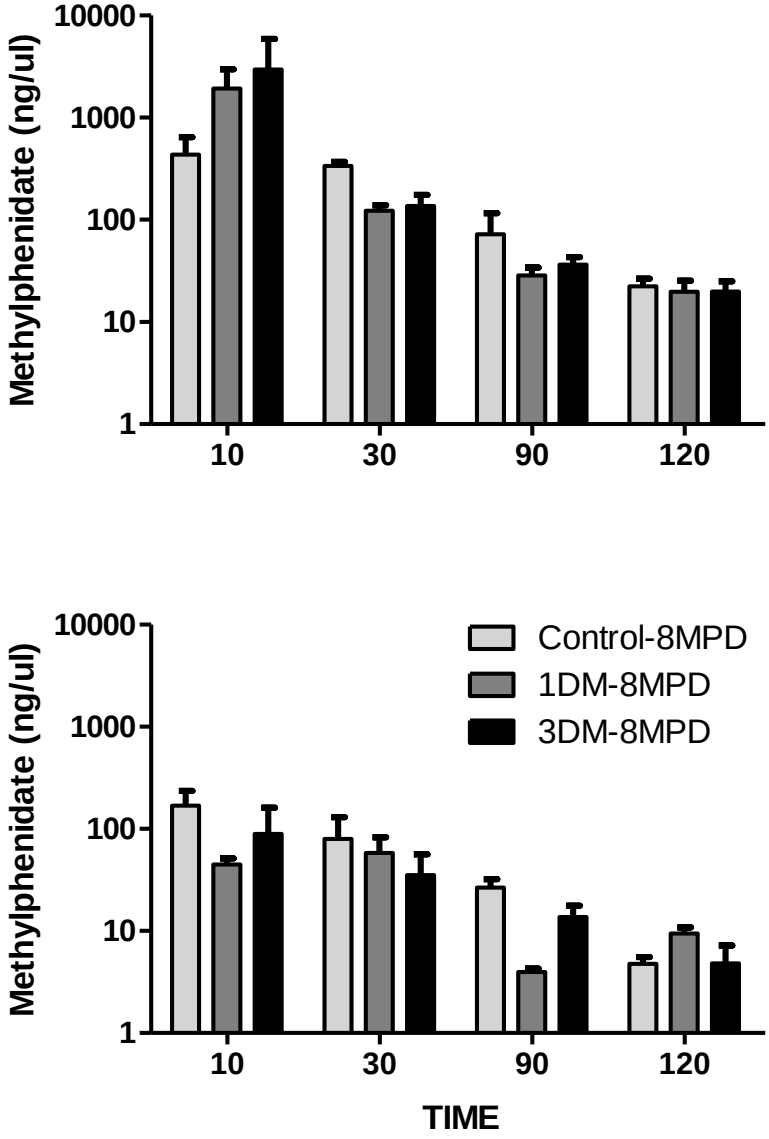

Figure 44. Plasma distribution of MPD in male and female mice following 8mg/kg MPD. Mean plasma MPD AUC values (ng/ml) plotted across time in plasma in male (top panel) and female (bottom panel) following oral administration of 8mg/kg MPD. n=3/trt/timepoint/gender.

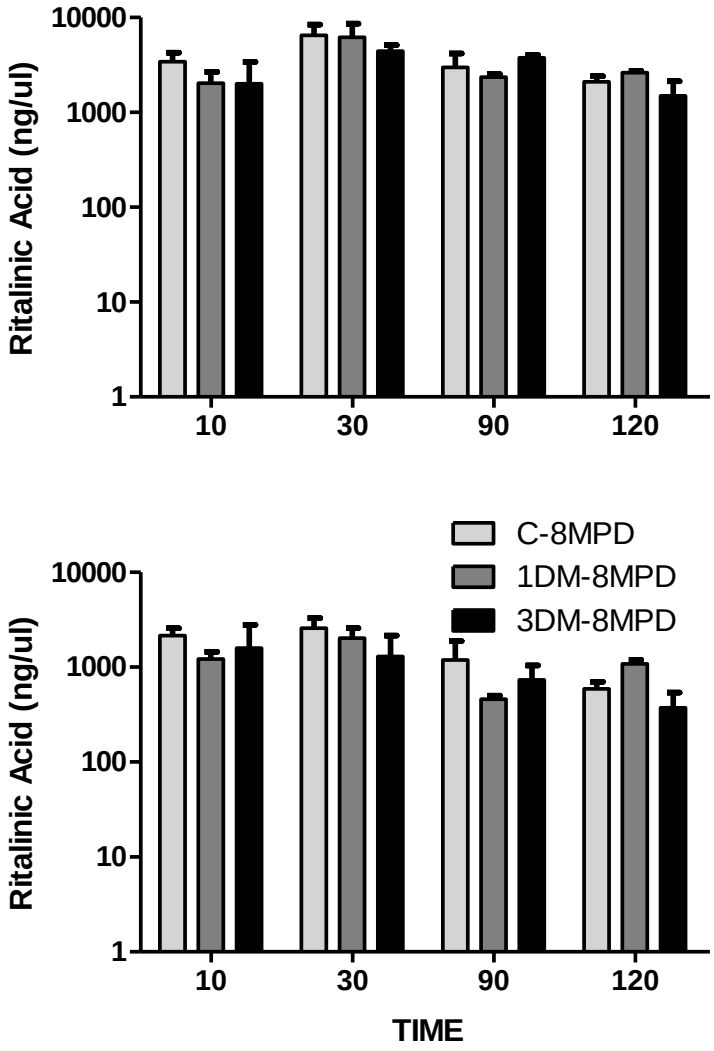

Figure 45. Plasma distribution of RA in male and female mice following 8mg/kg MPD. Mean plasma RA AUC values (ng/ml) plotted across time in plasma in male (top panel) and female (bottom panel) following oral administration of 8mg/kg MPD. n=3/trt/timepoint/gender.

Table 6. Pharmacokinetic parameters for MPD and RA of male and female frontal cortex tissue exposed developmentally to either 0, 1 or 3mg/kg DM. Data shown represent mean ± SEM (n=3).

		4mg/kg MPD-FC		
DM Dose	Male (MPD)	Male (RA)	Female (MPD)	Female (RA)
0	6.25	.68	1.32	3.36
3	2.78	.54	2.02	2.5
		8mg/kg MPD-FC		
DM Dose	Male (MPD)	Male (RA)	Female (MPD)	Female (RA)
0	13.32	4.51	4.72	1.05
3	.56	.32	1.19	.69

Table 6. Pharmacokinetic profile of MPD and RA of male and female frontal cortex tissue (data represent ng/ml).

Table 7. Pharmacokinetic parameters for MPD and RA in male and female mouse brain tissue exposed developmentally to either 0, 1 or 3mg/kg DM. Data shown represent mean ± SEM (n=3).

		4mg/kg MPD-STR		
DM Dose	Male (MPD)	Male (RA)	Female (MPD)	Female (RA)
0	5.15	.37	1.29	3.36
3	2.17	.59	0.42	2.5
		8mg/kg MPD-STR		
DM Dose	Male (MPD)	Male (RA)	Female (MPD)	Female (RA)
0	10.76	0.21	4.92	N/A
3	3.53	0.45	1.41	N/A

Table 7. Pharmacokinetic profile of MPD and RA in male and female mouse STR tissue (data represent ng/ml).

DISCUSSION

This study describes the measurement of MPD and its major metabolite RA in plasma and brain of control mice and those developmentally exposed to deltamethrin. These data are consistent with most previous research which (Chan et al., 1980; Doerge et al., 2000; Hudziak et al., 2005) suggests that MPD undergoes substantial first-pass metabolism and/or deesterification. It has been reported that it is completely absorbed (Faraj et al., 1974). However, it's unlikely that the entire dose reaches the systemic circulation unchanged. Indeed, low absolute bioavailability has been reported in rats, monkeys (Faraj et al., 1974) and children (Chan et al., 1980).

The pharmacokinetic profile of MPD in DM-exposed mice appears to differ slightly from that of humans. The $t_{1/2}$ of MPD averaged 2.11hr in adults (Wargin et al., 1983) and 1.88 (Chan et al., 1980), 2.56 (Hungund et al., 1979) and 2.43hr (Wargin et al., 1983) in children. Here, we report $t_{1/2}$ values ranging from 16.23-25.77min in males given 8mg/kg MPD and from 25.79-60.82min in females given 4mg/kg MPD, the dose and time corresponded with the reversal of impulsive-like behavior. Accordingly, mice likely clear MPD much faster. Moreover, systemic clearance of MPD could not be determined here since it was administered orally. Further, since the longest timepoint examined was 120min, calculation of elimination rates would be imprecise. However, RA clearance can be inferred to be less than that of MPD due to its considerably larger AUC values. The clinical significance of this finding is negligible since RA has very little or no pharmacological activity (Patrick et al., 1981). There is no clear evidence of DM-dependent kinetics in the present experiments. Although, variability in AUC values within and across DM exposures, complicates analysis of these data, there was evidence

that male mice developmentally exposed to deltamethrin had lower brain levels of MPD. The C_{max} was slightly higher in females following both doses of MPD than what is observed in humans (7.8-10.8ng/mL) following oral administration of therapeutic doses of MPD (Doerge et al., 2000). Nevertheless, these values were within the range and are considered clinically relevant. Unexpectedly, the obtained male C_{max} values were 10 and upwards of 100 times (4 and 8mg/kg MPD respectively) that of the females. This raises the possibility of enzyme inhibition in males or enzyme induction in females, an assertion that has yet to be explored. Analysis of liver enzyme function in response to MPD in DM-exposed animals would provide clues. Also, given the variability in these data, replication is warranted. Overall, the pharmacokinetic parameters determined for MPD and RA in mice following oral administration of MPD, including peak plasma levels and elimination half-lives, were comparable to those previously reported for human children and adults. This establishes an important correlation between this animal model and humans.

CHAPTER 6:

GENERAL DISCUSSION

The mechanisms underlying ADHD have yet to be elucidated. While genetic factors appear to play a significant role in pathogenesis of ADHD, recent evidence suggests that environmental influences during development result in disease later in life (Heindel, 2007). Indeed, epidemiological studies have determined that myriad environmental factors such as maternal nicotine use, neonatal hypoxia, lead exposure, low birth weight and prematurity increase ADHD risk (Banerjee et al., 2007; Braun et al., 2006; Gusella et al., 1984; Kotimaa et al., 2003; Linnet et al., 2003; Milberger et al., 1996; Milberger et al., 1998; Needleman et al., 1979; Nigg et al., 2007; Sprich-Buckminster et al., 1993; Thapar et al., 2003; Weissman et al., 1999) . Notably, exposure of pyrethroids in pregnant women has been confirmed (Berkowitz et al., 2003; Whyatt et al., 2003; Whyatt et al., 2007). Also, our recent epidemiological data revealed that children aged 6-15 with detectable levels of pyrethroid metabolites in their urine were more than twice as likely to be diagnosed with ADHD (Richardson et al., 2009). Specifically, we determined the odds ratio of ADHD diagnosis for children with levels of the pyrethroid pesticide metabolite 3-PBA in their urine above the limit of detection was 2.3, suggesting that exposure to pyrethroid pesticides significantly increases the risk of being diagnosed with ADHD (Richardson et al., 2009). Despite these data, little research has been conducted to identify the mechanism by which pyrethroids, specifically DM, affect the dopamine system and whether exposure might contribute to behavioral

abnormalities associated with ADHD. This research was performed in an effort to elucidate the mechanism of DM and its relevance to ADHD risk using a mouse model of developmental DM exposure. The objectives of this study were to determine whether perinatal DM exposure in mice results in impulsive-like behavior that is ameliorated by methylphenidate treatment as well as to characterize the pharmacokinetic profile of methylphenidate in murine tissue. Another objective was to determine the contribution of dopamine receptor subtypes on hyperactivity and impulsive-like behavior in DM-exposed mice and quantitatively determine DM-induced changes in dopamine transporter and receptor levels and their neuroanatomical location.

MAJOR FINDINGS

DM and Timing Behavior and Dopamine Receptors

Findings from this study demonstrate that DM exposure in pregnant mice produces impulsive-like behavior in the offspring on a FI60s schedule of reinforcement. Perinatal DM exposure dose-dependently increased rate of responding in both genders. The highest dose of DM (3mg/kg) produced robust increases in rate and run rate in males (292 and 181% respectively). In females, DM produced more modest increases (74 and 98%) in rate and run rate respectively. These data are consistent with the profile of effects observed in ADHD patients (Darcheville et al., 1992, 1993) and the gender difference reflects the clinical ADHD population in that males are diagnosed at a much higher rate than females. Furthermore, 3mg/kg DM decreased PRP by 33 and 38% and IRT by 66 and 80% in males and females respectively. Exposure of 1mg/kg DM also

produced decreases in PRP and IRT. Administration of both ETI and SCH23390 ameliorated many of these impulsive-like behaviors. Finally, our autoradiographic and homogenate binding data suggest a prominent role for D1 receptors in ADHD.

Reversal of DM-Induced Impulsive-Like Behavior by MPD

This study demonstrated that developmental exposure to DM alters the pattern of responding on a multiple FRWAIT schedule of reinforcement. Specifically, DM exposure significantly increased response rates (males: 167%; females: 101%), FR resets (males: 167%; females: 103%) and number of wait reinforcers (males: 38%; females: 28%). Early resetting kept the wait duration from incrementing to longer intervals which is evident by significantly reduced wait time in both genders exposed to DM (males: 35%; females: 21%). This strategy maximizes the number of reinforcers earned. However, this was at a high response cost as DM mice emitted more responses per reinforcer (53% and 20% respectively). Like the clinical ADHD population, MPD successfully ameliorated impulsive-like behavior. For instance, rate decreased in both genders. In addition, MPD returned RESETS and WAITSR to control levels in females. These data indicate that like the clinical ADH D population, MPD successfully ameliorates DM-induced impulsive-like behavior.

Characterization of MPD in Murine Plasma and Tissue

The SPE and LC/MS method utilized in this dissertation allowed simultaneous quantification of MPD and RA simultaneously in both plasma and brain tissue of mice. The amount of RA detected in plasma samples of both genders was 10-fold higher than

MPD at 10 and 30min post-MPD and roughly 100-fold higher than MPD at the two later timepoints (90 and 120min). The difference between MPD and RA increased across time as plasma levels of parent compound decreased and metabolite levels were elevated at the early timepoints and remained elevated at 120min. Indeed, the plasma distribution profiles of MPD showed levels decreased across time following both doses of MPD in males and females. One of the most intriguing aspects of the data is the observance that lower levels of MPD are found in the brain of mice developmentally exposed to deltamethrin.

Proposed Mechanism of Action

A model for the action of MPD on the dopaminergic system in DM-exposed mice is depicted in figure 42. Depicted on the left is a striatal neuron in DM-exposed mice prior to MPD exposure. It illustrates a hypodopaminergic state characterized by low levels of DA along with high levels of DAT and inhibited presynaptic D2 receptors. Indeed, our lab has shown that DAT levels are elevated in mice exposed developmentally to DM. MPD, acting as an indirect DA agonist, increases the accumulation of DA in the extracellular space by blockade of DAT. It is posited that increased extracellular DA concentrations occurs not only by blocking DAT, which recaptures less DA back up into the cell, but also by disinhibition of D2 autoreceptors on the presynaptic terminal and activation of D1 receptors on the postsynaptic terminal. This is manifest behaviorally as decreased RATE and FRRESETS and increasing WAIT. Acute administration of the selective D2 receptor agonist, quinpirole, may confirm the disinhibition of autoreceptors. L-DOPA, in particular, enhances dopamine release and effectively ameliorates

Parkinson's symptoms by correcting an overt dopamine deficit. Given that ADHD is also hypothesized to result from a dopamine deficit, it follows that L-DOPA may alleviate the symptoms. Yet, when given to ADHD patients, L-DOPA as well as quinpirole are completely ineffective (Pliszka, 2005). This suggests that ADHD is caused by more than a simple 'hypodopaminergic' state.

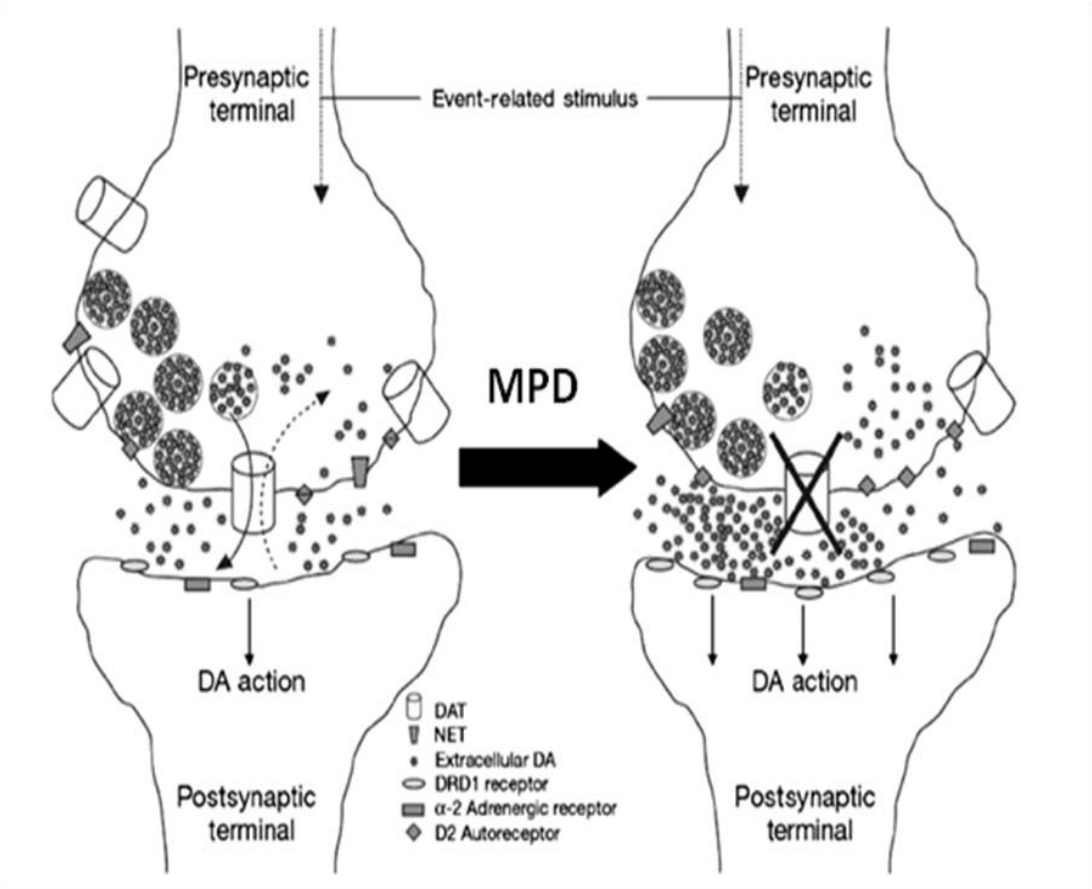

Figure 46. Model of MPD action on the dopaminergic system (adapted from (Wilens, 2009). Synapse on the left depicts a hypothesized striatal neuron in a mouse perinatally exposed to DM before MPD treatment. It shows relatively low levels of DA and high levels of DAT. The D2 autoreceptors remain inhibited. The figure on the right represents a neuron following MPD treatment which depicts blockade of DAT and increased accumulation of DA in the extracellular space. MPD is believed to increase extracellular DA concentration via blockade of DAT disinhibition of D2 autoreceptors on the presynaptic terminal and D1 receptor activation on the postsynaptic neuron, thereby amplifying DA activity (Wilens, 2009)

The Role of Gender

The consistently disparate effects between male and female mice across assays in these experiments are intriguing and may reflect sex differences in dopaminergic function. During both the perinatal and pubertal stages of development, release of gonadal steroids organize many sex differences, producing alterations in brain excitability and morphology that endure across the lifespan (Waddell et al., 2010). Estrogen has been implicated as a potential mediator of sex differences in behavior (Johnson et al., 2010). Most psychiatric and neurological disorders have opposing gender risk profiles. For instance, females are more likely to progress rapidly into drug addiction and more likely to relapse than males (Brady et al., 1999). Males have a higher incidence of Parkinson's disease and ADHD (Baldereschi et al., 2000; Waddell et al., 2010; Wooten et al., 2004). In general, males are more likely to be diagnosed with disorders which manifest in early development such as ADHD and learning disabilities (*Diagnostic and Statistical Manual of Mental Disorders-Text Revision*, 2000) while females are more likely to develop mood disorders, such as depression, that manifest during or following puberty (*Diagnostic and Statistical Manual of Mental Disorders-Text Revision*, 2000). Sexual dimorphisms in dopaminergic function represent a potential mediator of the divergent risk profiles.

Studies in adult rats reveal that females have an exaggerated locomotor response to stimulant drugs such as cocaine or amphetamine compared to males (Festa et al., 2004; Walker et al., 2001). They also exhibit a greater place preference to stimulant drugs and have a greater proclivity to self-administer them than do males (Lynch, 2006; Roth et al., 2004). Furthermore, electrically-stimulated striatal DA uptake and release is enhanced in females compared to males (Becker, 1999; Walker et al., 2006; Walker et al., 2000).

Estrogen-mediated modifications on dopaminergic function are implicated. Indeed, ovariectomy in rodents attenuates locomotor, self-administration and dopaminergic responses to stimulants which can then be reversed with exogenous estrogen replacement (Becker, 1999; Chin et al., 2002; Di Paolo, 1994; Festa et al., 2004; Walker et al., 2001). Also, ovariectomy decreases and estrogen replacement restores, DA receptor and transporter levels (Le Saux et al., 2006; Morisette et al., 2008). Clearly, estrogen modulates several aspects of dopaminergic function from presynaptic regulation of release to postsynaptic receptor sensitivity (Johnson et al., 2010).

Evidence from laboratory animals as well as humans indicates that estrogen not only modulates dopaminergic function, but also enhances DA cell survival (Johnson et al., 2010). In women, estrogen replacement therapy reduces the risk of Parkinson's disease as well as reduces symptoms related to early onset Parkinson's disease in postmenopausal patients (Benedetti et al., 2001; Saunders-Pullman et al., 1999). In non-human primates, gonadectomy produces a significant loss in DA neurons which immediate, but not delayed estrogen replacement prevents (Leranth et al., 2000).. Estrogen has also been shown to offer protection from neurotoxicant exposure. For instance, administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine (MPTP) to adult rats produces significantly greater DA cell loss (90 and 60% in males and females respectively) and motor impairment in males than in females (Tamas et al., 2005). In order to determine the role of estrogen and its receptors (α and β), Johnson et al. (2010) examined DA cell number in ovariectomized and hormone-replaced female rats and mice before and following vehicle, 17β-estradiol (E_2) or estrogen receptor (ER) agonists propyl-pyrazole-triol (ERα agonist) or diarylpropionitrile (ER_β agonist). They found that

ovariectomy reduced the number of DA cells (as measured by decreased tyrosine hydroxlyase-immunoreactive [TH-IR] cell body number) in both rats and mice. Replacement with E_2 as well as both ER agonists prevented or attenuated the loss in both rats and mice (Johnson et al., 2010). Further examination of mice lacking either ER_α or ER_β revealed that those lacking ER_α but not ER_β had fewer TH-IR cell counts than the wild-type mice suggesting that ER_α plays a more prominent role in DA cell survival (Johnson et al., 2010).

Estrogen is likely not the only hormone interacting with the developing brain. While the main focus of previous studies has been on the neuroprotective effects of estrogen on dopaminergic pathways (Dluzen, 2000; Sawada et al., 1998; Sawada et al., 2000; Walker et al., 2000), little research has examined the role of testosterone on these pathways. Early studies show robust increases in striatal catecholamine synthesis (Engel et al., 1979), DA metabolites (Dluzen et al., 1989) and in vitro DA release (Bitar et al., 1991). It has been suggested that during normal development in rodents, high levels of testosterone during late gestation and early postnatal development increases cell death (Geschwind et al., 1985; Goodman, 1991; Lyon et al., 1991). Recently, Johnson et al. (2010) examined the number of TH-IR cell bodies in the SNpc and VTA using stereological analysis and revealed that TH-IR cell number increased in both regions following castration in both rats and mice (Johnson et al., 2010). Replacement with either testosterone or the non-aromatisable analogue dihydrotestosterone (DHT) reduced the number of TH-IR in both the SNpc and VTA of rats but only the SNpc of mice (Johnson et al., 2010), They argued that the increase in TH-IR cell number following castration is not due to increased TH expression since there was not a concomitant,

proportional decrease in TH-immunonegative cells following castration or replacement with testosterone or DHT (Johnson et al., 2010). In orchidectomized mice, testosterone replacement lowers potassium-stimulated DA output compared to the those that did not receive testosterone replacement (Shemisa et al., 2006). This effect was reversed with reserpine (a vesicular monoamine transport blocker), in that potassium-stimulated DA output was significantly greater in the group receiving testosterone replacement suggesting that testosterone may modulate storage and uptake of DA within the vesicles (Shemisa et al., 2006).

While controversial, it has been suggested that males are at a higher risk for learning disabilities and ADHD because testosterone slows brain development, thereby rendering males vulnerable to environmental insults for a protracted period of time, resulting in increased variability in behavioral outcome (Geschwind et al., 1985; Lyon et al., 1991; Morris et al., 2004). Consistent with this, increased levels of testosterone during the late prenatal and early postnatal period leads to increased neural lateralization as a consequence of increased cell death in the right hemisphere as well as slower development of the left hemisphere (Geschwind et al., 1985; Goodman, 1991). Functional brain imaging of children diagnosed with ADHD reveals reduced volume of the right hemisphere and corpus callosum (Seidman et al., 2005).

Environmental Exposures and ADHD

There is a growing literature that suggests that environmental influences during development alter the dopamine system and may contribute to behaviors associated with ADHD. Epidemiologic studies have found correlations between maternal smoking,

(Milberger et al., 1996; Milberger et al., 1998), neonatal hypoxia (Linnet et al., 2003), low birth weight (Braun et al., 2006) and ADHD. In fact, the prenatal and postnatal ramifications of maternal smoking have been particularly well studied (Gusella et al., 1984; Hill et al., 2000; Kotimaa et al., 2003; Milberger et al., 1998; Thapar et al., 2003; Weissman et al., 1999). Gusella and Fried (1984) showed that prenatal nicotine exposure resulted in motor decreases and lowered verbal comprehension in 13 month olds. Milberger et al. (1998) reported a 2.7 fold increased risk for ADHD associated with maternal smoking while Weissman et al. (1999) found a 2-fold increase in ADHD risk associated with prenatal tobacco use. A dose-response relationship (OR 1.30) between maternal smoking and hyperactivity has also been demonstrated (Kotimaa et al., 2003).

The current literature confirms the notion that pregnancy and delivery complications predispose children to ADHD (Sprich-Buckminster et al., 1993). Complications such as preeclampsia, poor maternal health, advanced maternal age, prolonged gestation, labor duration, fetal distress and low birth weight are all implicated in ADHD (Banerjee et al., 2007). Low birth weight, in particular is associated with ADHD. The association of prenatal smoking exposure with ADHD was confounded by family variables but low birth weight independently predicted ADHD (Nigg et al., 2007).

Several environmental agents have been linked to ADHD. Pesticides, in particular, may be considered prime candidates. First, pesticides are one of the few environmental agents that are produced specifically to be introduced to the environment. Secondly, pesticides, in particular insecticides, often target the nervous system and there is often significant overlap of the molecular targets of pesticide action in both target and non-target species such as humans (NRC, 1993).

Exposure to environmental toxicants such as lead (Braun et al., 2006; Needleman et al., 1979) and manganese (Collipp et al., 1983) have also been linked to ADHD-like behavior in children. It was found that children with elevated dentine lead levels exhibited disorganized and inattentive behavior (Needleman et al., 1979). Behavioral and neurochemical research in both rodents (Brockel et al., 1998) and primates (Rice, 2000) support these findings. Together, these studies demonstrate that developmental exposure to lead alters the dopamine system and produces impulsive-like behavior and in some cases hyperactivity as well. However, most children diagnosed with ADHD do not have high blood lead levels and conversely, many children with high lead exposure do not develop ADHD (Banerjee et al., 2007). Therefore, developmental exposure to other environmental agents that affect the dopamine system may be expected to contribute to behaviors associated with ADHD, although a specific environmental "trigger" has not been identified.

Future Studies

Epidemiological data implicates developmental pesticide exposure as a risk factor for ADHD (Richardson et al., 2009). Data presented in this dissertation, clearly demonstrate that developmental DM exposure in mice, produces behavior patterns that recapitulates those in ADHD children, making this exposure paradigm a potential animal model of ADHD. The current prevailing model of ADHD proposes a hypodopaminergic state based on the success of stimulant treatment, which increases extracellular dopamine levels. However, the dopamine-deficit theory of ADHD oversimplifies the dopamine theory of reward. For instance, imaging studies that have monitored D2 receptor binding

have shown that extracellular dopamine influences the availability of D2 receptors in such a way that an increase in extracellular dopamine would produce a concomitant decrease in D2 binding. While a blunted response to MPD in ADHD subjects has been shown and may reflect lower dopamine release compared to controls, it also possible that could reflect higher baseline dopaminergic tone (Volkow et al., 2007). Indeed, 6-week old SHR rats, have been shown to exhibit a higher basal extracellular dopamine level as well as a more robust MPD-induced increase in extracellular release than control (Carboni et al., 2003). Furthermore, the dopamine system does not exist in a vacuum; other neurotransmitters (norepinephrine and GABA) interact with it. To more fully elucidate the dynamics of extracellular dopamine would provide more information than the static measurements of tissue levels of DA and its metabolites or receptor number. There are a couple of approaches that could be implemented to monitor neurotransmitter dynamics in DM-exposed mice are microdialysis and cyclic voltammetry. Microdialysis can be conducted in freely moving animals to obtain information during behavioral test sessions. Steady state extracellular dopamine levels could be determined following D1/D2 antagonist and agonist and MPD treatment could also be determined. However, the temporal resolution using microdialysis is poor. On the other hand, cyclic voltammetry would afford greater temporal resolution by simultaneous monitoring of electrically evoked release and clearance of monoamines with millisecond resolution. In general, microdialysis probes are located far from release sites and measures extrasynaptic neurotransmitter levels (Jones et al., 1999). Voltammetric electrodes are located closer to release sites and allow for assessment of synaptic and perisynaptic neurotransmitter release and clearance (Jones et al., 1999). The parameters of

extracellular dynamics obtained by these techniques complement each other and would provide extremely valuable information (Jones et al., 1999). Interestingly, our lab has recently shown, using microdialysis, that extracellular DA in the accumbens is decreased by about 25% in males exposed to DM, but not females.

We examined plasma and tissue levels as long as 2hr following MPD administration. Further studies replicating this method should include longer timepoints and larger samples in order to elaborate the pharmacokinetic profile and obtain more accurate determination of clearance and reduce variability. Further, since MPD produced disparate behavioral responses in males and females and in control and treated mice, the role of liver enzymes would provide greater insight into potential gender and treatment differences in metabolism and whether these impact MPD levels in the brain. Recent data from the Richardson laboratory have demonstrated that developmental DM exposure increases liver carboxylesterase, which may explain the decreased levels observed in the mice developmentally exposed to DM.

In order to more adequately explore the role of gender in on DM-induced changes in behavior, future experiments could include groups of female mice which either did or did not have ovariectomy and either receive estrogen replacement or not. This would allow for better experimental control over the role of estrogen as a potential neuroprotector. Given that males were more greatly impacted than females, further research should also explore the role of testosterone replacement in sham or gonadectomized males.

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CURRICULUM VITAE
Michele Marie Taylor
Education

Oct 2012	DOCTORATE OF PHILOSOPHY. Joint Graduate Program in Toxicology. Rutgers, The State University of New Jersey and University of Medicine and Dentistry New Jersey. Dissertation Title: "Characterization of Impulsive-Like Behavior Produced by Developmental Deltamethrin Exposure: Role of Dopaminergic Dysfunction". Advisor: Jason Richardson, PhD
June 1996	MASTER OF ARTS. Western Michigan University. Kalamazoo, MI. (Major: Psychology).
April 1993	BACHELOR OF ARTS. Western Michigan University. Kalamazoo, MI. (Majors: Psychology and Sociology).

Positions Held

Nov 2010 to Present	CONTRACTOR, United States Environmental Protection Agency, National Health and Environmental Effects Research Laboratory
Oct 1999 to Aug 2002	RESEARCH PSYCHOLOGIST, United States Environmental Protection Agency, National Health and Environmental Effects Research Laboratory
Oct 1997 to Oct 1999	BIOLOGICAL SCIENCES LABORATORY TECHNICIAN, United States Environmental Protection Agency, National Health and Environmental Effects Research Laboratory
May 1996 to April 1997	VISITING SCIENTIST, Pharmacia and Upjohn, CNS Diseases Research Division
April 1995 to Aug 1995	SENIOR LAB TECHNICIAN, International Research and Development Corporation (currently MPI), Behavioral Neuroscience Division
April 1994 to Aug 1994	SENIOR LAB TECHNICIAN, International Research and Development Corporation (currently MPI), Small Animal Toxicology Division
Aug 1994 to June 1996	RESEARCH ASSISTANT, Behavioral Pharmacology Lab, Western Michigan University
April 1991 to June 1996	RESEARCH ASSISTANT, Physiology/Psychopharmacology Lab Western Michigan Universit

Publications

Baker, A., Taylor, M. M., and Richardson, J. R. (2011). Environmental Factors in Attention Deficit-Hyperactivity Disorder. In: Developmental Neurotoxicology Research: Principles, Models, Techniques, Strategies and Mechanisms. (W. Slikker, Jr., and C. Chang, eds). John Wiley and Sons, 257-274.

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