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Ventral Tegmental Area Neuronal Responses To Methylphenidate Correlated To Behavioral Responses Of Adolescent And Adult Rats

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VENTRAL TEGMENTAL AREA NEURONAL RESPONSES TO METHYLPHENIDATE
CORRELATED TO BEHAVIORAL RESPONSES OF ADOLESCENT AND
ADULT RATS

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ADULT RATS

A
THESIS

Presented to the Faculty of
The University of Texas
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Graduate School of Biomedical Sciences
In Partial Fulfillment
of the requirements
for the degree of
MASTER OF SCIENCE

By
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Houston, Texas
December 2013

Dedications

This thesis is dedicated to my family, my mother Jeanine, my father Tom and my brother Tommy. Without my family's endless support I would not be in the position that I am today. I am thankful for their tireless love, support and encouragement throughout my life.

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I would to thank my parents and brother for their love and support through all the moments of my life. Thank you both for giving me everything I needed to reach my goals and follow my dreams.

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Zachary Ryan Jones, B.S.

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Abstract

Methylphenidate (MPD) is a psychostimulant used in the treatment of attention deficit hyperactivity disorder and more recently is being used as a cognitive enhancement and recreational drug. Its therapeutic effects are not fully understood, nor are the long term effects of the drug on brain development. The ventral tegmental area (VTA) is a site of psychostimulant action thought to be involved in behavioral sensitization. The aim of this study was to first determine if there are differences in neuronal responses between individuals, with respect to neuronal firing patterns in the ventral tegmental area (VTA), and whether these differences are responsible for the conflicting reports on behavioral responses with chronic MPD exposure. Additionally, we wanted to determine if the same dose of MPD can cause both behavioral sensitization and behavioral tolerance and to examine if there are intrinsic differences in the VTA neuronal activity between the behaviorally sensitized and tolerant animals. One hundred and thirteen adolescent animals and 51 adult rats were divided into 4 groups: saline, 0.6, 2.5, and 10.0 mg/kg MPD and had their neuronal and behavioral activity recorded concomitantly following one of the chosen MPD doses. In order to determine if there are neuronal differences between these two groups the VTA neuronal activity was evaluated based on the animals' behavioral response to chronic MPD exposure. In conclusion, acute MPD exposure elicits dose related increases in behavioral activity and following chronic MPD exposure, it was shown that the same repetitive MPD dose can elicit either behavioral sensitization or tolerance in both adolescent and adult animals. Differences were observed between the adolescent and adult for both the neuronal and behavioral data for each dose of MPD tested.

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1 Introduction

The History of MPD and its Uses

Methylphenidate (MPD) is a psychostimulant most commonly used in the treatment of attention deficit hyperactivity disorder (ADHD) in adolescents and adults (Challman and Lipsky, 2000; Garland 1998; Lee et al., 2012; Solanto 1998). Methylphenidate (Ritalin) was first synthesized in 1944 (U.S. Pharmacist, 2002) in 1954 its testing on humans had begun (U.S. Pharmacist, 2002). During the year 1957, Ciba Pharmaceutical Company began marketing MPD for the treatment of narcolepsy, chronic fatigue, psychosis, and depression (U.S. Pharmacist, 2002). In 1960, MPD was marketed as Ritonic, and was used to treat and improve the patients' mood and to help maintain cognitive vitality (U.S. Pharmacist, 2002). During the 1970's and 80's, MPD was starting to be used for the treatment of ADHD. The symptoms of ADHD, as described in the Diagnostic and Statistical Manual for Mental Disorders (DSM IV), are as follows: inattention, hyperactivity, and impulsivity for a period of at least six months that causes impairment both at home and at school or work (DSM IV 1994 4th Ed.). This disorder can cause children to have trouble concentrating and misbehave in school and can also create social misconduct in the affected individuals (Newcorn, 2000). From the years 1990-1999, there was a 500% increase in the use of MPD in the United States (U.S. Pharmacist, 2002) and the daily doses administered to patients had increased from 75 million in 1990 to 360 million in 1998 (Woodworth, 2000). It was estimated that the United States consumes 85% of the world's supply of MPD (Woodworth, 2000). In addition, with the 2000 US census population of over 281 million individuals, it was approximated that one out of five hundred randomly chosen people are currently prescribed with Ritalin (Woodworth, 2000). Today, MPD is still the pharmacotherapy of choice and gold standard in the treatment of ADHD.

However, due to the overabundance of adolescents being prescribed MPD there has become a high risk of nonmedical use and abuse by patients and their peers and family members. According to the National Survey on Drug Use and Health, nonmedical use of a medication is defined as;

“The use of prescription type psychotherapeutic drugs not prescribed for the respondent by a physician or the use of a drug only for the experience or the feeling it caused” (www.oas.samhsa.gov).

Drug abuse is defined as
“The use of a drug for a nontherapeutic effect, and habitual use of drugs to alter one’s mood, emotion, or state of consciousness” (www.oas.samhsa.gov).

Recently, the recreational use of MPD by adolescent and young adults has risen dramatically (Arria and Wish, 2006; Imbert et al., 2013) and there have been a large number of studies supporting the assertion that MPD is abused at a high rate by adolescents and adults (McCabe et al., 2007; Teter et al., 2006; Wilens et al., 2005). One such study focusing on undergraduate college students, found that over 30% of students that were tested had taken non-prescribed MPD either to get high or to aid in their studying (Teter et al., 2006; Wilens et al., 2005). In a 2006 study of college students, McCabe et al. found that 16% of those tested had abused stimulants for the cognitive enhancement effect, with 96% of them preferring Ritalin/MPD as their drug of choice (McCabe et al., 2007). Likewise, a study involving 700 participants aged 12 to 44 found that 22.9% had given some of their prescription to someone else, 26.9% had borrowed someone else’s, and 39.4% were willing to share their medication with family members (Goldsworthy et al., 2008). This study also reported that 20.8% of subjects between the ages of 18 and 25 have borrowed prescription medication to relax or feel good compared to only 10% of those aged 36 to 44 , which indicates that young people are more likely to use and abuse MPD. Additionally, this study also reported that 34.1% of adolescents said they were more likely to use Ritalin/MPD if it came from someone they were close to or who was familiar with the drug (Goldsworthy et al., 2008). These statistics on nonmedical MPD use highlights the importance of studies which examine the short and long term effects of MPD exposure to further expand the long term effects of the drug on the brain and overall health.

The behavioral effects following short term MPD exposure have been well documented (Brands et al., 1998). However, the data for more long term MPD exposures have not been fully

explored and are still not completely understood. Some of the short term behavioral effects which occur with low doses of MPD are wakefulness, heightened alertness, appetite suppression, and several negative effects such as impairment of voluntary movement, headache, and vomiting (Brands et al., 1998). In addition, with higher doses of MPD, there are many short term behavioral effects which include agitation, exhilaration, muscle twitching, excitation, dilation of pupils, increased blood pressure, other sympathetic responses, confusion, hallucinations, increased pulse rate, delirium, paranoia, repetition of movements and meaningless tasks and even seizures followed by coma can occur (Borcherding et al., 1990; Masand and Tesar, 1996). There have been a few studies on the long term effects of low and high dose MPD exposure. One such study with the use of positron emission tomography (PET) scans discovered that the extended use of MPD can constrict blood flow to the brain which may cause an increase in blood pressure (Wang et al., 1994). Among a number of other behavioral studies examining the long term effects of MPD use have come up with conflicting results (Lambert, 1999; Piazza et al., 1989; Robinson and Berridge, 1993; Wilens et al., 2005). One such study reported that MPD treatment provided protection to adolescents from later drug dependence (Wilens et al., 2005), while another study found the opposite to be true, namely that MPD use created a certain susceptibility to drug use later in life (Lambert, 1999; Piazza et al., 1989; Robinson and Berridge, 1993). Additionally, another longitudinal study reported no direct correlation between adolescent or youth stimulant use and drug use later on in the individual's life (Barkley et al., 2003). With the amount of contradictory information among the literature, we believe that recording both behavior and neuronal activity concomitantly will give us a better idea as to why there are so many differing views. We want to first determine if there are differences between the adolescent response to MPD and the adult response to MPD. Following this, further research can be done to test the same animal throughout adolescence into adulthood to examine whether the longitudinal response to MPD has changed.

Due to the increased prescription of MPD to young children who do not have fully developed brains, it is important to understand the way this drug affects adolescents compared to

adults. The current research will help show how a stimulant like MPD affects a developing brain in young subjects, versus how it affects a brain that is fully developed. Many changes are still occurring in young developing brains with synaptic outgrowth and pruning which help to shape the adult brain. What effects that drugs like MPD have on the adolescent brain is still largely unknown. MPD has a chemical structure similar to amphetamine and methamphetamine and has neuropharmacological characteristics to cocaine (Kallman and Isaac, 1975; Patrick and Markowitz, 1997; Teo et al., 2003; Volkow, 2001). Therefore, due to its close similarities to these drugs of abuse, MPD poses the risk as a drug of addiction. MPD has a similar mechanism of action to cocaine in that it acts as an indirect agonist of dopamine (DA) by binding with high affinity to the dopamine transporter (DAT), blocking the DA reuptake back into the presynaptic terminal. This in turn causes increased levels of extracellular DA in the synaptic cleft giving the postsynaptic longer exposure to the released DA increasing the rate of postsynaptic binding. MPD is absorbed and metabolized via de-esterification to ritalinic acid and is usually found in the urine within 48 hours of use (Faraj et al., 1974; Wang et al., 1997; Wargin et al., 1983). Because the psychostimulant is concentrated in the catecholaminergic systems, and has free flow across the blood brain barrier, the subsequent concentration of MPD in the brain exceeds the plasma levels. In addition, MPD also has a rapid uptake in the brain like cocaine, except MPD has a much lower rate of clearance (Morse et al., 1995). This may explain why i.v. or intranasal administration of MPD has higher mortality rates than both cocaine and amphetamines for higher doses (Gatley et al., 1999; Volkow et al., 1995, 1996, 1999). Most users who recreationally use MPD to get high are not aware of the dangers associated with their actions, thus there is a great danger in the misuse of the drug.

The dosage and route of administration of MPD plays a large role in the experience of the user, due to the fact that the behavioral responses and the neurochemical reactions to the drug are dependent upon both the length of time it takes for the drug to reach its peak level in the periphery and how long the drug is in the system (Kuczenski and Segal., 2002). When MPD is administered orally, which is the normal route for ADHD patients; it is absorbed through the intestinal tract having

a half-life around 1 hour and shows peaks in the blood approximately 60-90 minutes after ingestion (Garland 1998; Gerasimov et al., 2000; Kuczenski and Segal 2002; Solanto 1998, 2000; Stewart & Badiani 1993). Following intravenous (i.v) injection or intraperitoneal (i.p.) administration, peak levels of MPD in the periphery are reached at approximately 8-20 minutes and 15-28 minutes, respectively (Gerasimov et al., 2000; Kuczenski and Segal 2002; Stewart & Badiani 1993). The ability to reach peak levels rapidly (8-30 minutes) is one of the main factors responsible for both the euphoric/pleasurable responses and the adverse effects, such as a sensitization response.

ADHD in Adolescents and Adults

It has been estimated that worldwide, about 5% of children and 4% of adults are affected with ADHD and in the U.S. around 8.7% of the children tested meet the criteria for ADHD (Froehlich et al., 2007; Polanczyk and Rohde, 2007; Wilens et al., 2005). Therefore, it is not uncommon for MPD to be prescribed to children as early as elementary school, a time when the brain is still going through development (Lakhan and Kirchgessner, 2012). In fact, the percentage of children between the ages of 4–17 years who have been diagnosed with ADHD has increased from 7.8% in 2003 to 9.5% in 2007, representing a 21.8% increase in just 4 years (Center for Disease Control and Prevention, 2010). Because so many children are being prescribed psychostimulants at young ages, more research needs to be carried out to better understand the way that these drugs affect young brains. The current literature on adolescent methylphenidate studies is lacking, and it remains unclear how exactly drugs like methylphenidate affect young developing brains and whether that is different than its effects in adult brains. Between 5 and 15 years of age, the human brain goes through a process of over production of synaptic connections and receptors followed by their pruning and competitive elimination, which may serve as a factor in the development of behavior disorders (Andersen and Teicher, 2000; Brenhouse and Andersen, 2011; Huttenlocher, 1974; Rakic et al., 1986).

Behavioral and chemical studies have reported that MPD affects the young differently than adults (Brandon et al., 2003; Canese et al., 2010; Dafny and Yang, 2006; Yang et al., 2011). However, there have not been any studies done recording both neuronal and behavioral recordings concomitantly. MPD exposure has been shown to produce an age dose dependent increase in extracellular levels of DA and in norepinephrine (NE) levels (Kuczenski and Segal, 2001). Walker et al. (2010) reported that the extracellular DA levels in adolescents treated with MPD are higher than those in adults who have been treated with MPD. In anesthetized animals, MPD was reported to affect neuronal functions differently in the young compared to adults (Gronier et al., 2010). A functional MRI study has reported drastically different responses to MPD between adolescent and adults (Canese et al., 2009). Thus it is possible to assume that MPD efficacy will be different in young populations when compared to adults. Therefore it is important to study the role of MPD in adolescents as well as adults to examine if differences exist between the two when treated with MPD.

Behavioral Sensitization and Tolerance Following MPD Administration

Chronic exposure to psychostimulants such as MPD can result in the initiation and alteration of biochemical, molecular, and morphological configuration as well as behavioral changes which can lead to plasticity in the CNS (Chao and Nestler, 2004; Dafny and Yang, 2006; Dietz et al., 2009; Kim et al., 2009; Nestler 2004; Robison and Nestler, 2011). MPD has been shown to cause different behavioral responses when subjects are given the same dose of the drug. There are some studies which report that MPD elicits behavioral sensitization, whereas there are other studies which report that the same dose of MPD results in behavioral tolerance, causing further conflicts in the literature (Barron et al., 2004; Eckerman et al., 1991; Gaytan et al., 1996, 2000; Yang et al., 2003; 2006, 2007). The aim of the this study was to first determine if there are differences in neuronal responses between individuals, with respect to neuronal firing patterns in the ventral tegmental area (VTA), and whether these differences are responsible for the conflicting reports on behavioral responses with

chronic MPD exposure. I hypothesized that animals will exhibit different overall neuronal responses depending on whether that animal exhibits behavioral sensitization or behavioral tolerance.

Furthermore, I hypothesize that an increase or a decrease in neuronal responses directly correlates to behavioral sensitization or tolerance, respectively.

Several reviews suggest that behavioral tolerance and sensitization are phenomena in animals that represent an enduring response to medications even after the discontinuation of drug use, and are used as a model for drug craving and dependence (Laasko et al., 2002; Kalivas et al., 1998; Kauer, 2004; Robinson and Berridge, 1993; Wolf, 1998). It has been previously shown that stimulants such as cocaine, amphetamine, and MPD are able to cause dose dependent behavioral sensitization in animals (Algahim et al., 2009; Bergheim et al., 2012; Gaytan et al., 1996, 1999; Kalivas et al., 1988; Tang et al., 2009; Yang et al., 2003, 2011). Behavioral sensitization is defined as the increased amplification of activity resulting from repetitive administration of psychostimulants (Chao and Nestler, 2004; Gaytan et al., 1997; Kalivas and Stewart, 1991). Behavioral sensitization occurs in two phases: induction and expression. In the induction phase, transient changes occur following repetitive psychostimulant administration that subsequently result in increased behavioral activity. The expression phase is characterized by persistent long-lasting neuronal changes that result in sustained augmented behavioral responses despite cessation of psychostimulant use (Pierce and Kalivas, 1997). Thus, there is an increased behavioral activity response observed when animals are given a rechallenge exposure of the drug following a period when the drug is no longer in the system.

Generally it is believed that behavioral sensitization occurs due to synaptic plasticity in the reward circuit, including the CNS structures: the nucleus accumbens (NAc), prefrontal cortex (PFC), caudate nucleus (CN), and the VTA.

The induction phase is thought to occur at glutamatergic synapses of the dopamine (DA) neurons in the ventral tegmental area (VTA) (Kalivas and Weber, 1988; Vezina, 1993; Perugini and Vezina, 1994; Pert, 1998). The expression of behavioral sensitization is suggested to be due to repetitive (chronic) psychostimulant exposure causing increased glutamate transmission and a decrease of D1 DA to GABAergic neurons in the VTA (Bonci and Williams, 1996; Pierce

and Kalivas, 1997; Kalivas and Duffy, 1993). Reprinted from *Jones, Z.; Dafny, N. Acute and chronic dose-response effect of methylphenidate on ventral tegmental area neurons correlated with animal behavior. J. Neural Transm. 2013 In Press.* with kind permission of Springer Science and Business Media. Copyright © 2013, Springer Science and Business Media.

The Role of the Ventral Tegmental Area (VTA) in Response to MPD

The VTA is an extremely important area of the brain when it comes to drug use and has a large role in the behavioral effects of psychostimulants. The VTA is a midbrain structure that is rich in both DA and gamma amino-butyric acid (GABA) neurons (Grace and Onn; 1989) which project to the NAc and PFC and the latter structures also send neurons back to the VTA, thus there is communication back and forth between these structures (Figure 1). Both DA and GABA neurons in the VTA get input mediated by GABA and glutamate. The primary afferent neurons which are excitatory to the VTA and contain glutamate come from the PFC, while those containing GABA originate in the NAc (Beckstead et al., 1979; Johnson and North, 1992; Kalivas and Duffy, 1995). In addition, the VTA gets other excitatory inputs from the amygdala, laterodorsal tegmental nucleus and the bed nucleus of the stria terminalis (Kauer, 2004). The dopaminergic neurons in the VTA co-express GABA-ergic or glutamatergic markers and it is thought that these DA neurons are activated by the glutamatergic inputs that originate in the PFC and specifically that these neurons are involved in the induction of behavioral sensitization following repetitive exposure of psychostimulants (Scheggi et al., 2002; Tang et al., 2009; Wanchoo et al., 2010). The DA neurons in the VTA that express the glutamatergic markers do so for both the metabotropic and ionotropic receptors which in turn include the NMDA and AMPA subtypes. In a morphological study, it has been shown that about two-thirds of the neurons in the VTA are dopaminergic, while the remaining neurons are mostly GABA (Nair-Roberts et al., 2008).

Subsequently, it has been shown that the mesoaccumbens projection which is formed by the ascending VTA DA neurons to the nucleus accumbens (NAc) have been implicated with the induction of behavioral sensitization and that the VTA plays an integral role in this phenomena (Joyce and Rayport, 2000; Kalivas et al., 1993; Wolf, 1998). For example, in studies that have used

amphetamine and DA D1 and NMDA receptor antagonists have shown that the VTA is responsible for the induction phase of sensitization following repetitive exposure to psychostimulants and perhaps the control of relapse, dependence and drug craving (Kalivas and Weber, 1988; Kalivas and Stewart, 1991). In another study, it was shown that with repeated injection of amphetamine into the VTA, sensitization can be produced (Vezina, 1993; Perugini and Vezina, 1994). After such treatment, it is also possible to become sensitized to systemic morphine injections (Vezina and Stuart, 1990). On the other hand, if DA D1 receptor antagonists or NMDA receptor antagonists are injected into the VTA, then behavioral sensitization cannot be observed (Kalivas and Stuart, 1991). Therefore, it is directly shown that the VTA is absolutely crucial to the development of behavioral sensitization. Chronic stimulant use has been reported to be responsible for structurally altering areas of the brain affected by MPD such as the CN and VTA following acute and repeated administration of MPD. These alterations include the regulation of the transcription factors CREB and Δ Fos B, both implicated in drug dependence (Chao and Nestler, 2004; Nestler 2004, 2008). Δ Fos B gene expression is increased by the upregulation of ERK which has been reported to induce long-term neuroadaptations in the brain (Shi and McGinty, 2010; Zhang et al., 2004). Δ Fos B elevation is correlated with increased reliance to the behavioral effects and increased motivation for the drug (Nestler, 2001; 2004; 2008). D1 receptor inhibition by blockade or ablation results in an increase in phosphorylation of cAMP response element binding protein (CREB), inhibiting the excitatory responses (Ferguson et al., 2010). Due to the importance of the VTA in stimulant action like that of MPD, the VTA was chosen as the target structure of this study.

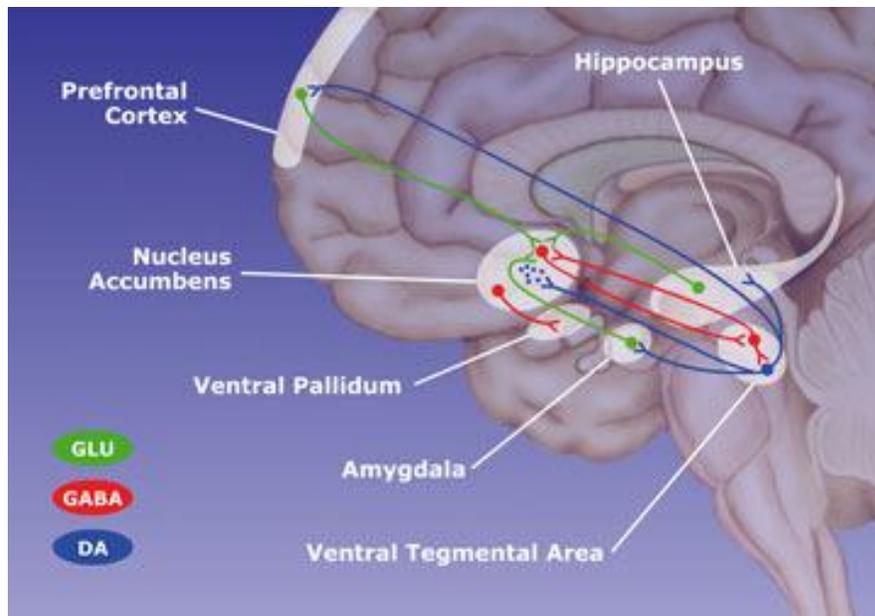


Figure 1 shows the location of the VTA and its connections to other areas of the reward circuit. The VTA sends connections to the prefrontal cortex as well as the nucleus accumbens and in turn receives input from those areas. Rights for reuse of figure obtained through Elsevier

In this study, we strove to compare the neuronal responses and behavioral responses to acute and chronic MPD administration and to determine whether there are any relationships which can be made between these two types of responses. We hypothesize that animals exposed to the same dose of MPD will exhibit different behavioral responses in that some animals will exhibit behavioral sensitization while others will exhibit behavioral tolerance to the same dose of drug. Also, we hypothesized that when these animals are separated based on their behavioral responses of sensitization or tolerance, their neuronal responses will be statistically significantly different. Therefore, the aim of this study was to examine the dose response effects of acute and chronic MPD on VTA neurons of non-anesthetized freely moving animals previously implanted with permanent electrodes and to evaluate the VTA neuronal activity based on the animals' behavioral response to chronic MPD administration. We chose to investigate this hypothesis using a rodent model, as the relationship between drug doses (milligrams of hydrochloride salt/kilogram of body weight) and percentage occupancy of the dopamine transporter (DAT) is identical for cocaine and MPH in both rodents and humans (Gatley et al., 1999). Previous neurophysiological studies targeting the reward circuit independent of the animals behavioral activity show that 2.5 mg/kg MPD modulates the neuronal activity of certain CNS sites such as the VTA, NAc, PFC, and CN following both acute and chronic MPH exposure (Chong et al., 2012; Claussen and Dafny, 2012; Salek et al., 2012). We believe that finding a correlation between the neuronal and behavioral properties of animals in response to acute and chronic MPD will give us insight into why conflicting results are observed across both animal and human studies that investigate the long term adaptations occurring in response to chronic MPD exposure and will further the knowledge of how MPD affects the VTA.

2 Materials and Methods

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2.1 Animals

Male Sprague-Dawley rats (N=51 adult, N=113 adolescent) at post natal of about 50 days (adult) and 32 days (adolescent) were purchased (Harlan, Indianapolis, IN, USA) and were allowed 3-4 days of acclimation in our vivarium room on a 12 hour light/dark schedule (lights on 6:00am). Food and water were given ad libitum. The animals were housed individually in clear acrylic standard cages that served as both home cage and test cage for this study. The experiment was approved by our Animal Welfare Committee and carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2 Surgeries

Prior to surgery, two Nickel-Chromium Teflon coated (fully insulated except at tips) 60 μ in diameter wire were twisted to make two recording electrodes for each VTA hemisphere. Each of the four wires was secured to a 1.0 cm copper connector pin (A-M systems, INC.). On the day of surgery, the rats were anesthetized with an intraperitoneal (i.p.) injection of 25 mg/kg pentobarbital. The animal's head was shaved and lidocaine hydrochloride topical gel was applied to the shaved area. The animal was then placed in a stereotaxic apparatus where an incision was made on the scalp and the muscle and connective tissue was removed to expose the skull. Bilateral holes were drilled above the VTA at 2.3 mm anterior to lambda and 1 mm lateral to midline with a depth of 7.6 to 8.0 mm in the adolescent rat using the Sherwood and Timiras (1970) brain atlas coordinates. Bilateral holes were drilled above the VTA at 6.0 mm posterior to bregma and 0.5 mm lateral from midline with a depth of 8.5 mm, using Paxinos and Watson (1986) brain atlas coordinates for the adult animals. Six anchor screws were inserted in the skull at vacant spots to secure the implanted electrodes and the head plug.

Electrodes were then inserted individually into the brain from the skull with neuronal activity monitored by Grass emitter Hi Z Probe connected to a Grass P511 series amplifier. When a 3:1 signal to noise ratio spike activity was obtained, the electrode was permanently secured to the skull using web glue cyanoacrylate surgical adhesive. When the neuronal activity exhibited less than a 3:1 signal to noise ratio spike activity, the electrode was lowered in 5-10 μm increments until a 3:1 ratio activity was observed (Chong et al., 2012; Claussen and Dafny, 2012; Dafny, 1980; 1982; Dafny and Terkel, 1990; Salek et. al., 2012). Similar procedures were followed for the second twisted electrode which was implanted into the VTA of the opposite hemisphere. The two copper pins from each twisted electrode from the four recording electrodes, were inserted into Amphenol plugs which were secured to the skull using dental acrylic cement creating the skull cap. Animals were allowed 3 to 5 days recovery after electrode implantation during which they were placed daily with their home cage in the experimental apparatus and connected to the wireless (telemetric) head stage transmitter (Triangle BioSystems Intl (TBSI); Durham, NC, USA) for acclimation for at least 2 hours/day to the behavioral and electrophysiological recording systems. At the first experimental day, the adolescent animal's weight is around 100-120 grams and around 39-45 days post natal, while the adult animal's weight was between 200 and 220 grams and at about post natal 62-64 days.

2.3 Drugs

Three methylphenidate hydrochloride (MPD) (obtained from Mallinckrot Hazelwood, MO, USA) doses of 0.6, 2.5, and 10.0 mg/kg, were used; the MPD doses were calculated as a free base and were dissolved in 0.9% isotonic saline solution. Control injections consisted of 0.8 ml isotonic saline solution (0.9% NaCl) administered i.p. All injections were equalized to a volume of 0.8 ml with 0.9% saline to keep injection volumes the same for all of the animals and for all the MPD doses.

Previous MPD dose response experiments, testing behavioral and neurophysiological sensory evoked potential procedures, from 0.1 mg/kg to 40.0 mg/kg MPD administration, found that behavioral effects of MPD were observed from the 0.6 mg/kg MPD dosage (Algham et al., 2009;

Gaytan et al., 1996, 2000; Lee et al., 2009; Podet et al., 2010; Yang et al., 2003, 2006a; 2006b; 2006c; 2006d; 2007). Therefore 0.6, 2.5, and 10.0 mg/kg MPD dosages were selected for this study.

There are no universally recognized MPD dosage guidelines or blood levels to achieve optimum dose treatment. A study of 289 patients treated with MPD (White and Yadao, 2000) reported that the range of doses ingested was from 0.06 to 29.3 mg/kg. Approximately 2 to 3 mg/kg i.p. MPD in rodent achieved plasma levels similar to those achieved in clinical use (Gatley et al., 1999; Gerasimov et al., 2000). Drug effects in rodents often require higher doses (on mg/kg basis) than humans because rodents exhibit a more rapid metabolism (Gatley et al., 1999).

2.4 Experimental Protocol

On experimental day one (ED1), rats with their home cage were placed in a Faraday testing box to reduce noise during the recording session. The wireless (TBSI); (Fan et al., 2011) head stage was connected to the electrode pins of the skull cap and the animals were allowed to acclimate for 30 minutes prior to the recording session. This time was used to prepare the recording software parameters and the injections of both saline and MPD. After acclimation, the animals received a saline injection of 0.8 ml (standardized for all injections) and the neuronal and behavioral activity was recorded to obtain the baseline activity for one hour followed by either saline, or 0.6, 2.5 or 10.0 mg/kg MPD injection, and the behavioral and the neuronal activity recordings were resumed for an additional hour post injection (Table 1). The wireless TBSI head stage sent neuronal activity signals from the 4 recording electrodes to a receiver that was connected to a Cambridge Electronic Design (CED) Cambridge England analog-to-digital converter (Micro1401-3; CED) which collected and stored the recorded data on a PC using Spike 2.7 CED software. On ED2 through ED6 animals received either saline or daily MPD injections similar to ED1 injection in their home cage without recording. On ED7 through ED9 the animals underwent washout in which no injections were given proceeded by ED10 in which a saline injection was given and the neuronal and behavioral activity was recorded for an hour post saline injection followed by a rechallenge administration of either

saline or MPD similar to ED1 and recordings were resumed for an additional one hour just as on ED1 (Table 1).

Table 1

	Experimental Days			
Treatment	Day 1	Days 2-6	Days 7-9	Day 10
Saline	Saline/Saline	Saline	Washout	Saline/Saline
0.6 mg/kg MPD	Saline/0.6 mg/kg MPD	0.6 mg/kg MPD	Washout	Saline/0.6 mg/kg MPD
2.5 mg/kg MPD	Saline/2.5 mg/kg MPD	2.5 mg/kg MPD	Washout	Saline/2.5 mg/kg MPD
10.0 mg/kg MPD	Saline/10.0 mg/kg MPD	10.0 mg/kg MPD	Washout	Saline/10.0 mg/kg MPD

Table 1 summarizes the experimental protocol. Four groups of animals were used: saline, 0.6, 2.5 and 10.0 mg/kg MPD. On experimental day 1 (ED1), animals are given an initial dose of saline and recordings were taken for one hour followed by one of the four designated doses and recordings were resumed for an additional hour post injection. On days 2-6, the animals are only given an injection of the specified dose. Days 7-9 are washout days where the animal gets no injection of any kind. On ED10, the animals are given another dose of saline for one hour followed by the designated dose for one hour, identical to that given on ED1.

2.5 Behavioral Recording System

Locomotor activity was recorded concomitantly with neuronal activity, using an open field computerized animal activity system (Opto-M3, Columbus Instruments, Columbus, OH). The animal's home cage (40 cm in length, 20 cm in width) fit into the recording apparatus allowing the recording of the animals in his home cage. The cage and the recording system were located inside a Faraday box to reduce noise and any outside interference. The open field system 16 and 8 infrared beams and their sensors on the opposite side set at 5 cm above the floor of the cage. The open field assay has been previously described in detail (Gaytan et al., 1996, 1997a, b, 2000; Yang et al., 2006a, 2007, 2011). In short, the activity monitoring system checked each of the sensor beams at a 100 Hz frequency to determine whether beams were interrupted. The interrupted beams were compiled by the software and downloaded to a PC every 10 min, (i.e. 6 bins/hr). The program organized the beam interruption into different locomotor movement indices, such as horizontal activity (HA) which records the overall locomotor activity that used to assess the overall amount of locomotor activity and the number of stereotypic activity (NOS) which counts the number of repetitive movement episodes with at least one second interval before the beginning of another episode of movements. The 6 bin counts following each hour were used for the statistical analysis and to produce temporal graphs and histograms for total activity/hr for both the saline (baseline activity) and the activity after MPD administration for ED1 and ED10.

2.6 Electrophysiological apparatus

A Triangle BioSystems International (TBSI) telemetric head stage weighing 4.5 g was attached to the electrode pins of the skull cap of the rat. The head stage sent neuronal activity signals (sampling rates up to 200 kHz) to a receiver connected to the Cambridge Electronic Design (CED) analog-to-digital converter (Micro 1401-3; CED, Cambridge, UK) which stored the data on a PC using the Spike 2 version 7 software for offline analysis.

2.7 Data analysis

2.7.1 Behavioral Analysis

The locomotor activity was recorded and summed in 10 minute bins for 60 minutes after saline injection proceeding another 60 minutes of behavioral activity counts recorded following MPD injection (12 bins total) on ED1 and again on ED10 following saline and MPD exposure (Table 1).

1) Behavioral analysis of HA of activity following acute MPD was compared to the activity after saline (control) at ED1; 2) the activity post saline at ED10 was compared to the activity after saline on ED1 and 3) the chronic MPD effect was determined by comparing the activity following MPD on ED10 to the activity after MPD at ED1 using the Critical Ratio CR Test; $(C.R.) = \frac{E-C}{\sqrt{E+C}} = \pm 1.96 =$ $p < 0.05$ and the student paired t-test (Chong et al., 2012; Claussen and Dafny, 2012; Salek et al., 2012; Yang et al., 2012; Yang et al., 2006a; 2006b; 2006d; 2007). Where for example E represents the HA after MPD administration at ED1, and C represents the HA after saline injection of ED1.

When a CR value more than 1.96 was obtained it indicated that the drug elicited significantly increased activity i.e. behavioral sensitization and when a CR value more than -1.96 was obtained it indicated that the drug elicited significant attenuation as compared to the initial drug effects i.e. behavioral tolerance.

Based on the above analysis each rat was individually classified as expressing either behavioral sensitization or tolerance and thus two sub groups were created: sensitized and tolerant/non-sensitized category. These rat subgroups were analyzed again as a group using analysis of variance (ANOVA: treatment days and drug doses). Any statistical significance was determined with the post hoc Fischer's LSD method. Statistical significance was set at $P < 0.05$ for all comparisons.

2.7.2 Spike sorting

The Spike 2 version 7 software (CED) was used for spike sorting. The data was captured by the program (sampling rates up to 200 kHz) and processed using low and high pass filters (0.3-3 kHz). There were two window discriminator levels, one for positive-going spikes and one for

negative-going spikes. The spikes with peak amplitudes within the window were used to create templates using 1000 waveform data points. The parameters that were used to capture a spike pattern allows the extraction of templates that provide high-dimensional reference points which can be used to perform accurate spike sorting, despite some movement artifacts noise, false threshold crossing and waveform overlap. All temporally displaced templates are compared with the incoming spike event to find the best fitting to the selected template amplitude that yields the minimum residue variance. When the distance between the template and waveform exceeds some threshold (80%), the waveforms are rejected. This means that the spike sorting accuracy in the reconstructed data is about 95%. All of the parameters of spike sorting for each electrode at ED1 were stored and reused for the activity sorting at ED10 aiming to count the same spike amplitude and pattern at ED1 and ED10 following saline and MPD administration from the same electrode. Spikes with peak amplitudes outside these limits and spikes that didn't fit the template were rejected.

2.7.3 Electrophysiological Data evaluation

Once the neuronal activity was sorted and counted, the activity post saline and MPD exposure was exported to a spreadsheet to produce sequential firing rate graph spikes/sec and counting the total neuronal activity. The initial 60 minutes following saline injection at ED1 was used as the ED1 baseline activity. Similar calculations were done for ED10, i.e. 60 min post ED10 saline for the ED10 baseline activity and 60 min post MPD at ED10, the effect of MPD rechallenge. The activity post MPD at ED1 compared to ED1 baseline activity was used to determine the MPD effect. The baseline activity on ED10 was compared to the baseline activity on ED1 to find out whether the six consecutive daily injection and the three washout days resulted in the baseline alteration, and lastly, the effect of drug administration on ED10 was compared to the effects of drug administration on ED1 to find out whether sensitization or tolerance was expressed.

Multiple methods were employed to find out whether MPD elicits significant effect on VTA neuronal activity as follows: the mean firing rate after MPD treatment needs to be at least two standard errors (S.E.) difference from the control mean firing rate. Firing rates were evaluated also

for normality assumptions to determine parametric or non-parametric methods. To evaluate differences between the above comparisons, the firing rates were determined to not hold normality assumptions, so we assessed also differences in firing rates using the Critical Ratio test. Critical Ratio test ((C.R.) = $\frac{E-C}{\sqrt{E+C}} = \pm 1.96 = p < 0.05$) (C = control, E = activity after drug exposure) for the first comparison. For the second comparison, E was the baseline at ED10 and C is the baseline at ED1, while for the third comparison E was the data post MPD exposure at ED10 and C the data post MPD exposure at ED1 (Chong et. al., 2012; Claussen and Dafny 2012; Dafny, 1975; 1982; Salek et. al., 2012; Yang et al. 2006a; 2006b; 2006c).

In addition, the natural log odds ratio and an ANOVA statistical tests were used: the natural logs ratio was utilized to determine the likelihood that VTA neuronal populations recorded from animals expressing behavioral sensitization are the same or different from the VTA neuronal populations recorded from animals expressing behavioral tolerance to repetitive MPD exposure. This was done for the data obtained following the initial (acute) MPD injection compared to ED1 baseline, the baseline neuronal activity at ED10 compared to the baseline activity at ED1, and the recording following rechallenge of MPD at ED10 compared to the effect of initial MPD injection at ED1. To compensate for the smaller values observed on certain days for varying doses, 0.5 was added to all numbers for computation of the odds ratio. A number of 1 and higher in the odds ratio test indicates a significantly higher likelihood of different responses to MPD exposure (increases or decreases in the neuronal activity) in the neuronal populations recorded from behaviorally sensitized animals compared to recordings from animals expressing behavioral tolerance. Conversely a number smaller than 1 represents a less likely chance of differences between the two VTA neuronal populations (Morris and Gardner, 1988) in response to MPD exposure.

The ANOVA statistical test with statistic value ($p < 0.05$) was used to compare the difference in response to MPD exposure between the neuronal population recorded from animals expressing behavioral sensitization to the VTA neuronal population recorded from animals expressing

behavioral tolerance to acute and chronic MPD exposure, as well as to compare the adult sensitized animals to the adolescent sensitized animals and the same for the tolerant animals.

2.7.4 Histological verification

Upon completion of the recording at ED10, the animals were euthanized with sodium pentobarbital. The rat was then perfused intracardially with 10% formaldehyde solution containing 3% potassium ferrocyanide. A 2mA DC current was passed through the tip of each electrode for 40 seconds to create a small lesion to identify the electrode location; the brain was extracted from the skull and placed in 10% formaldehyde for several days. The brains were sliced in 40-60 μ m sections and the position of the electrode tip was identified by the location of the electrolytic lesion in the adolescent rat by using the Sherwood and Timiras brain atlas (1970) (Figure 2) and in the adult animal using the Rat Brain atlas (Paxinos and Watson, 1986) (Figure 3).

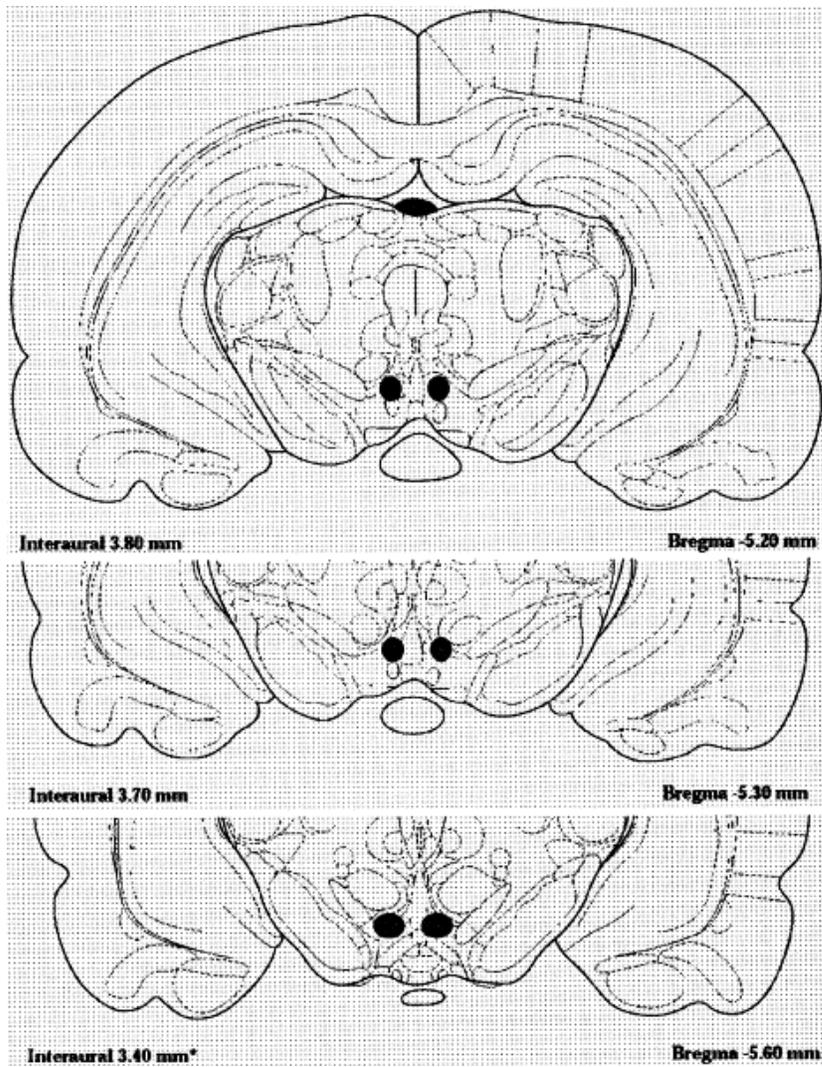


Figure 2. Histologically verified electrode tip placement in the VTA using the coordinates given in the Sherwood and Timiras rat brain atlas 1970. The black spots are the ideal locations of the electrode tips. Rights for reuse of figure obtained through Elsevier.

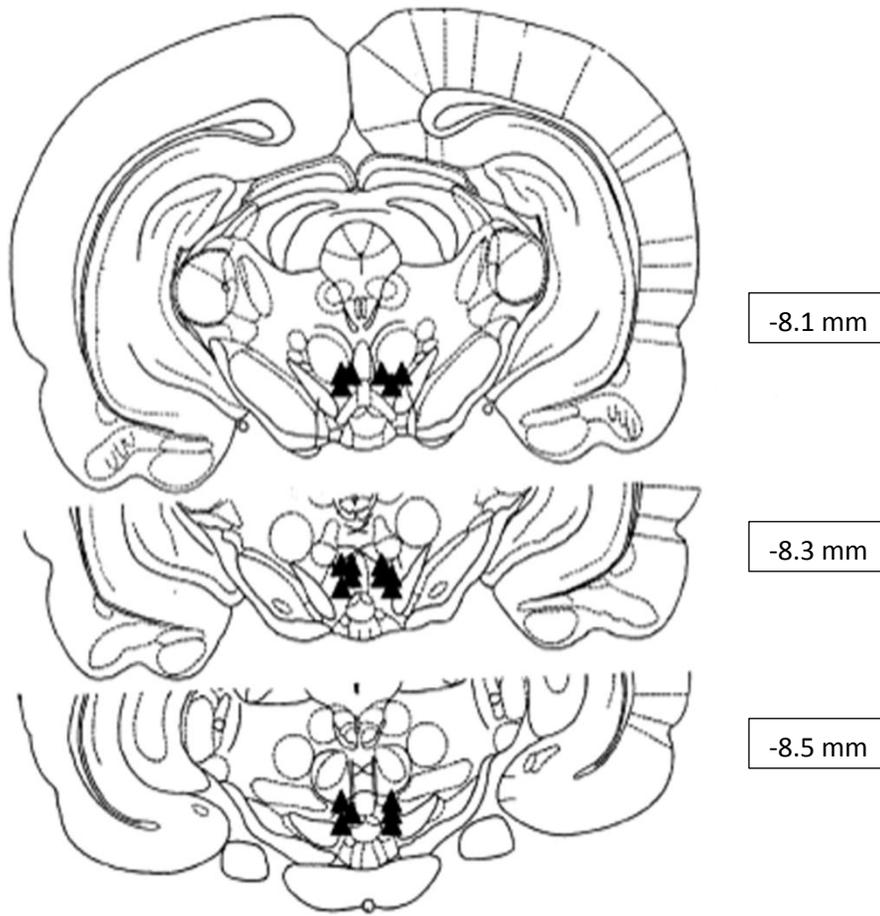


Figure 3 reconstructs histologically verified electrode tip placement in the adult VTA using the plate from Paxinos and Watson Rat Brain Atlas 1986. The black triangles indicate areas that are considered VTA for histological purposes. Rights for reuse of figure obtained through Elsevier

3 Results

3.1 Adolescent

A total of 113 adolescent rats with electrodes confirmed to be in the VTA were used in this experiment; 11, 28, 31, and 43 rats were treated with saline (control), 0.6, 2.5, or 10.0 mg/kg MPD respectively and 307 VTA units were recorded, 26, 81, 99, and 101 after saline, 0.6, 2.5, and 10.0 mg/kg MPD respectively.

3.2 Behavioral Results Adolescent

The overall effect of the acute MPD administration resulted in a dose response dependent increase in locomotor activity. In addition, the baseline activity at ED10 after six daily MPD exposures and three washout days compared to experimental day 1 (ED1) was increased. In general the locomotor activity of all the animals after MPD rechallenge on experimental day 10 (ED10) compared to the recording of ED1 after the initial MPD administration for the dose of 2.5 mg/kg MPD elicited behavioral tolerance and in the higher MPD dose of 10.0 mg/kg dose the group expressed behavioral sensitization as determined by a significant increase in behavioral activity on ED10 following MPD compared to the behavioral activity following MPD on ED1. Eleven animals were treated only with saline. The locomotor activity (HA) from ED10 compared to ED1 following chronic injection of saline exhibited similar locomotor activities with minor non-significant fluctuations in the locomotor indices evaluated following single and multiple saline injections. This showed that neither saline, needle injection nor handling had any effect on behavioral activity (Figure 4). Therefore any significant changes from baseline after drug treatment were due to the effects of MPD. For the adolescent behavioral figures, Sensitized= summarizes the total distance of animals expressing behavioral sensitization and Tolerance= summarizes the total distance of the individual animals that express behavioral tolerance.

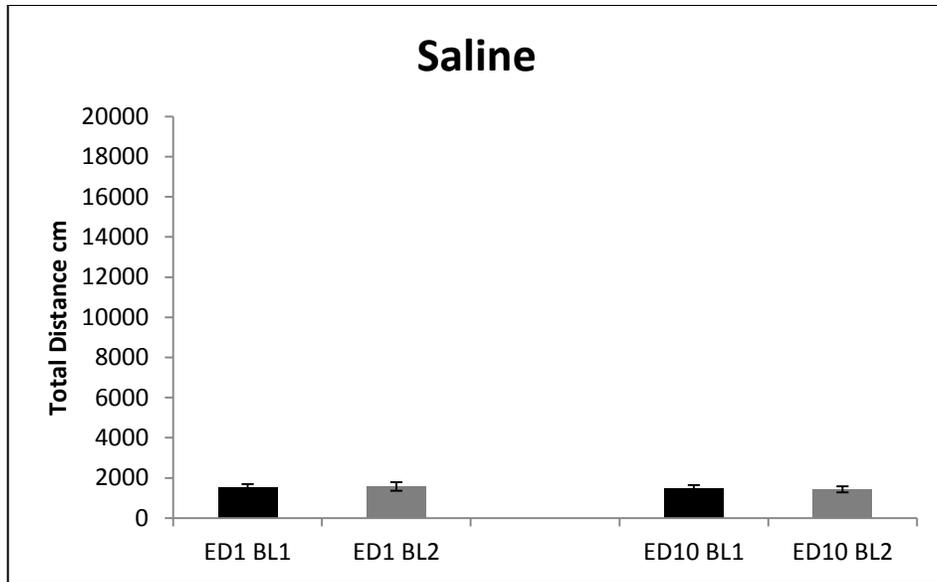


Figure 4 summarizes the total distance activity of the control groups (N=11) after injection of saline on experimental day 1 (ED1) and ED10. For each experimental day, saline was first injected and the behavioral activity was recorded for one hour. Following this, another injection of saline was given and again the animals were recorded for another hour. Comparison between the 2nd injections to the first for both days, showed no significant differences.

3.2.1 Adolescent 0.6 mg/kg MPD

Twenty-eight animals were treated with 0.6 mg/kg MPD and when grouped together, no significant changes in behavioral activity were noted for either the acute or the chronic exposures to MPD or when comparing the baseline activity on ED10 after six daily 0.6 mg/kg MPD exposures and three washout days, to the baseline activity recorded on ED1 using the ANOVA test (Fig 5 All). When the animals were separated based on their individual responses to chronic MPD using the C.R. test, 14 individual animals failed to respond significantly to the initial (acute) MPD exposure at ED1, but expressed significant ($p < 0.05$) increases in their locomotor activity using the ANOVA test i.e. they expressed behavioral sensitization to MPD rechallenge exposure at ED10 (Fig 5 0.6 mg/kg MPD Sensitized). Fourteen individual animals exhibited behavioral tolerance to 0.6 mg/kg MPD rechallenge at ED10 compared to MPD at ED1 using the C.R. test. These animals at ED1 exhibited significant ($p < 0.05$) increases in their behavioral activity following acute MPD exposures and on ED10 exhibited a significant ($p < 0.05$) decrease in their activity in response to the MPD rechallenge compared to activity post MPD at ED1 using the ANOVA test (Fig 5 0.6 mg/kg MPD Tolerance). We were able to show that 0.6 mg/kg MPD can induce both behavioral sensitization and behavioral tolerance to different animals.

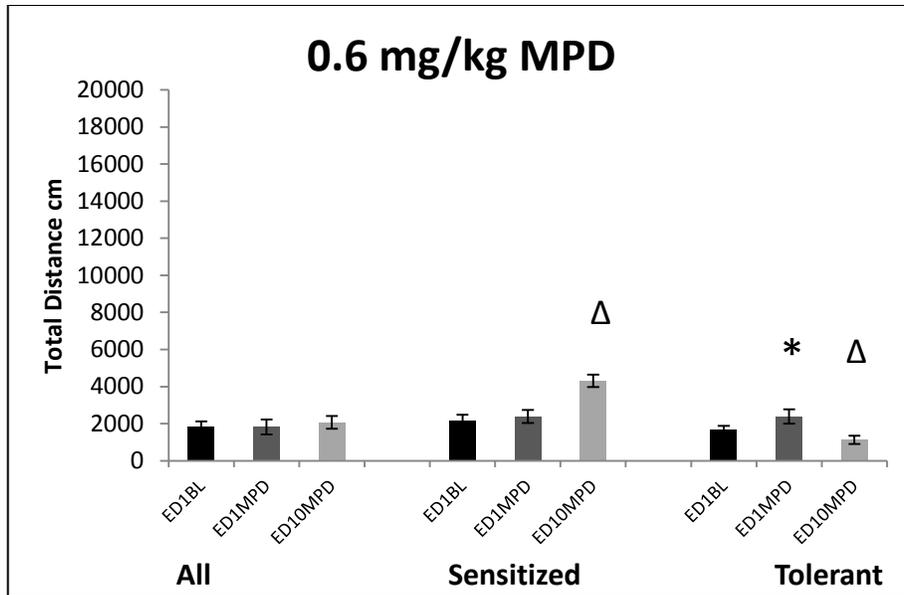


Figure 5 summarizes the behavioral activity following acute and chronic injection of 0.6 mg/kg MPD. The activity for all the animals grouped together (N=28) is shown on the left with comparisons between the ED1 baseline activity, the ED1 MPD injection, and the ED10 MPD injection compared to that post MPD on ED1. For the middle and right sides of the histogram, the animal groups are broken down into two groups: those animals exhibiting behavioral sensitization (N=14) and those exhibiting behavioral tolerance (N=14). For these two groups, the same comparisons were made as the 'all' group. All= summarizes the total distance of all the animals. *= indicates significant ($p < 0.05$) difference between the effect of the initial MPD exposure at ED1 to their baseline activity. Δ= indicates significant difference between the ED10 activity post MPD to the activity of ED1 post MPD exposure.

3.2.2 Adolescent 2.5 mg/kg MPD

Thirty-one animals were treated with acute and chronic 2.5 mg/kg MPD. Acute MPD exposure elicited significant ($p < 0.05$) increases in activity while repetitive (chronic) 2.5 mg/kg MPD elicited significant ($p < 0.05$) decreases in activity at ED10 compared to the activity elicited by the initial MPD exposure at ED1 i.e., tolerance to MPD rechallenge (Fig 6 2.5 mg/kg MPD All). When the animals were separated by their individual behavioral responses to MPD, 22 out of the 31 animals exhibited individually behavioral sensitization using the C.R. test and as a group using the ANOVA test had significant ($p < 0.05$) increases to acute administration of MPD, and following rechallenge dose of 2.5 mg/kg MPD on ED10 exhibited further significant ($p < 0.05$) increase compared to that observed on ED1 i.e. expressing behavioral sensitization (Fig 6 2.5 mg/kg MPD Sensitized). Nine individual animals each exhibited behavioral tolerance using the C.R. test. These animals showed a significant ($p < 0.05$) increase in activity to acute administration of MPD, and a significant decrease in activity following rechallenge to MPD on ED10 compared to the effect of MPD on ED1 using the ANOVA test (Figure 6 2.5 mg/kg Tolerance). Those animals that exhibited tolerance to MPD rechallenge at ED10 responded to acute MPD significantly ($p < 0.05$) higher than the animals that expressed behavioral sensitization (Fig 6 2.5 mg/kg MPD).

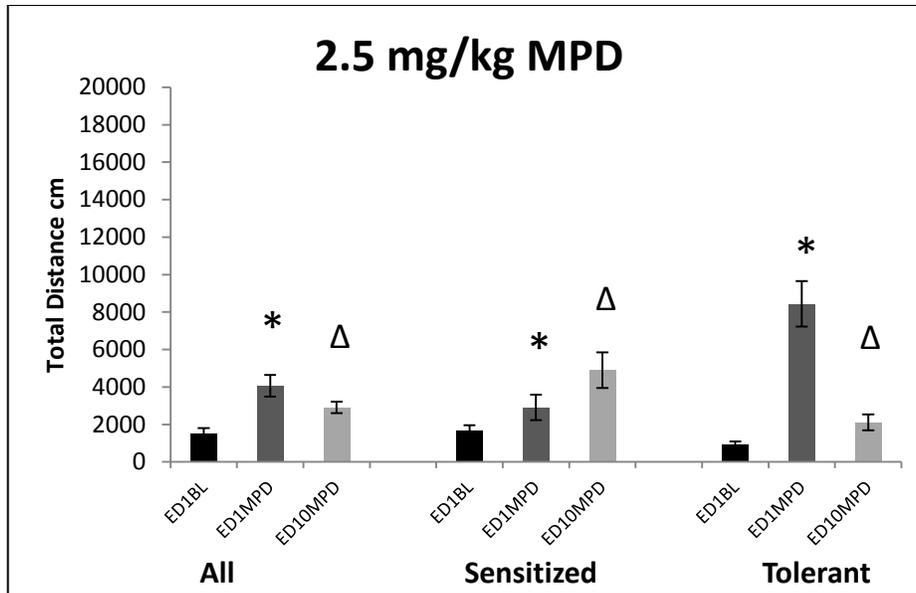


Figure 6 summarizes the behavioral activity following acute and chronic injection of 2.5 mg/kg MPD. The activity for all the animals grouped together (N=31) is shown on the left with comparisons between the ED1 baseline activity, the ED1 MPD injection, and the ED10 MPD injection compared to that post MPD on ED1. For the middle and right sides of the histogram, the animal groups are broken down into two groups: those animals exhibiting behavioral sensitization (N=22) and those exhibiting behavioral tolerance (N=9). For these two groups, the same comparisons were made as the 'all' group. All= summarizes the total distance of all the animals. *= indicates significant ($p < 0.05$) difference between the effect of the initial MPD exposure at ED1 to their baseline activity. Δ = indicates significant difference between the ED10 activity post MPD to the activity of ED1 post MPD exposure.

3.2.3 Adolescent 10.0 mg/kg MPD Behavior

Forty-three animals were treated with 10.0 mg/kg MPD and when grouped together, these animals exhibited a significant ($p < 0.05$) increase in locomotor activity to the acute administration of MPD on ED1. Following MPD rechallenge at ED10, these animals exhibited further significantly ($p < 0.05$) increased locomotor activity using the ANOVA test (Fig 7, 10.0 mg/kg MPD, All). When the animals were separated based off of their individual responses to 10.0 mg/kg MPD using the C.R. test, 37 animals that individually exhibited behavioral sensitization responded significantly ($p < 0.05$) to acute MPD exposure by increasing their behavioral activity and had further significant ($p < 0.05$) increases in their locomotor activity following rechallenge MPD at ED10 compared to ED1 activity post MPD exposure using the ANOVA test (Fig 7 10.0 mg/kg MPD Sensitized). Six individual animals responded to acute MPD exposure by increasing their locomotor activity and the MPD exposure at ED10 resulted in significantly ($p < 0.05$) less activity compared to the initial MPD exposure using the ANOVA test i.e., these animals expressed behavioral tolerance to MPD rechallenge at ED10 compared to the initial acute MPD exposure at ED1 (Fig 7 10.0 mg/kg MPD Tolerance).

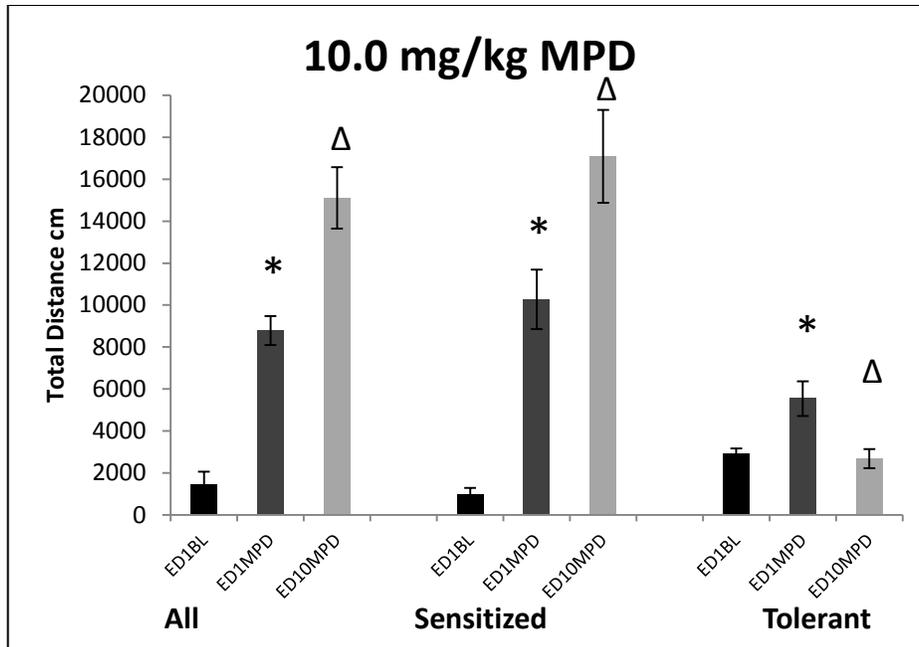


Figure 7 summarizes the behavioral activity following acute and chronic injection of 10.0 mg/kg MPD. The activity for all the animals grouped together (N=43) is shown on the left with comparisons between the ED1 baseline activity, the ED1 MPD injection, and the ED10 MPD injection compared to that post MPD on ED1. For the middle and right sides of the histogram, the animal groups are broken down into two groups: those animals exhibiting behavioral sensitization (N=37) and those exhibiting behavioral tolerance (N=6). For these two groups, the same comparisons were made as the 'all' group. All= summarizes the total distance of all the animals. *= indicates significant ($p < 0.05$) difference between the effect of the initial MPD exposure at ED1 to their baseline activity. Δ= indicates significant difference between the ED10 activity post MPD to the activity of ED1 post MPD exposure.

3.3 Adults

Parts of this chapter are based on *Jones, Z.; Dafny, N. Acute and chronic dose-response effect of methylphenidate on ventral tegmental area neurons correlated with animal behavior. J. Neural Transm. 2013 In Press.* Reprinted from Jones and Dafny, 2013 with kind permission of Springer Science and Business Media. Copyright © 2013, Springer Science and Business Media.

A total of 51 rats with 111 electrodes confirmed to be in the VTA were used in this experiment.

Twenty, 14, 7, and 10 rats were treated with saline, 0.6, 2.5, and 10.0 mg/kg MPD respectively and 361 units were recorded, 76, 132, 62, and 91 after saline, 0.6, 2.5, and 10.0 mg/kg MPD respectively.

3.4 Behavioral Results Adult

Control: Twenty animals were treated only with saline. Following acute and repetitive saline injection, all animals expressed similar locomotor activity following the 2nd saline injection compared to the first on ED1. This showed that saline and handling had no effect on the rats' behavioral activity. Therefore the activity after saline injection at ED1 can be used and was used as the control for the MPD effects, i.e., any significant changes due to drug exposure were due to the effects of MPD.

3.4.1 Adult 0.6 mg/kg MPD

Fourteen animals were treated with 0.6 mg/kg MPD, the ANOVA test showed no significant changes in locomotor activity after acute or chronic 0.6 mg/kg MPD or when the baseline activity at ED10 (after 6 daily 0.6 mg/kg MPD and 3 washout days) was compared with ED1 baseline (Fig 8Aa).

When these 14 animals were divided based on their individual behavioral responses to chronic MPD exposure, 3 animals at ED10 following MPD exposure exhibited significant ($p < 0.05$) increases in their locomotion compared to the activity following MPD injection on ED1 (Fig 8 Ab). These 3 animals at ED1 responded to MPD by significant ($p < 0.05$) decreases by 52% in their locomotor activity compared to their ED1 baseline (Fig 8 Ab). The baseline locomotor activity at ED10 after 6 daily injections of 0.6 mg/kg MPD and 3 washout days was significantly ($p < 0.05$) elevated by 38%

compared to the baseline at ED1 (Fig 8 Ab). The remaining 11 animals treated with 0.6 mg/kg MPD showed reduction in their locomotor activity at ED10 post MPD compared to ED1 post MPD exposure, but this attenuation appears to be non-significant as a group. These animals did not respond significantly to acute 0.6 mg/kg MPD and their ED10 baseline behavioral activity compared to ED1 baseline behavioral activity was relatively unchanged (Fig 8 Ac).

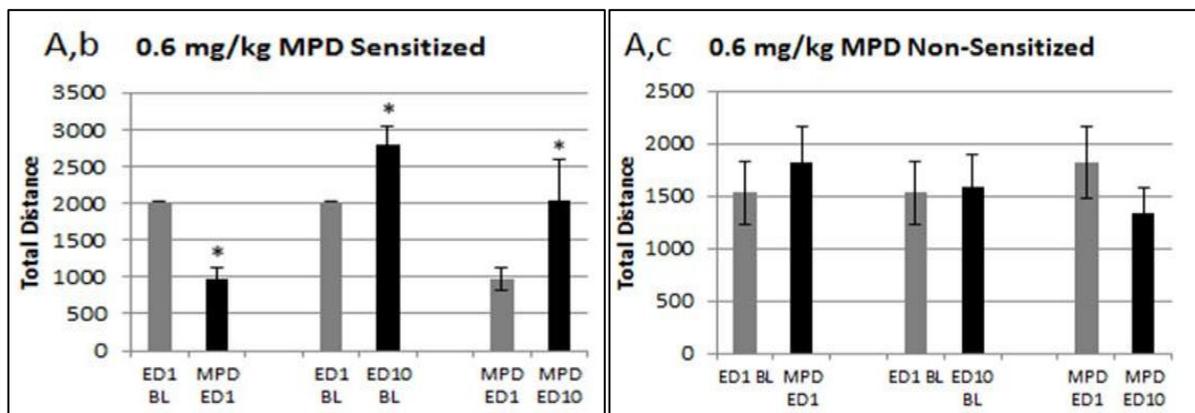
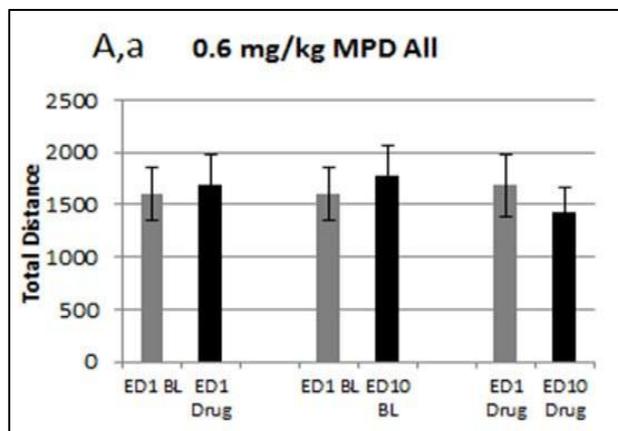


Figure 8 shows the horizontal activity (HA) traveled data following 0.6 mg/kg MPD dose. The top histogram (Aa) summarizes all the animals (N = 14). In addition, data was broken down into those animals that exhibited behavioral sensitization (N = 3) (Ab) and those animals that exhibited behavioral tolerance (N = 11) (Ac) to chronic MPD exposure respectively. For each group, the HA of ED1 after MPD exposure is compared to ED1 Baseline activity; the ED10 Baseline HA is compared to ED1 Baseline HA; and the HA activity on ED10 following MPD exposure is compared to the ED1 HA post MPD exposure. ED - experimental day; BL – baseline; MPD – methylphenidate; * - P < 0.05.

3.4.2 Adult 2.5 mg/kg MPD

Seven animals were treated with acute and chronic 2.5 mg/kg MPD, and when grouped together for analysis of their behavioral activity, it was observed (ANOVA test) that all the animals exhibited significant ($p < 0.05$) increases in their locomotion following the initial (acute) 2.5 mg/kg MPD exposure at ED1 (Fig 9 Bd). However, the activity post MPD exposure at ED10 compared to activity post MPD at ED1, as well as the baseline activity at ED10 compared to the baseline at ED1, was about the same, i.e. was not significantly different (Fig 9 Bd). When these seven rats were evaluated individually using the C.R. test and then grouped based on their individual responses to chronic MPD exposure, 3 animals exhibited behavioral sensitization (using the ANOVA test, $p < 0.05$). The acute 2.5 mg/kg MPD to these 3 animals elicited non-statistically significant increases in locomotion (Fig 9 Be). The baseline activity at ED10 of these 3 animals compared to baseline at ED1 exhibited non-significant attenuation, and there were increases in their locomotor activity at ED10 compared to ED1 post MPD exposure, but due to a large standard error and small N, it was not significant (Fig 9 Be). The remaining 4 rats that individually exhibited a decrease in their locomotor activity ($p < 0.05$) following chronic MPD exposure, i.e. exhibited behavioral tolerance. Their behavioral response to the initial (acute) MPD was robustly ($p < 0.05$) increased (Fig 9 Bf) and the baseline activity of this group at ED10 compared to ED1 baseline exhibited a non-significant difference. The activity following chronic MPD exposure at ED10 compared to the effect of MPD on ED1 (Fig 9 Bf) after 2.5 mg/kg MPD exposure as a group, exhibited a non-significant decrease in their behavioral activity.

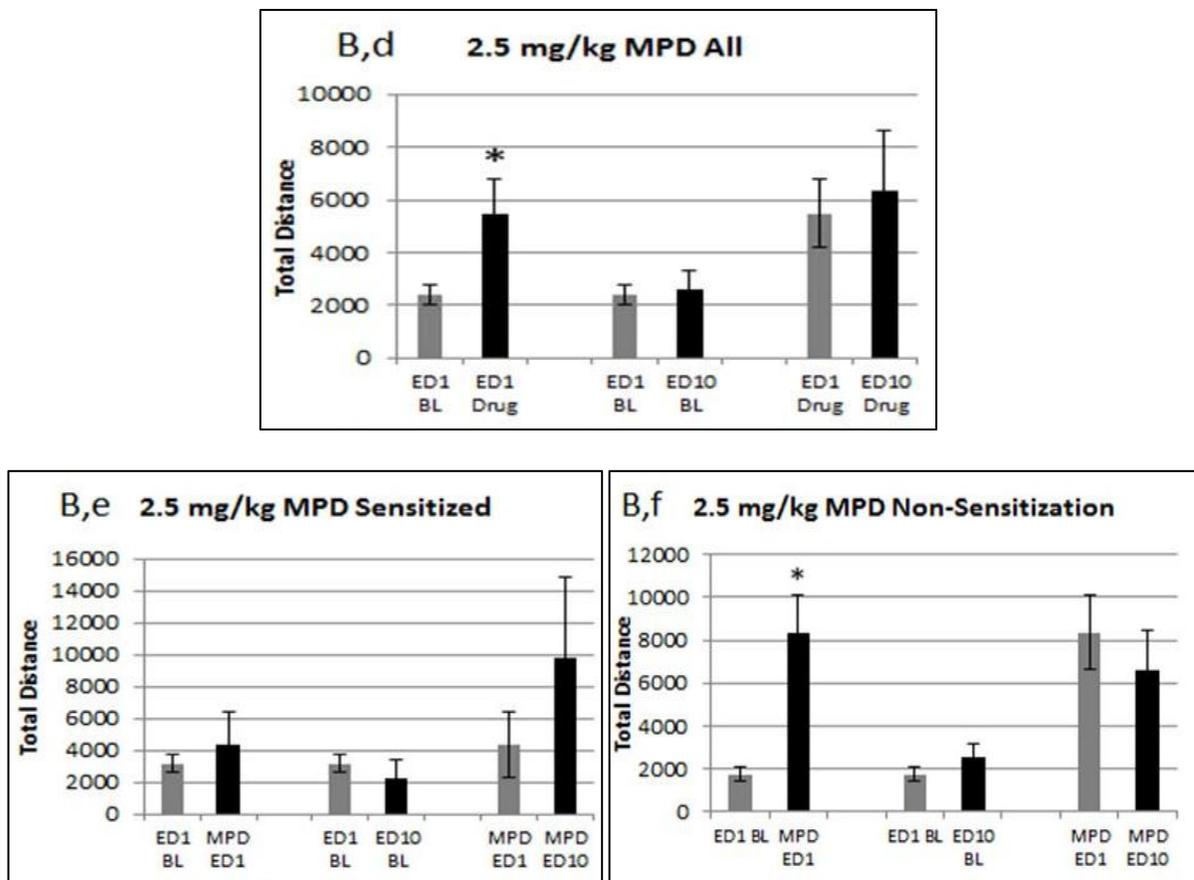


Figure 9 shows the horizontal activity (HA) traveled data following 2.5 mg/kg MPD dose. The top histogram (Bd) summarizes all the animals (N = 7). In addition, data was broken down into those animals that exhibited behavioral sensitization (N = 3) (Be) and those animals that exhibited behavioral tolerance (N = 4) (Bf) to chronic MPD exposure respectively. For each group, the HA of ED1 after MPD exposure is compared to ED1 Baseline activity; the ED10 Baseline HA is compared to ED1 Baseline HA; and the HA activity on ED10 following MPD exposure is compared to the ED1 HA post MPD exposure. ED - experimental day; BL – baseline; MPD – methylphenidate; * - P < 0.05.

3.4.3 Adult 10.0 mg/kg MPD

The 10 animals treated with 10.0 mg/kg MPD exhibited a significant ($p < 0.001$) increase in locomotor activity by 580% compared to their baseline arbitrarily set at 100% (Fig 10 Cg). The ED10 baseline activity remained the same compared to the initial baseline activity at ED1. MPD exposure at ED10 compared to the initial MPD exposure at ED1 elicits similar effects (Fig 10 Cg). When the animals were analyzed individually based on their chronic response to the drug to the acute effect, 5 of the animals exhibited significant ($p < 0.05$) behavioral sensitization (Fig 10 Ch), and 5 animals exhibited a significant ($p < 0.05$) reduction in their locomotor activity (Fig 10 Ch, Ci). Those individual animals that exhibit behavioral sensitization exhibited significantly ($p < 0.05$) increased responses to MPD in response to the acute dose of 10.0 mg/kg by 487% (Fig 10 Ch). The baseline activity at ED10 compared to ED1 baseline was increased, but non-significantly. The chronic effect of the drug at ED10 was compared to the initial MPD exposure at ED1, there was a significant ($p < 0.05$) increase in activity by 177% (Fig 10 Ch). The 5 animals that individually exhibited behavioral tolerance in response to 10.0 mg/kg MPD exhibited a significant ($p < 0.05$) increase in response to acute drug on ED1 by 665%. Their acute response to MPD was significantly ($p < 0.05$) higher compared to those animals in the sensitized group (Fig 10 Ci). The baseline activity of these 5 tolerant rats at ED10 was similar to ED1 baseline. In response to chronic MPD on ED10 compared to ED1 post MPD, the animals exhibited a decrease in activity, but this reduction in activity was not significant due to a large SE (Fig 10 Ci).

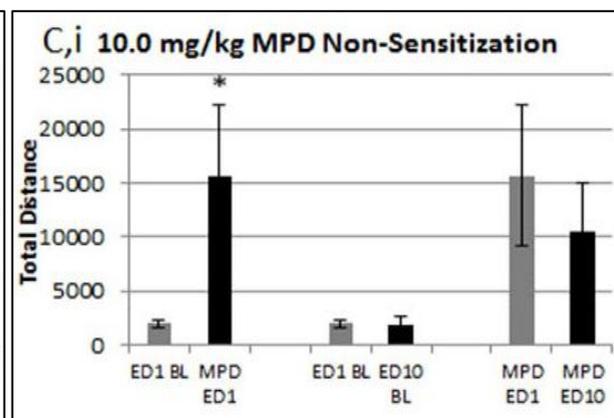
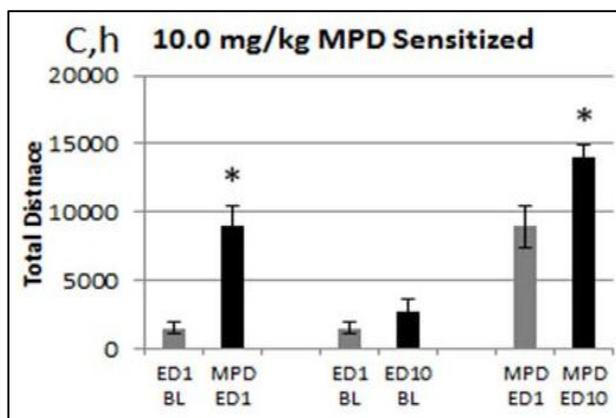
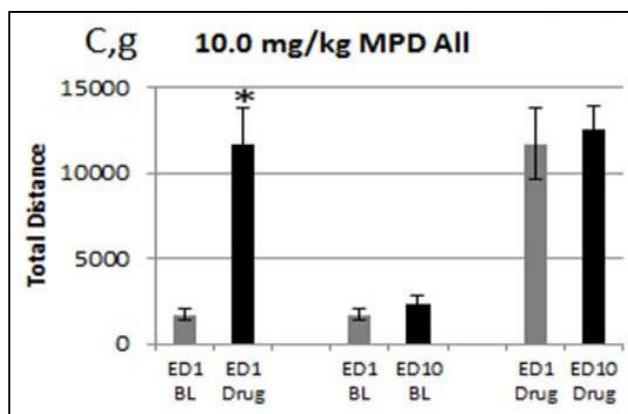


Figure 10 shows the horizontal activity (HA) traveled data following 10.0 mg/kg MPD dose. The top histogram (Cg) summarizes all the animals (N = 10). In addition, data was broken down into those animals that exhibited behavioral sensitization (N= 5) (Ch) and those animals that exhibited behavioral tolerance (N = 5) (Ci) to chronic MPD exposure respectively. For each group, the HA of ED1 after MPD exposure is compared to ED1 Baseline activity; the ED10 Baseline HA is compared to ED1 Baseline HA; and the HA activity on ED10 following MPD exposure is compared to the ED1 HA post MPD exposure. ED - experimental day; BL – baseline; MPD – methylphenidate; * - P < 0.05.

3.5 Comparison between Adolescent and Adult animal behavior

When comparing the adult to the adolescent behavioral following MPD administration using an ANOVA test, there were no significant differences between the two groups for all three doses for both ED1 and ED10. However, when they were separated out based on behavior, there were significant differences between the adult and adolescents. For the tolerant animals, there were significant differences between adults and adolescents for 2.5 and 10.0 mg/kg MPD on both ED1 and ED10 (F: 7.194, P: 0.05; F: 10.466, P: 0.03; F: 9.908, P: 0.01; F: 12.832, P: 0.007). For the sensitized animals, there were no significant differences most likely due to a low N, however, near significance was seen for the 0.6 and 10.0 mg/kg MPD doses on ED10.

3.6 Neuronal Activity Adolescent

Two hundred and eighty-one VTA units were recorded following acute and chronic MPD exposure. Fifty-nine percent (165/281) of the VTA units exposed to acute (0.6, 2.5, or 10.0 mg/kg) MPD responded significantly ($p < 0.05$) to the drug at ED1 by changing their firing rate compared to their baseline activity and the majority of the VTA responsive units, 73% (121/165), exhibited significant ($p < 0.05$) increases in their neuronal firing rates in response to the initial (acute) MPD exposure. Seventy percent (197/281) of the units exhibited significant changes in their baseline activity at ED10, after six daily MPD injections and 3 washout days (see Table 1), compared to ED1 baseline, and 62% (122/197) of them exhibiting an increase in their neuronal activity. At ED10, 68% (192/281) of the VTA units responded significantly ($p < 0.05$) to MPD rechallenge by changing their firing compared to the acute MPD exposure at ED1 and the majority of them, 62% (118/192), exhibited significant ($p < 0.05$) increases in their neuronal activity following MPD exposure at ED10 compared to the effect of MPD exposure at ED1.

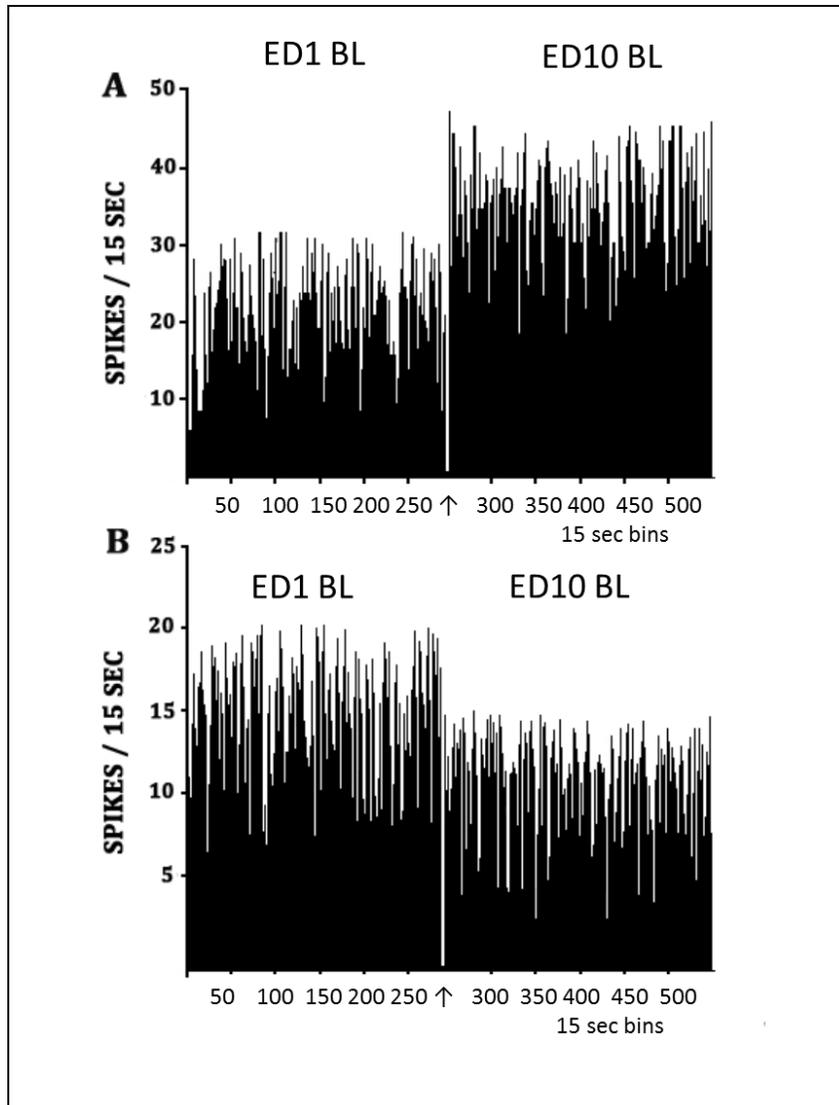


Figure 11 is a representative histogram showing the baseline (BL) neuronal firing rates of adolescent VTA neurons on ED1 and the baseline activity at ED10 after six daily MPD exposure and three washout days. In Part A, the baseline activity (BL) is shown for ED1 and then again on ED10. This animal's baseline activity was potentiated after the 6 days of MPD exposure and 3 washout days. In Part B, the baseline activity is shown for ED1 and again for ED10 expressing attenuation in their activity following 6 days of MPD exposure and 3 washout days.

3.6.1 Adolescent VTA Neuronal (0.6 mg/kg)

The neuronal activity following 0.6 mg/kg MPD was recorded from 81 VTA units in 28 animals. Only 38% (31/81) of the VTA units responded to acute 0.6 mg/kg MPD exposure and of those responding units, 55% (17/31) exhibited an increase in their firing rate. In addition, when comparing the baseline neuronal activity on ED10 to baseline activity at ED1, 51% (41/81) of the units exhibited a significant ($p < 0.05$) change in their firing rate, with 51% (21/51) exhibiting a significant ($p < 0.05$) decrease in their firing rate. Upon MPD rechallenge at ED10 compared to the activity post MPD on ED1, 47% (38/81) of VTA units responded significantly ($p < 0.05$) different by changing their firing rate, with 66% (25/38) of them exhibiting an increase in firing rate (Table 2A).

3.6.2 Adolescent VTA neuronal based on behavior (0.6 mg/kg)

Fifty four VTA units were recorded from 14 animals that exhibited behavioral tolerance. Following acute administration of MPD, 41% (22/54) of the VTA units responded significantly ($p < 0.05$) to the drug by changing their firing rate, with 55% (12/22) of these responding VTA units exhibiting a decrease in their firing rates. Comparing the baseline activity of ED10 after six daily MPD exposures and three washout days to that of ED1 shows that 59% (32/54) of the units changed their firing rate significantly ($p < 0.05$), with 56% (18/32) of those units exhibited an increase in their firing rates. Upon MPD rechallenge at ED10, 54% (29/54) of the VTA units responded with significant ($p < 0.05$) changes in their firing rate and from these responding units, 62% (18/29) of them exhibited significantly ($p < 0.05$) increased firing rates following MPD exposure on ED10 compared to that observed following MPD exposure at ED1 (Table 2C).

Twenty seven VTA units were recorded from 14 animals that exhibited behavioral sensitization to 0.6 mg/kg MPD. Only 33% (9/27) of the units responded to MPD administration on ED1 and on ED10 and of these responding VTA units, 78% responded with an increase in their neuronal activity (Table 2B). When the baseline activity on ED10 was compared to ED1 baseline activity, 33% (9/27) of the units exhibited significant ($p < 0.05$) change in their firing rate and of these VTA units, 66% exhibited an increase in their neuronal activity on ED10, after six daily MPD injections and 3

washout days, compared to ED1 baseline activity (Table 2B). Upon MPD rechallenge at ED10, 33% (9/27) of the VTA units responded with significant ($p < 0.05$) changes in their firing rate compared to the activity post MPD exposure on ED1 and of these responding units, 78% (7/9) of them exhibited further significantly ($p < 0.05$) increased firing rates (Table 2B).

0.6 mg/kg MPD

Subject Group		Acute (ED ₁)	Baseline (ED ₁₀ to ED ₁)	Rechallenge (ED ₁₀)
A) Total N= 81	↑	17 (21%)	20 (24.7%)	25 (30.9%)
	↓	14 (17.3%)	21 (25.9%)	13 (16%)
	↔	50 (61.7%)	40 (49.4%)	43 (53.1%)
B) Rats Exhibiting Behavioral Sensitization N= 27	↑	7 (25.9%)	6 (22.2%)	7 (25.9%)
	↓	2 (7.4%)	3 (11.1%)	2 (7.4%)
	↔	18 (66.7%)	18 (66.7%)	18 (66.7%)
C) Rats Exhibiting Non-Sensitized Behavior N= 54	↑	10 (18.5%)	14 (25.9%)	18 (33.3%)
	↓	12 (22.2%)	18 (33.3%)	11 (20.4%)
	↔	32 (59.3%)	22 (40.8%)	25 (46.3%)

Table 2 summarizes the VTA neuronal activity following 0.6 mg/kg MPD administration. In part A, all of the neuronal units are grouped together. The unit response is shown as either an increase in activity, decrease in activity or no change. Data is shown for the acute administration of MPD on ED1, the baseline activity on ED10 compared to ED1 baseline activity, and the rechallenge dose of MPD on ED10 after 6 daily injections and 3 washout days. In part B, the data is shown for the animals which exhibited behavioral sensitization, and in part C, the data is shown for the animals which exhibited behavioral tolerance.

3.6.3 Adolescent VTA Neuronal (2.5 mg/kg)

The neuronal activity following 2.5 mg/kg MPD was recorded for 99 VTA units from 31 animals. Fifty-five percent (54/99) of these units responded to the acute administration of the drug by significantly ($p < 0.05$) changing their firing rates, 59% (31/54) of these responding VTA units responded with a significant ($p < 0.05$) increase in their firing activity. When comparing the ED10 baseline activity to the ED1 baseline activity, 64% (63/99) of the units exhibited significant ($p < 0.05$) changes in their activity, with 68% (40/63) of them exhibiting a significant ($p < 0.05$) increase in their ED10 baseline activity compared to ED1 baseline activity. In response to MPD rechallenge on ED10, 64% (63/99) of the units responded significantly ($p < 0.05$) to the drug, the majority of these responding units 68% (43/63) responded to MPD rechallenge with further significant increases in their neuronal activity (Table 3A).

3.6.4 Adolescent VTA neuronal based on behavior (2.5 mg/kg)

Forty VTA units were recorded from 9 animals that exhibited behavioral tolerance. Following acute administration of MPD, only 40% (16/40) of the VTA units responded significantly to the drug with 56% (9/16) of these responding units exhibiting significant ($p < 0.05$) decreases in their firing rates. Comparing baseline activity on ED10 to that of ED1 baseline activity, 40% (16/40) of the units exhibited significant ($p < 0.05$) changes in their baseline firing rate after the six daily MPD exposures and three washout days, with half of them exhibiting decreases in their firing rates and the other half exhibiting an increase in their baseline firing rate (Table 3C). Upon MPD rechallenge at ED10, 45% (18/40) of the units responded with significant ($p < 0.05$) changes in their firing rates compared to the activity post MPD exposure on ED1 and of these responding VTA units, 61% (11/18) of them exhibited significantly ($p < 0.05$) increased firing rates following 2.5 mg/kg MPD at ED10 compared to MPD exposure at ED1 (Table 3C).

Fifty nine VTA units were recorded from 22 animals that exhibited behavioral sensitization to 2.5 mg/kg MPD. In response to acute exposure of 2.5 mg/kg MPD, 64% (38/59) of the units responded to drug administration by significantly ($p < 0.05$) changing their firing rate and of these 38

responsive VTA units, 63% (24/38) responded to MPD with significant ($p < 0.05$) increases in their neuronal activity. When baseline activity on ED10 was compared to ED1 baseline activity, 80% (47/59) of the units exhibited a significant ($p < 0.05$) change in their baseline activity after the six daily MPD exposures and three washout days and of these 47 VTA units, 68% (32/47) exhibited an increase in their ED10 baseline neuronal activity compared to ED1 baseline activity. When given a rechallenge dose of 2.5 mg/kg MPD on ED10, 76% (45/59) of the units responded significantly ($p < 0.05$) to the drug compared to the activity post MPD exposure on ED1 and of the 45 responding VTA units, 71% (32/45) exhibited significant ($p < 0.05$) increases in their firing rates to MPD rechallenge (Table 3B) (Figure 12).

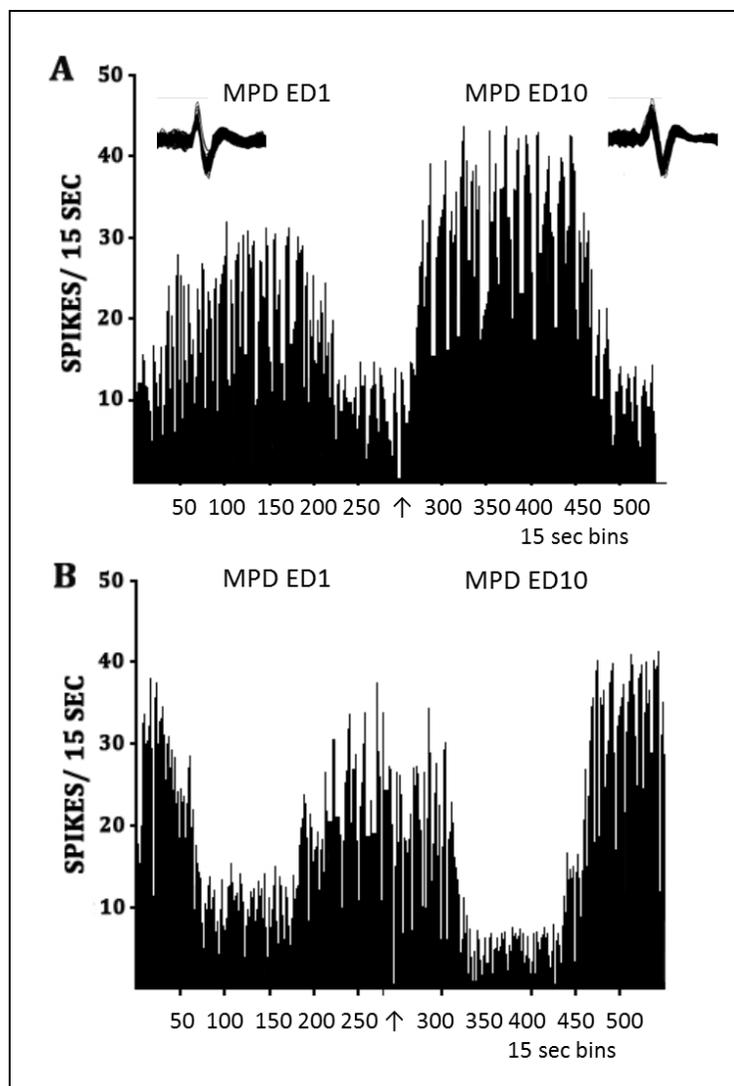


Figure 12 is a representative histogram showing the neuronal activity post MPD exposure on ED1 and ED10. In Part A, 2.5 mg/kg MPD rechallenge exposure at ED10 compared to ED1 elicited further potentiation as a result of 6 days of daily MPD exposure, and 3 washout days. Part B is VTA representative of units showing the neuronal activity post MPD on ED1 and again on ED10 where the neuronal activity is on ED10 attenuated following 6 days of MPD (2.5 mg/kg) exposure, followed by 3 washout days and MPD rechallenge compared to ED1 post MPD exposure. At the top is 20 superimposed analog neuronal spike activity, showing that the spike recorded post MPD on ED1 is similar to the spike post MPD rechallenge on ED10.

2.5 mg/kg MPD

Subject Group		Acute (ED ₁)	Baseline (ED ₁₀ to ED ₁)	Rechallenge (ED ₁₀)
A) Total N= 99	↑	31 (31.3%)	40 (40.4%)	43 (43.4%)
	↓	23 (23.2%)	23 (23.2%)	20 (20.2%)
	↔	45 (45.5%)	36 (36.4%)	36 (36.4%)
B) Rats Exhibiting Behavioral Sensitization N= 59	↑	24 (40.7%)	32 (54.2%)	32 (54.2%)
	↓	14 (23.7%)	15 (25.4%)	13 (22.1%)
	↔	21 (35.6%)	12 (20.4%)	14 (23.7%)
C) Rats Exhibiting Non- Sensitized Behavior N= 40	↑	7 (17.5%)	8 (20%)	11 (27.5%)
	↓	9 (22.5%)	8 (20%)	7 (17.5%)
	↔	24 (60%)	24 (60%)	22 (55%)

Table 3 summarizes the VTA neuronal activity following 2.5 mg/kg MPD administration. In part A, all of the neuronal units are grouped together. The unit response is shown as either an increase in activity, decrease in activity or no change. Data is shown for the acute administration of MPD on ED1, the baseline activity on ED10 compared to ED1 baseline activity, and the rechallenge dose of MPD on ED10 after 6 daily injections and 3 washout days. In part B, the data is shown for the animals which exhibited behavioral sensitization, and in part C, the data is shown for the animals which exhibited behavioral tolerance.

3.6.5 Adolescent VTA Neuronal (10.0 mg/kg)

The neuronal activity following 10.0 mg/kg MPD was recorded from 101 VTA units in 43 animals. The majority, 72% (73/101) of these VTA units following 10.0 mg/kg MPD exposure responded significantly ($p < 0.05$) by changing their firing rate, 91% (73/80) of the responding VTA units, exhibited a significant ($p < 0.05$) increase in their neuronal activity to MPD exposure (Table 4A). When comparing the baseline neuronal activity of ED10 to the baseline activity of ED1, 92% (93/101) of these units exhibited significant ($p < 0.05$) changes in their ED10 neuronal firing rate, and 67% (62/93) of these units exhibited a significant ($p < 0.05$) increase in their neuronal activity. When MPD rechallenge was given on ED10, 90% (91/101) of the VTA units responded by significantly ($p < 0.05$) changing their firing rate compared to the activity from acute MPD exposure at ED1, and of those responding units, 55% (50/91) of them exhibited a further significant ($p < 0.05$) increase in their activity compared to the initial effect of 10.0 mg/kg MPD at ED1 (Table 4A).

3.6.6 Adolescent VTA neuronal based on behavior (10.0 mg/kg)

Twenty VTA units were recorded from 6 animals that exhibited behavioral tolerance. Following acute administration of MPD, 65% (13/20) of these VTA units responded significantly ($p < 0.05$) to the drug by changing their firing rate with 62% (8/13) of these responding VTA units exhibiting significant ($p < 0.05$) increases in their firing rates following MPD exposure. Comparing baseline activity on ED10 to the baseline activity at ED1 reveals that 90% (18/20) of the units changed their firing rate significantly ($p < 0.05$) after the six daily MPD exposures and three washout days, with 56% (10/18) of them exhibiting a significant ($p < 0.05$) decrease in their neuronal firing rates. Upon MPD rechallenge at ED10, 85% (17/20) of the units responded with significant ($p < 0.05$) changes in their firing rate and of these responding VTA units, 59% (10/17) of them exhibited further significant ($p < 0.05$) decreases in their firing rates compared to the effect elicited by the initial MPD exposure (Table 4C).

Eighty one VTA units were recorded from 37 animals that exhibited behavioral sensitization. In response to acute exposure of MPD, the majority of these units, 83% (67/81) responded to the drug

administration by significantly ($p < 0.05$) changing their firing rate and of these responding units, 97% (65/67) responded significantly ($p < 0.05$) with an increase in their neuronal activity. When the baseline activity on ED10 was compared to the ED1 baseline activity, 93% (75/81) of the VTA units exhibited significant ($p < 0.05$) changes in their firing rate at ED10 and of these 75 VTA units, 72% (54/75) exhibited significant ($p < 0.05$) increases in their neuronal activity at ED10 compared to ED1 baseline neuronal activity. When given a rechallenge dose of 10.0 mg/kg MPD on ED10, 91% (74/81) of the VTA units responded significantly ($p < 0.05$) to the drug by changing their firing rate and of these responding 74 VTA units, 58% (43/74) exhibited further significant ($p < 0.05$) increases in their firing rate at ED10 when compared to the effect of MPD on ED1 (Table 4B) (Fig 13).

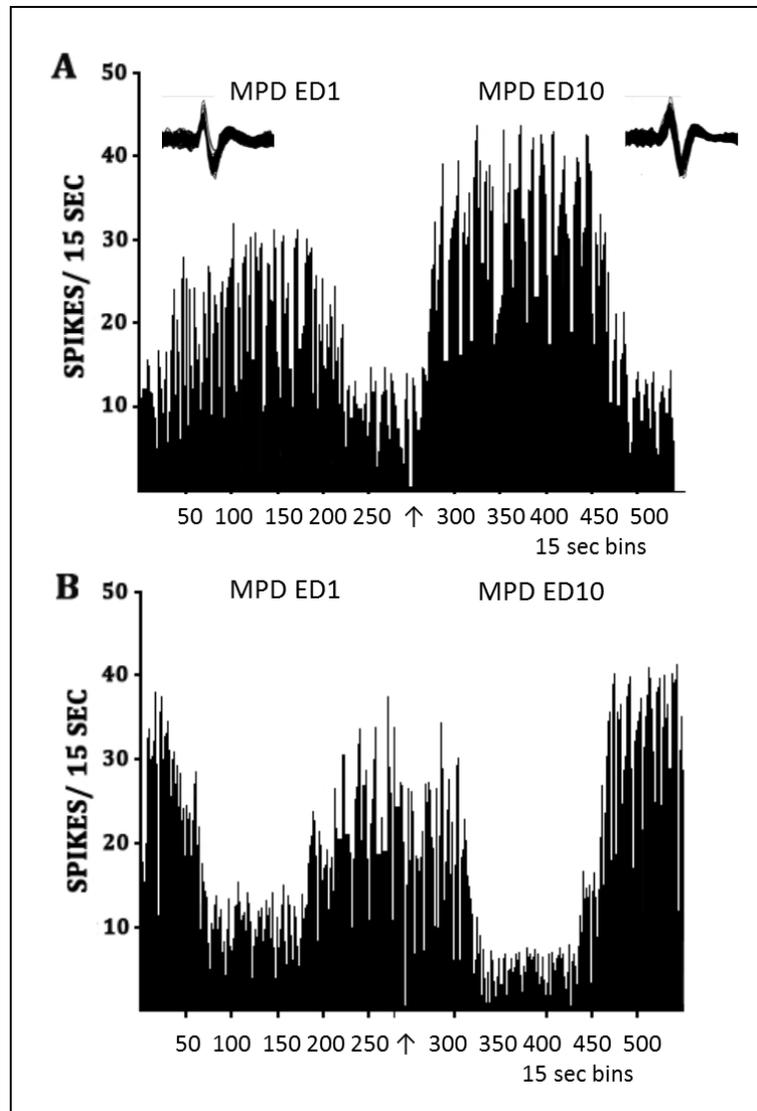


Figure 13 is a representative histogram showing VTA neuronal activity post saline injection i.e. the baseline (BL) activity and the activity post 10.0 mg/kg MPD administration. The drug was injected at 60 minutes post saline. In Part A, VTA neuronal activity following acute MPD exhibits a potentiation of neuronal activity. In contrast, Part B shows VTA neuronal activity units exhibiting attenuation following MPD administration.

10.0 mg/kg

Subject Group		Acute (ED ₁)	Baseline (ED ₁₀ to ED ₁)	Rechallenge (ED ₁₀)
A) Total N= 101	↑	73 (73.3%)	62 (61.4%)	50 (49.5%)
	↓	7 (6.9%)	31 (30.7%)	41 (40.6%)
	↔	21 (20.8%)	8 (7.9%)	10 (9.9%)
B) Rats Exhibiting Behavioral Sensitization N=81	↑	65 (80.2%)	54 (66.7%)	43 (53.1%)
	↓	2 (2.5%)	21 (25.9%)	31 (38.3%)
	↔	14 (17.3%)	6 (7.4%)	7 (8.6%)
C) Rats Exhibiting Non-Sensitized Behavior N= 20	↑	8 (40%)	8 (40%)	7 (35%)
	↓	5 (25%)	10 (50%)	10 (50%)
	↔	7 (35%)	2 (10%)	3 (15%)

Table 4 summarizes the VTA neuronal activity following 10.0 mg/kg MPD administration. In part A, all of the neuronal units are grouped together. The unit response is shown as either an increase in activity, decrease in activity or no change. Data is shown for the acute administration of MPD on ED1, the baseline activity on ED10 compared to ED1 baseline activity, and the rechallenge dose of MPD on ED10 after 6 daily injections and 3 washout days. In part B, the data is shown for the animals which exhibited behavioral sensitization, and in part C, the data is shown for the animals which exhibited behavioral tolerance.

3.7 Neuronal Activity Adult

A total of 321 units were histologically confirmed to be recorded from the VTA and exhibited similar amplitude and wave form at ED1 and ED10. Thirty-six VTA units were recorded following acute and repetitive saline injection. Two units showed decreased activity at ED1; the baseline activity of one VTA unit at ED10 compared to ED1 baseline exhibited a decrease in activity, and one VTA unit exhibited an increase in activity at ED10 following saline injection compared to ED1 baseline activity. This observation revealed that the saline injections and animal handling did not alter the neuronal activity of VTA neurons.

Two hundred and eighty-five VTA units were recorded following acute and chronic MPD exposure. Seventy-six percent (217/285) of the VTA units exposed to acute (0.6, 2.5 or 10.0 mg/kg) MPD responded significantly ($p < 0.05$) to MPD at ED1 and the majority, (61%; 133/217) of these responding units exhibited significant ($p < 0.05$) increases in their neuronal firing rates. Ninety-six percent (274/285) of the total units expressed significant changes in their baseline activity at ED10 compared to ED1 with 65% (178/274) exhibiting a decrease in their baseline activity. At ED10 95% (272/285) of the VTA units responded significantly ($p < 0.05$) to MPD rechallenge compared to activity post MPD exposure on ED1 where the majority, 60% (163/272), exhibited a significant ($p < 0.05$) decrease in their neuronal activity.

3.7.1 Adult VTA Neuronal (0.6 mg/kg)

The neuronal activity of 132 VTA units were recorded after acute and chronic administration of 0.6 mg/kg MPD; 71% (93/132) of the VTA units responded significantly ($p < 0.05$) to the initial exposure of the drug (Table 5A Acute) by changing their firing rates compared to their baseline firing rate. Most of the units, 94% (124/132), exhibited significant ($p < 0.05$) changes in their ED10 baseline activity after six daily MPD injections and three washout days compared to their ED1 baseline (Table 5A Saline). At ED10, upon MPD rechallenge compared to the initial MPD exposure

at ED1, 91% (120/132) of the VTA units exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 5A Rechallenge).

Of the 93 responsive VTA units to the initial (acute) MPD exposure on ED1 (Table 6A), the majority, 57% (53/93), exhibited a significant ($p < 0.05$) decrease in firing rate in response to acute MPD exposure (Table 6A Acute). Of the VTA units that exhibited significant change at ED10 baseline compared to ED1 baseline activity, the majority 60% (75/124) showed a significant ($p < 0.05$) decrease in baseline activity (Table 6A Saline). Following rechallenge of 0.6 mg/kg MPD at ED10 compared to the initial MPD injection at ED1, the majority of the responding units, 52% (62/120), responded to the drug by attenuating their neuronal activity (Table 3A Rechallenge). The 27 VTA units that did not respond to MPD exposure on ED1, exhibited a significant ($p < 0.05$) change in their ED10 baseline as well as in response to MPD rechallenge at ED10 after the six daily injections and three washout days (Table 6A All).

3.7.2 Adult VTA neuronal based on behavior (0.6 mg/kg)

The neuronal activity of 110 VTA units was recorded from animals that exhibited behavioral tolerance following acute and chronic 0.6 mg/kg MPD administration. The majority, 67% (74/110) of them responded to acute administration of MPD (Table 5A Tolerance Acute), while 94% (103/110) exhibited significant ($p < 0.05$) changes in their ED10 baseline compared to their ED1 baseline (Table 5A Tolerance Saline). At ED10 upon rechallenge with 0.6 mg/kg MPD compared to post MPD given at ED1, the majority 89% (98/110) of these VTA units exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 5A Tolerance Rechallenge).

Of the responding 74 VTA units to acute MPD on ED1, the majority, 54% (40/74) exhibited a significant ($p < 0.05$) increase in neuronal firing rate in response to MPD (Table 6A Tolerance Acute; Fig 14B). Of the 103 VTA units that exhibited significant change at ED10 baseline compared to ED1 baseline activity, the majority, 59% (61/103) showed a significant ($p < 0.05$) decrease in their neuronal activity (Table 6A Tolerance Saline). Upon rechallenge of 0.6 mg/kg MPD on ED10

compared to the initial effect of MPD at ED1, 51% (50/98) of the responsive VTA units exhibited a significant ($p < 0.05$) increase in their neuronal activity (Table 6A Rechallenge). The 24 units that did not respond to 0.6 mg/kg MPD on ED1 (Table 5A Tolerance Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity compared to ED1 baseline activity as well as to MPD rechallenge at ED10 after six daily MPD injections and three washout days compared to ED1 post MPD administration.

The neuronal activity of 22 VTA units was recorded from animals that exhibited behavioral sensitization following acute and chronic 0.6 mg/kg MPD administration. The majority of them, 86% (19/22), responded significantly ($p < 0.05$) to acute administration of MPD and all 19 responsive VTA units exhibited a decrease in their neuronal activity following 0.6 mg/kg MPD exposure (Table 5A Sensitization Acute). Most of these sensitized VTA neuronal units, 95% (21/22), exhibited significant ($p < 0.05$) changes in their ED10 baseline compared to ED1 baseline, and the majority of them, 67% (14/21) exhibited attenuation in their ED10 baseline compared to ED1 baseline (Table 5A Sensitization Saline). At ED10 upon rechallenge with MPD compared to post MPD given at ED1, all of the VTA units, (22/22) exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 5A Sensitization Rechallenge) and 64% (14/22) of these responding VTA units exhibited a significant ($p < 0.05$) decrease in their activity following 0.6 mg/kg MPD exposure on ED10 compared to 0.6 mg/kg MPD exposure on ED1.

The 3 VTA units that did not respond to 0.6 mg/kg MPD dose on ED1 (Table 5A Sensitization Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity as well as to MPD rechallenge at ED10 after six daily MPD injections and three washout days.

A	0.6 mg/kg MPD All			0.6 mg/kg MPD Exhibiting Behavioral Sensitization			0.6 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD D1	Saline (BL10 to BL1)	Rechallenge MPD
Increase ↑	40	49	58	0	7	8	40	42	50
Decrease ↓	53	75	62	19	14	14	34	61	48
No Change ≠	39	8	12	3	1	0	36	7	12
N =	132	132	132	22	22	22	110	110	110

Table 5 summarizes the neuronal recording results 0.6 mg/kg. The left, middle, and right columns summarize the data from all the animals, from those animals expressing behavioral sensitization, and from those animals expressing behavioral tolerance respectively. The arrows pointing upwards summarizes the VTA units exhibiting significant increases in neuronal unit activity whereas a downward arrow implies a decrease in neuronal unit activity and (≠) shows the number of units that did not respond. The percentages shown next to the numbers summarizes the percentage of units that responded i.e. total responsiveness. BL- baseline; BL1 – baseline of ED1; BL10 – baseline of ED10; Rechallenge – MPD exposure at ED10.

A	0.6 mg/kg MPD All			0.6 mg/kg MPD Exhibiting Behavioral Sensitization			0.6 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10
Increase ↑	40 (43%)	49 (40%)	58 (48%)	0 (0%)	7 (33%)	8 (36%)	40 (54%)	42 (41%)	50 (51%)
Decrease ↓	53 (57%)	75 (60%)	62 (52%)	19 (100%)	14 (67%)	14 (64%)	34 (46%)	61 (59%)	48 (49%)
N =	93	124	120	19	21	22	74	103	98

Table 6 summarizes the electrophysiological results of only the units that responded significantly to the treatment from Table 5. The units that did not respond to MPD are not represented. Each column for each dose summarizes the acute and chronic effects of the drug. Acute MPD on ED1 after drug exposure is compared to ED1 Baseline activity; the ED10 Baseline is compared to ED1 Baseline activity and the ED10 post MPD is compared to the ED1 post MPD exposure. The same is shown for each dose broken down into all the recordings (All) and to those animals that exhibited behavioral sensitization and to those animals that exhibited behavioral tolerance. The arrow pointing upwards implies a significant increase in neuronal unit activity whereas a downward arrow implies a significant decrease in neuronal unit activity following MPD exposure. The percentages shown next to each group is the percentage of units that responded with an increase or decrease in activity (total responsiveness arbitrarily set as 100%).

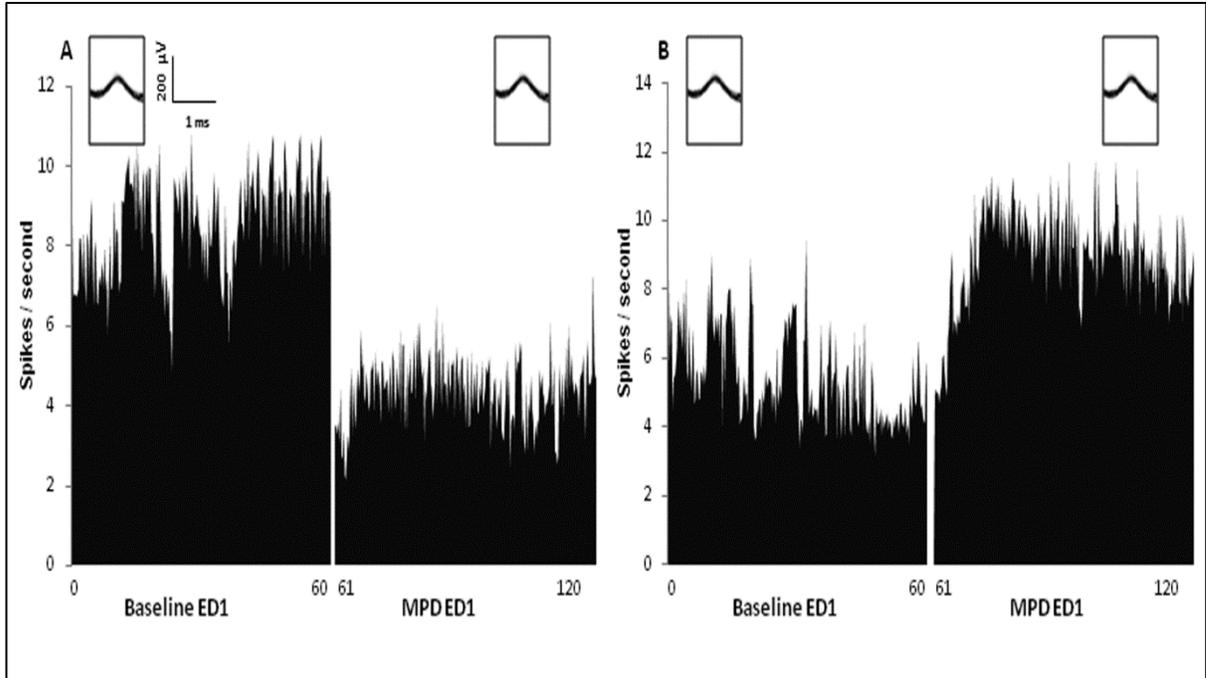


Figure 14 A representative of sequential frequency firing rates of two VTA units following acute 0.6 mg/kg MPD. Both histograms show the neuronal unit activity post saline for 60 minutes, followed by the neuronal unit activity after MPD on ED1 for the following 60 minutes. The left histogram (14A) shows the VTA unit exhibited a decrease in firing rate activity to acute MPD administration compared to the baseline activity, while the right histogram (14B) shows the VTA unit exhibited an increase in firing rate activity to acute MPD administration compared to the baseline activity.

3.7.3 Adult VTA Neuronal (2.5 mg/kg)

The neuronal activity of 62 VTA units were recorded after acute and chronic administration of 2.5 mg/kg MPD; 84% (52/62) of the VTA units responded significantly ($p < 0.05$) to the initial exposure of the drug (Table 7B Acute) and of these, the majority 79% (41/52) exhibited a significant ($p < 0.05$) increase in firing rate in response to the initial MPD exposure (Table 7B Acute). The baseline activity of ED10 was altered significantly ($p < 0.05$) in 95% (59/62) of the VTA units compared to ED1 baseline (Table 7B Saline; Fig 15) activity as a result of six daily MPD exposures and three washout days. Of the above VTA units, the majority 53% (31/59), showed a significant ($p < 0.05$) decrease in their neuronal activity (Table 7B Saline). At ED10, upon MPD rechallenge compared to the initial MPD exposure at ED1, 98% (61/62) of the VTA units exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 7B Rechallenge), of which the majority 52% (32/61) of the VTA units responded to the drug by significantly ($p < 0.05$) attenuating their neuronal activity (Table 7B Rechallenge). The 9 VTA units that did not respond to MPD exposure on ED1, exhibited a significant ($p < 0.05$) change in their ED10 baseline as well as in response to MPD rechallenge at ED10 after the six daily MPD injections and three washout days (Table 7B All).

3.7.4 Adult VTA neuronal based on behavior (2.5 mg/kg)

The neuronal activity of 38 VTA units was recorded from animals that exhibited behavioral tolerance following acute and chronic 2.5 mg/kg MPD administration. The majority, 90% (34/38), responded to acute administration of MPD (Table 7B Tolerance Acute), of which 91% (31/34) exhibited a significant ($p < 0.05$) increase in neuronal firing rate in response to MPD exposure (Table 8B Tolerance Acute). The ED10 baseline activity compared to ED1 baseline activity was significantly ($p < 0.05$) changed for 95% (36/38) of the VTA units (Table 7B Tolerance Saline) and of these units, 64% (23/36) showed a significant ($p < 0.05$) increase in their neuronal baseline activity (Table 8B Tolerance Saline). At ED10 upon rechallenge with MPD compared to post MPD given at ED1, all of the VTA units (38/38) exhibited significant ($p < 0.05$) changes in their neuronal activity

(Table 7B Tolerance Rechallenge) of which 63% (24/38) exhibited a significant ($p < 0.05$) increase in their neuronal activity. The 4 units that did not respond to the 2.5 mg/kg MPD dose on ED1 (Table 7B Tolerance Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity as well as MPD rechallenge at ED10 after six daily MPD injections and three washout days.

The neuronal activity of 24 VTA units was recorded from animals that exhibited behavioral sensitization following acute and chronic 2.5 mg/kg MPD administration. The majority, 75% (18/24), responded to acute administration of MPD (Table 7B Sensitization Acute) and of these 18 responding VTA units, 56% (10/18) exhibited a significant ($p < 0.05$) increase in neuronal firing rate in response to MPD (Table 8B Sensitization Acute). The ED10 baseline activity compared to ED1 baseline was changed significantly ($p < 0.05$) by 96% (23/24) of the VTA units (Table 7B Sensitization Saline) of which the majority, 78% (18/23), showed a significant ($p < 0.05$) decrease in their neuronal activity (Table 8B 2.5 mg/kg Sensitization Saline). At ED10 upon rechallenge with MPD compared to post MPD given at ED1, the majority of the VTA units, 96% (23/24) exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 7B Sensitization Rechallenge), of which the majority of these responsive units, 78% (18/23) exhibited a significant ($p < 0.05$) decrease in their neuronal activity. The 5 units that did not respond to the 2.5 mg/kg MPD dose on ED1 (Table 8B Sensitization Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity as well as MPD rechallenge at ED10 after six daily MPD injections and three washout days.

B	2.5 mg/kg MPD All			2.5 mg/kg MPD Exhibiting Behavioral Sensitization			2.5 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD
Increase ↑	41	28	29	10	5	5	31	23	24
Decrease ↓	11	31	32	8	18	18	3	13	14
No Change ≠	10	3	1	6	1	1	4	2	0
N =	62	62	62	24	24	24	38	38	38

Table 7 summarizes the neuronal recording results 2.5 mg/kg. The left, middle, and right columns summarize the data from all the animals, from those animals expressing behavioral sensitization, and from those animals expressing behavioral tolerance respectively. The arrows pointing upwards summarizes the VTA units exhibiting significant increases in neuronal unit activity whereas a downward arrow implies a decrease in neuronal unit activity and (≠) shows the number of units that did not respond. The percentages shown next to the numbers summarizes the percentage of units that responded i.e. total responsiveness. BL- baseline; BL1 – baseline of ED1; BL10 – baseline of ED10; Rechallenge – MPD exposure at ED10.

B	2.5 mg/kg MPD All			2.5 mg/kg MPD Exhibiting Behavioral Sensitization			2.5 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10
Increase ↑	41 (79%)	28 (47%)	29 (48%)	10 (56%)	5 (22%)	5 (22%)	31 (91%)	23 (64%)	24 (63%)
Decrease ↓	11 (21%)	31 (53%)	32 (52%)	8 (44%)	18 (78%)	18 (78%)	3 (9%)	13 (36%)	14 (37%)
N =	52	59	61	18	23	23	34	36	38

Table 8 summarizes the electrophysiological results of only the units that responded significantly to the treatment from Table 7. The units that did not respond to MPD are not represented. Each column for each dose summarizes the acute and chronic effects of the drug. Acute MPD on ED1 after drug exposure is compared to ED1 Baseline activity; the ED10 Baseline is compared to ED1 Baseline activity and the ED10 post MPD is compared to the ED1 post MPD exposure. The same is shown for each dose broken down into all the recordings (All) and to those animals that exhibited behavioral sensitization and to those animals that exhibited behavioral tolerance. The arrow pointing upwards implies a significant increase in neuronal unit activity whereas a downward arrow implies a significant decrease in neuronal unit activity following MPD exposure. The percentages shown next to each group is the percentage of units that responded with an increase or decrease in activity (total responsiveness arbitrarily set as 100%).

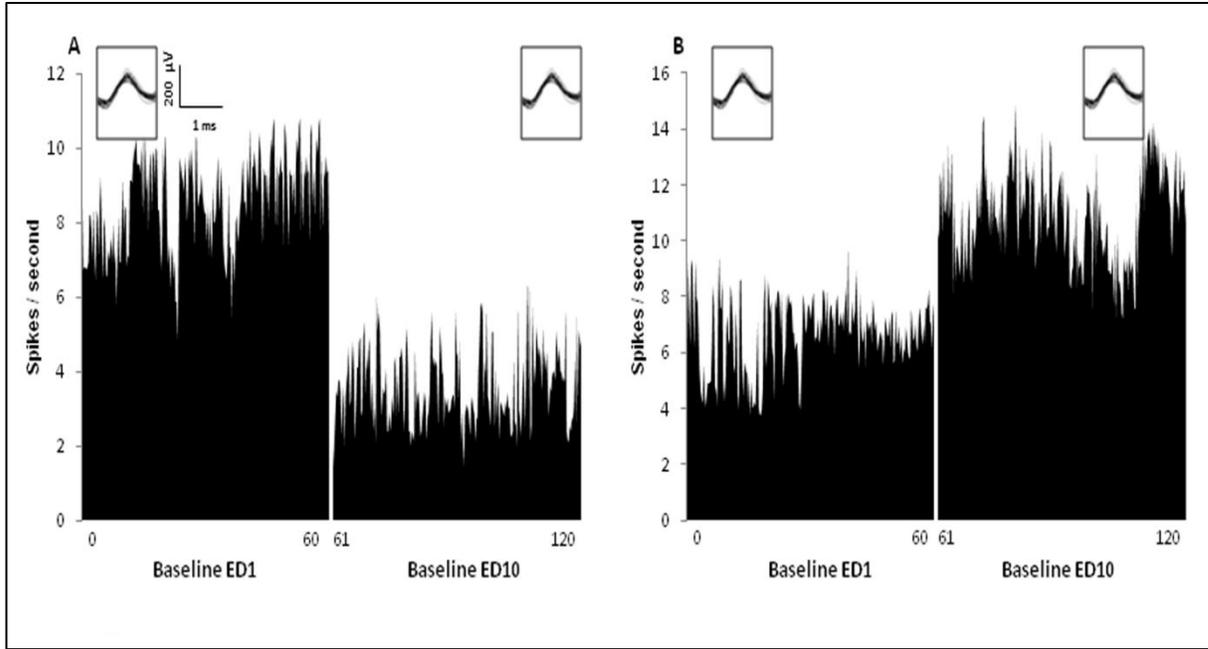


Figure 15 A representative of sequential frequency firing rates of the baseline of two VTA units at experimental day 1 (ED1) compared to the baseline at ED10 following six daily 2.5 mg/kg MPD injections and three washout days. The left histogram (15A) shows the VTA unit that exhibited a decrease in its baseline firing rate activity at ED10 following six daily 2.5 mg/kg MPD injections and three washout days compared to the baseline activity on ED1, while the right histogram (15B) shows the VTA unit exhibited an increase in its baseline firing rate activity at ED10 following six daily 2.5 mg/kg MPD injections and 3 washout days compared to the baseline activity on ED1.

3.7.5 Adult VTA Neuronal (10.0 mg/kg)

The neuronal activity of 91 VTA units were recorded after acute and chronic administration of 10.0 mg/kg MPD; 79% (72/91) of the VTA units responded significantly ($p < 0.05$) to the initial exposure of the drug (Table 9C Acute) and of these, the majority, 72% (52/72), exhibited a significant ($p < 0.05$) increase in firing rate in response to MPD exposure (Table 10C Acute). All of the units (91/91) exhibited significant ($p < 0.05$) changes in their ED10 baseline compared to their ED1 baseline of which, the majority 79% (72/91) showed a significant ($p < 0.05$) decrease in their baseline activity (Table 10C Saline). At ED10, upon MPD rechallenge compared to the initial MPD exposure at ED1, 100% (91/91) of the VTA units exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 9C Rechallenge), of which the majority 76% (69/91) of the VTA units responded to the drug by attenuating their neuronal activity (Table 10C Rechallenge). The 19 VTA units that did not respond to MPD exposure on ED1, exhibited a significant ($p < 0.05$) change in their ED10 baseline as well as in response to MPD rechallenge at ED10 after the six daily MPD injections and three washout days (Table 9C All).

3.7.6 Adult VTA neuronal based on behavior (10.0 mg/kg)

The neuronal activity of 51 VTA units was recorded from animals that exhibited behavioral tolerance following acute and chronic 10.0 mg/kg MPD administration. The majority, 69% (35/51) of them responded to acute administration of MPD (Table 9C Tolerance Acute), of which 91% (32/35) exhibited a significant ($p < 0.05$) increase in their neuronal firing rate in response to MPD exposure (Table 10C Tolerance Acute). All of the units (51/51) exhibited significant ($p < 0.05$) changes in their ED10 baseline activity compared to ED1 baseline (Table 9C Tolerance Saline) of which the majority, 88% (45/51) showed a significant ($p < 0.05$) decrease in their neuronal activity (Table 10C Tolerance Saline). At ED10 upon rechallenge with MPD compared to post MPD given at ED1, all of the VTA units (51/51) exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 9C Tolerance Rechallenge) and 84% (43/51) of these exhibited a significant ($p < 0.05$) decrease in their neuronal activity. The 16 units that did not respond to the 10.0 mg/kg MPD dose on

ED1 (Table 9C Tolerance Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity compared to their ED1 baseline neuronal firing rate as well as following MPD rechallenge at ED10 after six daily MPD injections and three washout days, compared to the initial MPD exposure. The neuronal activity of 40 VTA units was recorded from animals that exhibited behavioral sensitization following acute and chronic 10.0 mg/kg MPD administration. The majority, 93% (37/40) responded significantly ($p < 0.05$) to acute administration of MPD (Table 9C Sensitization Acute) and of these, 54% (20/37) exhibited a significant ($p < 0.05$) increase in neuronal firing rate in response to MPD (Table 10C Sensitization Acute). All of the units (40/40) exhibited significant ($p < 0.05$) changes in their ED10 baseline firing rate compared to ED1 baseline firing rate (Table 9C Sensitization Saline), of which the majority 67% (27/40) showed a significant ($p < 0.05$) decrease in neuronal activity (Table 10C Sensitization Saline). At ED10 upon rechallenge with MPD compared to post MPD given at ED1, all of the VTA units (40/40) exhibited significant ($p < 0.05$) changes in their neuronal activity (Table 9C Sensitization Rechallenge) of which 65% (26/40) exhibited a significant ($p < 0.05$) decrease in their neuronal activity (Table 10C Sensitization Rechallenge). The 3 units that did not respond to the 10.0 mg/kg MPD dose on ED1 (Table 9C Sensitization Acute), exhibited a significant ($p < 0.05$) change in their ED10 baseline activity as well as following MPD rechallenge at ED10 after six daily MPD injections and three washout days.

C	10.0 mg/kg MPD All			10.0 mg/kg MPD Exhibiting Behavioral Sensitization			10.0 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD	Acute MPD	Saline (BL10 to BL1)	Rechallenge MPD
Increase ↑	52	19	22	20	13	14	32	6	8
Decrease ↓	20	72	69	17	27	26	3	45	43
No Change ≠	19	0	0	3	0	0	16	0	0
N =	91	91	91	40	40	40	51	51	51

Table 9 summarizes the neuronal recording results 0.6 mg/kg. The left, middle, and right columns summarize the data from all the animals, from those animals expressing behavioral sensitization, and from those animals expressing behavioral tolerance respectively. The arrows pointing upwards summarizes the VTA units exhibiting significant increases in neuronal unit activity whereas a downward arrow implies a decrease in neuronal unit activity and (≠) shows the number of units that did not respond. The percentages shown next to the numbers summarizes the percentage of units that responded i.e. total responsiveness. BL- baseline; BL1 – baseline of ED1; BL10 – baseline of ED10; Rechallenge – MPD exposure at ED10.

C	10.0 mg/kg MPD All			10.0 mg/kg MPD Exhibiting Behavioral Sensitization			10.0 mg/kg MPD Exhibiting Behavioral Tolerance		
	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10	Acute MPD ED1	Saline (BL ED10 to BL ED1)	Rechallenge MPD ED10
Increase ↑	52 (72%)	19 (21%)	22 (24%)	20 (54%)	13 (33%)	14 (35%)	32 (91%)	6 (12%)	8 (16%)
Decrease ↓	20 (28%)	72 (79%)	69 (76%)	17 (46%)	27 (67%)	26 (65%)	3 (9%)	45 (88%)	43 (84%)
N =	72	91	91	37	40	40	35	51	51

Table 10 summarizes the electrophysiological results of only the units that responded significantly to the treatment from Table 9. The units that did not respond to MPD are not represented. Each column for each dose summarizes the acute and chronic effects of the drug. Acute MPD on ED1 after drug exposure is compared to ED1 Baseline activity; the ED10 Baseline is compared to ED1 Baseline activity and the ED10 post MPD is compared to the ED1 post MPD exposure. The same is shown for each dose broken down into all the recordings (All) and to those animals that exhibited behavioral sensitization and to those animals that exhibited behavioral tolerance. The arrow pointing upwards implies a significant increase in neuronal unit activity whereas a downward arrow implies a significant decrease in neuronal unit activity following MPD exposure. The percentages shown next to each group is the percentage of units that responded with an increase or decrease in activity (total responsiveness arbitrarily set as 100%).

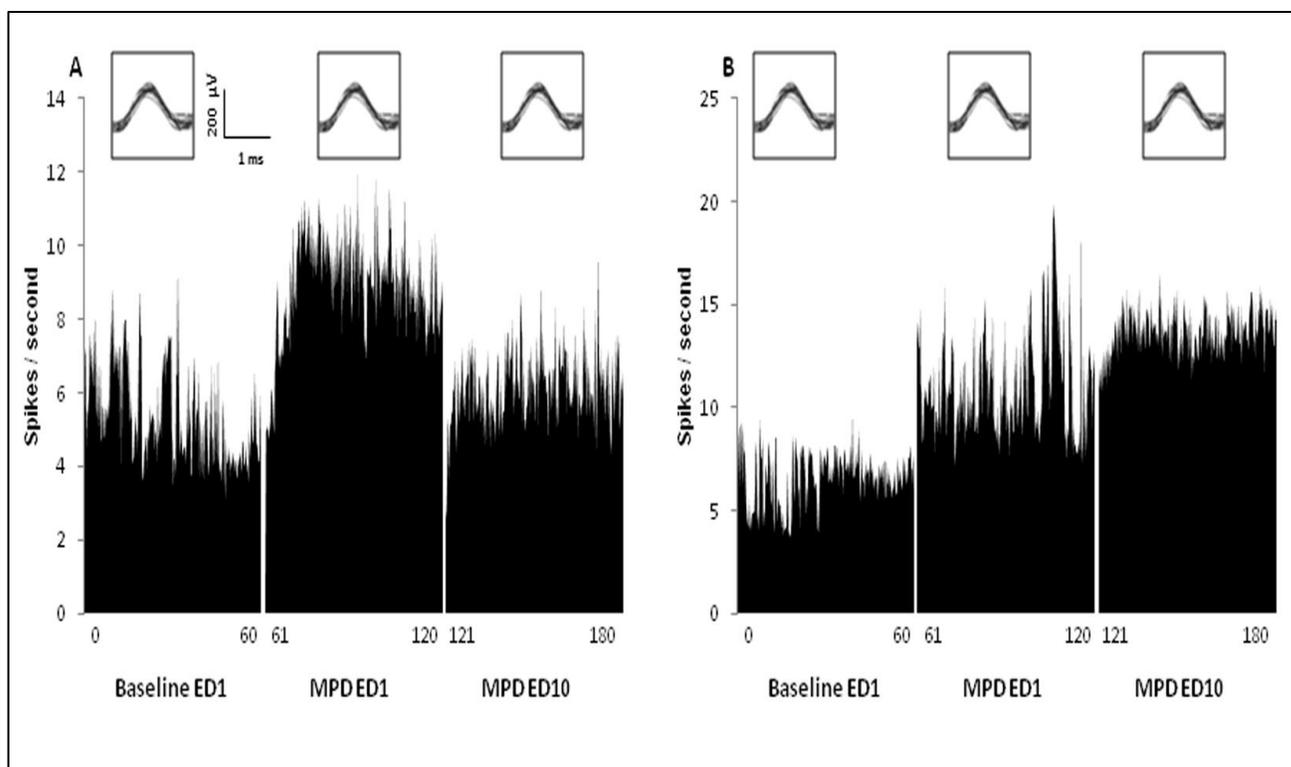


Figure 16 A representative of sequential frequency firing rates of two VTA units following acute and chronic MPD administration. Both histograms show the neuronal unit activity after 60 minutes of saline i.e. baseline activity, followed by an additional 60 minutes post 10.0 mg/kg MPD on ED1, and finally by another 60 minutes post 10.0 mg/kg MPD administration on ED10. In 16A, the acute effect of MPD on ED1 elicits an increase in VTA neuronal unit firing rate compared to the baseline activity. The effect of MPD rechallenge on ED10, after six daily MPD injections and three washout day of no drug, results in a lower response to the drug when compared to the effect of the drug on ED1. This neuronal unit activity was classified as neurophysiological tolerance. In 16B, the acute effect of 10.0 mg/kg MPD on ED1 results in an increase in neuronal unit activity compared to the ED1 baseline activity. The effect of MPD administration on ED10 after six daily MPD injections and three washout days results in a further increase in neuronal unit activity compared to the initial MPD effects on ED1. This type of response was classified as neurophysiological sensitization.

3.8 Statistical Comparisons

3.8.1 Adolescent behavioral tolerance compared to sensitized (0.06 mg/kg)

The natural log odds ratio statistical test shows that for 0.6 mg/kg the neuronal recordings obtained from animals expressing behavioral sensitization were more likely (1.09) to show an increase in neuronal activity during initial (acute) MPD exposure than those animals expressing behavioral tolerance. Additionally, for the baseline neuronal activity of ED10 compared to baseline neuronal activity recorded at ED1 and the neuronal activity recorded on ED10 following rechallenge with MPD compared to the neuronal activity on ED1 following MPD, the odds ratio revealed that the behaviorally sensitized animals were more likely (1.21) than the behaviorally tolerant animals to show an increased neuronal activity on ED10. When comparing the effect of MPD on ED10, both the behaviorally sensitized and tolerant animals were about as likely to have an increase in activity. When using a one-way ANOVA comparing the VTA units of those sensitized animals to those animals who are tolerant, there were no significant differences between the two groups both for ED1 MPD as well as the chronic exposure to MPD on ED10.

3.8.2 Adolescent behavioral tolerance compared to sensitized (2.5 mg/kg)

The natural log odds ratio statistical test shows that for 2.5 mg/kg the neuronal recordings obtained from animals expressing behavioral sensitization were more likely (1.78, 1.86, 1.42) to show an increase in neuronal activity for all three comparisons, i.e. the initial (acute) MPD exposure, the baseline neuronal activity at ED10 compared to baseline activity at ED1 and the neuronal activity recorded at ED10 following MPD rechallenge compared to the neuronal activity on ED1 in response to the initial MPD exposure. When using a one-way ANOVA comparing the VTA units of those sensitized animals to those animals who are tolerant, there were no significant differences between the two groups both for ED1 MPD as well as the chronic exposure to MPD on ED10.

3.8.3 Adolescent behavioral tolerance compared to sensitized (10.0 mg/kg)

The natural log odds ratio statistical test shows that for 10.0 mg/kg the neuronal recordings obtained from animals expressing behavioral sensitization were more likely (2.12, 1.72, 1.14) to

show an increase in neuronal activity for all three comparisons, i.e. the initial (acute) MPD exposure, the baseline neuronal activity at ED10 compared to baseline activity at ED1 and the neuronal activity recorded at ED10 following MPD rechallenge compared to the neuronal activity on ED1 in response to the initial MPD exposure. When using a one-way ANOVA comparing the VTA units of those sensitized animals to those animals who are tolerant, there were no significant differences between the two groups both for ED1 MPD as well as the chronic exposure to MPD on ED10.

3.8.4 Adult behavioral tolerant to sensitized (0.06 mg/kg)

The natural log odds ratio statistical test shows that for 0.6 mg/kg the neuronal recordings obtained from animals expressing behavioral tolerance were more likely (3.82) to show an increase in neuronal activity during initial (acute) MPD exposure than those animals expressing behavioral sensitization. In addition, for both the baseline neuronal activity of ED10 compared to baseline neuronal activity recorded at ED1 and the neuronal activity recorded on ED10 following rechallenge with MPD administration compared to the neuronal activity on ED1 following MPD exposure, the odds ratio revealed that both the behaviorally sensitized and behaviorally tolerant animals were about as likely to show increased neuronal activity. When using a one-way ANOVA comparing the VTA units of those sensitized animals to those animals who are tolerant, there were no significant differences between the two groups both for ED1 MPD as well as the chronic exposure to MPD on ED10 for 0.6 mg/kg MPD.

3.8.5 Adult behavioral tolerant to sensitized (2.5 mg/kg)

The natural log odds ratio statistical test shows that for 2.5 mg/kg the neuronal recordings obtained from animals expressing behavioral tolerance were more likely (1.99, 1.77 and 1.74) to show an increase in neuronal activity for all three comparisons, i.e. the initial (acute) MPD exposure, the baseline neuronal activity at ED10 compared to baseline activity at ED1 and the neuronal activity recorded at ED10 following MPD rechallenge compared to the neuronal activity on ED1 in response to the initial MPD exposure. When using a one-way ANOVA comparing the VTA units of those

sensitized animals to those animals who are tolerant, there were no significant differences between the two groups both for ED1 2.5 mg/kg MPD, however on ED10 there was a significant ($p < 0.05$) difference between the two groups.

3.8.6 Adult behavioral tolerant to sensitized (10.0 mg/kg)

The natural log odds ratio statistical test shows that for 10.0 mg/kg the neuronal recording obtained from animals expressing behavioral tolerance were more likely (2.07) to show an increase in neuronal activity during the initial (acute) MPD exposure phase compared to the VTA units recorded from animals expressing behavioral sensitization to chronic MPD exposure. In addition, the odds ratio statistical test of the VTA units recorded from animals exhibiting behavioral tolerance showed that they are more likely (1.23, 1.03) than the behaviorally sensitized animals to exhibit a decrease in their baseline neuronal activity at ED10 compared to their ED1 baseline activity as well as for ED10 neuronal activity after MPD rechallenge compared to the neuronal activity on ED1 following MPD administration respectively. When using a one-way ANOVA comparing the VTA units of those sensitized animals to those animals who are tolerant, there were no significant differences between the two groups for ED1 10.0 mg/kg MPD, however there was a large significant difference ($p < 0.05$) between the two groups on ED10 to chronic 10.0 mg/kg MPD.

3.8.7 Comparison between Adolescent and Adult animals

When we compared the adult and adolescent animals based on their neuronal activity, there was a significant difference for 0.6 mg/kg MPD on ED1 and ED10 (F: 11.3, P: 0.001; F: 8.9, P: 0.003) for the tolerant animals. In addition, there was a significant difference for 2.5 mg/kg on ED10 only for the sensitized animals (F: 10, P: 0.002). And finally for the dose of 10.0 mg/kg, there was a significant difference between the adult and adolescent neuronal activity for both ED1 and ED10 and both the sensitized and tolerant animals (F: 11.3, P: 0.001; F: 64, P: 0.001; F: 6.9, P: 0.01; F: 6.4, P: 0.04).

4 Discussion

Methylphenidate (MPD) is one of the most prescribed medications for ADHD. However the its mechanism of action is not fully known and is still under study (Seeman and Madras, 1998; Solanto 1998; Vanderschuren et al., 2012). MPD has mechanistic similarities to several drugs of abuse including cocaine, and it also has a chemical structure similar to amphetamine and methamphetamine (Kallman and Isaac, 1975; Patrick and Markowitz, 1997; Teo et al., 2003). It is currently hypothesized that ADHD is mostly due to an imbalance in the DA levels in the brain and drugs like MPD that target the DA systems are effective therapeutic choices for such a disease (Izenwasser et. al., 1999; Massello and Carpenter, 1999; Patrick and Markowitz, 1997; Volkow and Swanson, 2008). MPD binds to the dopamine transporter (DAT) which prevents the reuptake of DA back into the presynaptic terminals from the synaptic cleft causing the extracellular DA to have a more lasting effect (Volkow et al., 2002). It has been noted that many addictive drugs can elicit sensitization, tolerance, withdrawal symptoms and dependence, through their action in the CNS neuronal circuits among them which the VTA plays a critical role (Pierce and Kalivas, 1995; Kalivas et al. 1993; Nestler, 2008).

In the current study, we recorded VTA neuronal unit activity concomitantly with the animal's behavioral locomotor activity both before and after the administration of several acute and chronic MPD doses. In order to eliminate any environment contributions to the recorded data which could affect the drug's effect such as novel condition, all the recordings were done in the animal's home cages. Doing so makes sure that any change from baseline activity is due to the drug's (MPD) effect and not outside stimulations. The main findings of the current study for the adolescent animals are that acute MPD elicits a dose response increase in locomotor activity. Similarly, observations were repeated by Chelaru et al., (2012) in adolescents and by Gaytan et al., (1997; 2000) and Yang et al., (2003) in adult rats. The same repetitive (chronic) MPD exposure of 0.6, 2.5, or 10.0 mg/kg elicits either behavioral sensitization in some animals or behavioral tolerance in other animals which confirms our hypothesis that the same chronic MPD dose will elicit behavioral sensitization in some

animals and behavioral tolerance in others. This dual observation is the rational as to why we evaluate the VTA neuronal activity based on the animals' behavioral response to chronic MPD.

The VTA neurons of adolescent rats responded to acute MPD exposure with a dose response characteristic, as the MPD dose was increased, more VTA units responded to the drug by changing their firing rate. The overall baseline activity at ED10 compared to the baseline neuronal activity at ED1 after six daily MPD exposures and three washout days showed dose response increases in the total number of units whose baseline was changed; i.e. with increasing the MPD dose from 0.6 to 10.0 mg/kg MPD, more VTA units expressed changes in their ED10 baseline activity compared to ED1 baseline. This change in ED10 baseline may be expressive of expectation to get the drug again or withdrawal expression from abrupt stopping of drug exposure (Algahim et al., 2009; Bergheim et al., 2012; Lee et al., 2011). In response to the chronic administration of MPD at ED10 more units responded by changing their firing rate compared to the initial MPD exposure. The majority of these units responded with significant differences in response to MPD rechallenge at ED10 compared to the responses observed at ED1 after MPD exposure.

Due to the different responses of the VTA neuronal activity following acute compared to chronic MPD administration, it is possible to classify that there are several types of responses; one type express neurophysiological sensitization (Fig 16B) and the other type express neurophysiological tolerance (Fig 16A). Those units that express both an increase in neuronal activity on ED1 following MPD exposure and a further increase in their neuronal activity on ED10 to MPD rechallenge and those VTA units that exhibit a decrease in neuronal activity to the initial MPD exposure on ED1 and a further decrease activity on ED10 to MPD rechallenge, are said to show neurophysiological sensitization. The units that did not respond significantly to MPD on ED1, but did respond significantly to MPD on ED10 can also be said to exhibit neurophysiological sensitization. Those VTA units that at ED1 responded to the drug by either increase or attenuation in

their neuronal activity and at ED10 failed to respond to MPD rechallenge or those VTA units that show the opposite effects at ED1 compared to ED10 are said to show neurophysiological tolerance.

Since the same dose of MPD either 0.6, 2.5, or 10.0 mg/kg elicits either behavioral sensitization or tolerance, the VTA neuronal recording from animals expressing behavioral sensitization to chronic MPD exposure were evaluated separately from the VTA units recorded from animals expressing behavioral tolerance. It was found that there were significant differences in response to MPD between these two groups of VTA neuronal populations. The VTA units recorded from behaviorally sensitized animals were much more likely to show an increase in firing response upon initial exposure to the drug. Conversely, the VTA units recorded from animals expressing behavioral tolerance were more likely to exhibit a decrease in the firing rate to acute MPD exposure. Similar results were observed with the chronic effect of the drug, with the exception of 0.6 mg/kg group which exhibited more increases in their firing activity in response to the drug from the behaviorally tolerant animals.

The main findings of the study confirm that the repetitive exposure to each dose of MPD elicited behavioral sensitization in some animals while eliciting behavioral tolerance in other animals. This confirmed our study aim that the same repetitive MPD dose can elicit both behavioral responses. Due to this effect of MPD at each of the doses, we evaluated the neuronal unit activity for all the animals first with no correlation to their behavioral responses to chronic MPD exposure, followed by a comparison based on their behavioral response. This was to determine if the VTA neurons recorded from animals expressing behavioral sensitization responded to acute or chronic MPD differently than those VTA units recorded from the animals expressing behavioral tolerance. With this evaluation, we were able to show that in general, the VTA neuronal population recorded from animals expressing behavioral sensitization did in fact respond differently to MPD compared to those VTA neuronal populations recorded from animals expressing behavioral tolerance.

Could it be possible that the same dose of MPD can elicit two separate phenomena? Castellanos et al., (1996) and Arnsten and Dudley (2005) reported such individual differences due to

varieties in phenotype and also drug metabolic rate between animals following MPD exposure. Additionally, Volkow and Swanson (2003) who used MRI scans in humans reported different responses to MPD in normal patients as well as patients who had ADHD which were due to differences in basal activity of DA in these individuals. In the current study, each shipment contained about four animals, which could make it possible that each shipment had animals with a different phenotype and metabolism rate of MPD, which may explain as to why we saw the different responses observed. This observation stresses the importance to evaluate each animal individually.

Overall, each of the MPD doses (0.6, 2.5 and 10.0 mg/kg) stimulated the majority of the VTA neuronal units' activity. The lowest number of VTA units that responded to acute MPD was observed following 0.6 mg/kg MPD, while 2.5 mg/kg MPD exhibited the highest rate of responsiveness, followed by 10.0 mg/kg MPD. Following 0.6 mg/kg MPD exposure, the majority of the VTA units responded to acute MPD by attenuating their firing rate, while following 2.5 and 10.0 mg/kg MPD, the majority of the VTA units responded by increasing their firing rate. Overall, the ED10 baseline activity when compared to the ED1 baseline showed a dose response increase in the total number of units that had a change in baseline; i.e. increasing the MPD dose from 0.6 mg/kg to 10.0 mg/kg MPD, the more units that expressed changes in their ED10 baseline compared to ED1 baseline following MPD rechallenge. This change in baseline activity could be the animal expressing either withdrawal or expectation of MPD (Algham et al., 2009; Bergheim et al., 2012; Lee et al., 2011). Upon the rechallenge dose of MPD on ED10 compared to MPD at ED1, the majority of the VTA units responded to MPD with a decrease in their neuronal activity. For the animals that expressed behavioral sensitization, there was a "U shape" in the number of units which responded to the MPD while the opposite was seen from the units recorded from animals expressing behavioral tolerance which showed a reverse "U shape". Likewise, the responses to MPD exposure recorded from animals expressing behavioral sensitization were significantly different when compared to those VTA units recorded from animals expressing behavioral tolerance.

There have been several molecular studies (Chao and Nestler, 2004; Kim et al., 2009; Nestler, 2004) done which have in essence shown similar observations to the current study using dose response protocols with cocaine, morphine and amphetamine. These studies showed dual observations which may provide a possible explanation why the same dose of MPD can cause some VTA units to have excitation and attenuation in other units, as well as behavioral sensitization in some animals and behavioral tolerance in others. In these studies with cocaine and methamphetamine, it was noted that some animals expressed the upregulation of the transcription factor Δ FosB or CREB levels during behavioral sensitization and behavioral tolerance respectively. The upregulation of CREB in the NAc as a result of chronic drug administration decreased the rewarding effects of cocaine and morphine (Chao and Nestler, 2004). One of the targets of CREB is the opioid peptide dynorphin which is expressed in NAc medium spiny neurons (MSN). Dynorphin release is known to contribute to dysphoria through a negative-feedback loop from the NAc to the VTA DA neurons which in turn inhibits their activity (Hyman and Malenka, 2001; Spanagel et. al., 1992). Therefore for the animals that exhibit behavioral tolerance in response to repetitive MPD exposures, it is possible that the upregulation of CREB is partly responsible for the attenuating effects of the drug and that the upregulation of CREB induces behavioral tolerance in addition to a decrease in neuropil density (Chao and Nestler, 2004; Dietz et al., 2009; Kim et al., 2009; Nestler, 2004; 2008). These molecular changings can cause a decrease in the rewarding aspects of MPD and other psychostimulants (Chao and Nestler, 2004).

Acute exposure to psychostimulants such as MPD and cocaine can induce the upregulation of Δ FosB (Moratalla et. al., 1996, Chao and Nestler, 2004). This upregulation of Δ FosB following cocaine exposure results with increased locomotion and is involved in eliciting behavioral sensitization in mice and is partly responsible for the motivational effects of cocaine (Kelz et. al., 1999; Chao and Nestler, 2004). Blocking Δ FosB is shown to have opposite effect (Peakman et. al., 2003). These opposite changes in activity could be attributed to the molecular and morphological plasticity, which takes place in the MSN density, as well as the upregulation of Δ FosB.

Consequently, animals which exhibited higher MSN density or expressed an upregulation of Δ FosB following chronic MPD exposure would show a further increase in activity to rechallenge MPD respectively. As a result, it is possible that the animals that expressed behavioral sensitization or behavioral tolerance to MPD in the current study could be experiencing these dual molecular transcription factor changes in the CNS circuitry; and can provide further explanation to the present observation why the same dose of MPD can cause both behavioral sensitization and tolerance or increase activity in some VTA units and decreases in their activity in response to MPD exposure in other VTA units.

VTA neurons contain both D1 and D2 DA receptors, which are affected by drugs like MPD and the glutamatergic afferent inputs from the PFC, in addition to other neurons in the VTA. DA D1 receptors have an excitatory effect when activated, while the DA D2 receptors have an inhibitory effect on other cells when they are activated. It is possible that the VTA neurons which exhibit mainly a decrease in their activity are being recorded from an area of the VTA that contains mostly DA D2 receptors whose activation gives the decrease in activity. Conversely, the cells which exhibited mainly an increase in activity could be recorded from an area of the VTA containing mostly DA D1 neurons. Furthermore to these units, the afferent input of glutamatergic neurons from the PFC to DA neurons in the VTA can be affected by MPD which in turn affect how they fire to the VTA and could also be partially responsible to the different responses to MPD.

It was reported that low doses of MPD mainly affect the DA D2 autoreceptors in the VTA which leads to the attenuation of DA release in response to a stimulus such as MPD (Seeman and Madras, 1998, 2002). These auto-receptors counter regulate the extracellular DA released from the same cell and are likely the reason that 0.6 mg/kg MPD elicited a decrease in the locomotor activity of some animals. The exposure to the higher doses of 2.5 mg/kg and 10.0 mg/kg MPD overcome the inhibitory effects of the presynaptic auto-receptors with increased DA levels in the cleft and post synaptic activation which causes amplification of the DA signals as observed in this study (Volkow et. al., 2005). For the animals which exhibited behavioral sensitization, the majority of their neuronal

responses to MPD elicited a decrease in activity on ED10 regardless of an increase or decrease in activity on ED1 following MPD administration. For the animals that expressed behavioral tolerance, different patterns were observed.

It is believed that MPD works in a biphasic action, which includes a “spontaneous” phasic and tonic release of DA. The phasic release of DA from synaptic terminals is large but brief, and activates the postsynaptic DA receptors evoking DA dependent behavioral responses (Seeman and Madras, 1998, 2002). The other mode of action, tonic DA release, from the VTA is regulated by the presynaptic NMDA receptors controlled by glutamatergic afferents from the PFC (Grace, 1991). During normal nerve impulses the basal levels of DA rise about 60-fold and quickly diminish back to normal levels. With the low doses of MPD (0.6 mg/kg), the resting level of DA rises about 6-fold and is thought to act mostly on DA D2 autoreceptors which reduce the impulses which triggered the DA release. At higher doses of MPD exposure (2.5 and 10.0 mg/kg), both the resting DA levels and the triggered DA output are increased significantly causing the wide spread stimulation of postsynaptic DA receptors which cause strong stimulation (Seeman and Madras, 1998, 2002) and can help to explain why low doses of MPD can cause lower levels of activation and the higher doses of 2.5 mg/kg and 10.0 mg/kg MPD provide increased levels of activity upon acute administration of the drug.

In several studies, it has been reported that psychostimulants such as MPD can elicit increases in neuropil (the density and the dendritic branching of the MSN) in the NAc and PFC, while at the same time in other animals, elicits a decrease in the neuropil levels following the same dose of stimulant (Kim et al., 2009; Nestler, 2008; Robinson and Kolb, 1997; 1999). We suggest that the animals which expressed the increases in their neuropil as a result of chronic psychostimulant exposure would respond to rechallenge drug administration by increasing their firing rate and those animals which elicited decreases in their neuropil would respond to MPD by decreasing their firing rate.

Based on the responses to acute and chronic MPD, we have classified two types of neuronal activity. The first type of neuronal unit activity expresses neurophysiological sensitization and the second type expresses neurophysiological tolerance. The units that express both an increase in neuronal activity on ED1 after MPD administration and yet a further increase in their neuronal activity on ED10 following the MPD rechallenge dose, as well as the VTA units that exhibit a decrease in their neuronal activity to MPD on ED1 and a further decrease activity on ED10 to MPD rechallenge, exhibit neurophysiological sensitization. Some units did not respond significantly to MPD administration on ED1, but did respond significantly on ED10 following MPD can also be said to exhibit this neurophysiological sensitization. Those VTA units who responded to the drug by either increase or attenuation in their neuronal activity at ED1 and failed to respond to MPD rechallenge on ED10, as well as those VTA units that show the opposite effects at ED1 compared to ED10 are said to show neurophysiological tolerance.

When we applied the one way ANOVA and odds ratio tests comparing the effect of MPD on the VTA neuronal units recorded from animals expressing behaviorally sensitization to those units recorded from behaviorally tolerant animals for each dose, we found that the neuronal population recorded in the VTA from animals that express behavioral sensitization to repetitive MPD exposure did in fact respond differently to MPD compared to those VTA units recorded in animals expressing behavioral tolerance. These two tests show the importance of evaluating the neuronal firing rates following psychostimulant exposure based off of the animal's behavior in order to find any correlations between drug dose and firing rates with behavioral activity. We observed differences between the adolescent and adult animals in how they responded to MPD. In general, both groups showed dose response effects to the drug. The adolescents seemed to have higher activation to low dose MPD, while the adults had more activity following higher doses of MPD. This helps to show that there are inherent differences between the two age groups in how they respond to MPD and that the drug affects younger individuals differently than older individuals. This could be due to the adolescent brains continuing to go through synaptic pruning and shaping of the adult brain as well as

different molecular, morphological, and metabolic differences that come with age. We highlighted the importance of looking at both groups of animals as well as shed light on the differences between them. It will be important in future experiments to further the knowledge of MPD's effects on both groups and why the changes in activity

In conclusion, the current study showed that the VTA neuronal population activity recorded from animals expressing behavioral sensitization to chronic MPD, responded to MPD exposure differently from those VTA units recorded in animals expressing behavioral tolerance and that the effect of MPD on VTA neurons is different from other psychostimulants, i.e. MPD has its own unique effect that needs to be elucidated. We were able to show differences between the adolescent and adult animals which show that MPD can have differing effects on each group. This study can help to explore those differences and shows the importance of looking at both adult and adolescent studies for drugs such as MPD due to their wide usage in society and the potential risks they pose. Additionally, this study shows the importance of looking at individual responses to the acute effect of the drug and the baseline levels of individuals as a way to gauge how they will react to psychostimulants such as MPD. Moreover, the observation that the same repetitive MPD dose elicited behavioral sensitization in some animals while causing behavioral tolerance in others is essential information that the professional practitioners and the MPD users need to consider.

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6 Vita

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