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Catherine M. Claussen

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By

Catherine Claussen, BS

APPROVED:

Supervisory Professor

Dr. Vicky Knutson

Dr. Michael Beauchamp

Dr. Jack Waymire

Dr. Patrick Dougherty

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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Catherine M. Claussen, BS

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Dedications

This thesis is dedicated to my family, my mother Sherry, Siblings Rickie, Randy and Jill and my father Randy who may be gone but never forgotten. Without my family I would not be where I am today. I am thankful for their endless love, support and encouragement.

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Abstract

Methylphenidate is currently a drug of abuse and readily prescribed to both adolescents and adults. Chronic methylphenidate (MPH) exposure results in an increase in DA in the motive circuit, including the caudate nucleus (CN), similar to other drugs of abuse. This study focuses on research aimed to elucidate if there are intrinsic underlying differences in the CN electrophysiological activity of animals exhibiting different chronic responses to the same dose of MPH. Behavioral and caudate nucleus (CN) neuronal activity following acute and chronic doses of MPH was assessed by simultaneously recording the behavioral and neuronal activity. The experimental protocol lasted for 10 days using four groups; saline, 0.6, 2.5 and 10.0mg/kg MPH. Initially, the study determined that animals exposed to the same dose of MPH exhibited either behavioral sensitization or behavioral tolerance. Therefore animals were classified into two groups (behaviorally sensitized/tolerant) and their neuronal activity was evaluated. Four hundred and fifty one units were evaluated. Overall, a mixture of increases and decreases in CN neuronal populations was observed at initial MPH exposure, and at ED10 baseline and ED10 rechallenge. When separated based on their behavioral response (sensitized/tolerant), significant differences in neuronal response patterns was revealed. Animals exhibiting sensitization were more likely to increase their neuronal activity at ED1 and ED10 baseline, expressing the opposite response at ED10 rechallenge. Furthermore, when neuronal populations recorded from those animals exhibiting behavioral sensitization were statistically compared to those from animals exhibiting behavioral tolerance significant differences were observed. Collectively, these findings tell us that animals exposed to the same dose of MPH can respond oppositely and moreover that there is in fact some intrinsic difference in the two population's neuronal activity. This study offers new insight into the electrophysiological differences between sensitized and tolerant animals.

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1 Introduction

The History of MPH and its Uses

Methylphenidate [Ritalin/MPH] was first synthesized in 1944 (U.S. Pharmacist, 2002), and by 1954, it was being tested on humans (U.S. Pharmacist, 2002). Initially in 1957, Ciba Pharmaceutical Company began marketing MPH as Ritalin for the treatment of chronic fatigue, depression, psychosis, and narcolepsy (U.S. Pharmacist, 2002). In 1960, MPH was marketed as Ritonic, which was used as a treatment to improve a patient's mood and to maintain cognitive vitality (U.S. Pharmacist, 2002). By the 1970's-80's, MPH was prescribed for the treatment of attention deficit hyperactivity disorder (ADHD). The symptoms of ADHD, as described in the Diagnostic and Statistic 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.). From 1990-1999, a 500% increase in the use of MPH in the United States was observed (U.S. Pharmacist, 2002) and the daily doses administered to patients increased from 75 million in 1990 to 360 million in 1998 (Woodworth, 2000). Shockingly, the United States alone consumes 85% of the world's supply of MPH (Woodworth, 2000). Based on the 2000 US census population of 281,421,906, approximately one out of five hundred randomly chosen people are currently prescribed Ritalin (Woodworth, 2000). MPH is still the pharmacotherapy of choice and top seller for the treatment of ADHD.

Due to the overabundance of adolescents being prescribed MPH, there is a high risk of nonmedical misuse and abuse by patients' 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).

A substantial number of studies are available to support the assertion that MPH is abused at a high rate by adolescents and adults (McCabe et al., 2007; Teter et al., 2006; Wilens et al., 2006). One such study focusing on undergraduate college students, found that over 30% of students that were tested had taken non-prescribed MPH either to get high or to aid in studying (Teter et al., 2006; Wilens et al., 2006). In a 2006 study of college students, McCabe et al. found that 16%of respondents abused stimulants for the cognitive enhancement effect, with 96% of them preferring Ritalin/MPH as their drug of choice (McCabe et al., 2007). Furthermore, a study involving 700 participants aged 12 to 44 found that 22.9% had loaned their prescription to someone else, 26.9% had borrowed someone else's medical prescription, 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 borrowed prescription medication to relax or feel good compared to only 10% of those aged 36 to 44 , indicating that younger people are more likely to misuse and abuse MPH. In addition, this study reported that 34.1% of adolescents said they were more likely to use Ritalin/MPH if it came from someone who was familiar with the drug (Goldsworthy et al., 2008). These statistics on non-medical MPH use highlight the importance of performing further studies to examine the short- and long-term effects of MPH exposure.

The behavioral effects following short-term MPH exposure have been well documented (Brands et al., 1998), however, the data for long-term MPH exposure has

not been fully investigated. The short-term behavioral effects associated with low doses of MPH include wakefulness, appetite suppression, impairment of voluntary movement, headache, heightened alertness,, and vomiting (Brands et al., 1998). The short-term behavioral effects for higher doses of MPH include exhilaration, dilation of pupils, agitation, muscle twitching, excitation, confusion, hallucinations, increased blood pressure, increased pulse rate, delirium, paranoia, seizures followed by coma, excessive repetition of movements and meaningless tasks (Borcherding et al., 1990; Masand and Tesar, 1996). Although there are few studies on the long-term effects of low and high dose MPH exposure, one study employing positron emission tomography (PET) scans found that extended use of MPH constricted blood flow to the brain (Wang et al., 1994), which may causes an increase in blood pressure. Other behavioral studies on long-term effects of MPH use yielded conflicting results (Wilens et al., 2003; Piazza et al., 1989; Lambert , 1999; Robinson and Berridge, 1993). For instance, one study reported that MPH treatment protected adolescents from later drug dependence (Wilens et al., 2003), another reported MPH use created vulnerability to later drug exposure (Piazza et al., 1989; Lambert, 1999; Robinson and Berridge, 1993), while yet another longitudinal study reported that there was no direct correlation between adolescent or youth stimulant use and later drug use (Barkley et al., 2003). Given the amount of conflicting results observed in the literature, we believe that recording both behavior and neuronal activity together will give us a better idea as to why these reports are conflicting.

A better understanding of the short- and long-term neural adaptations that occur following MPH exposure is crucial, especially since a large population of those consuming this drug are children with central nervous systems (CNS) that are not fully developed. What is known about the mechanism of MPH action and neural responses to acute and repetitive exposure is as follows: MPH has a chemical structure and neuropharmalogical characteristics similar to drugs with a high probability of abuse, such

as cocaine and amphetamines (Teo et al., 2003; Volkow, 2001). MPH has a similar mechanism of action as cocaine in that it acts as an indirect agonist by binding with high affinity to the dopamine transporter, blocking dopamine (DA) re-uptake to the presynaptic terminal, and thus increasing levels of extracellular DA in the synaptic cleft. MPH is absorbed and metabolized via de-esterification to ritalinic acid (Faraj et al., 1974; Wang et al., 1997; Wargin et al.,1983) and released into the urine within 48 hours. Since the psychostimulant is concentrated in catecholaminergic systems with free flow across the blood brain barrier, concentrations of MPH in the brain exceed plasma levels. MPH also has a similar rapid uptake in the brain as cocaine, however MPH has a much lower rate of clearance (Morse et al., 1995). This may explain why IV or intranasal administration of MPH yields higher mortality rates than cocaine or amphetamines (Gatley et al., 1999; Volkow et al., 1995, 1996, 1999).

It has also been shown that dose and route of MPH administration play a significant role in the experience of the user, as behavioral responses and neurochemical reactions to the drug are dependent upon the length of time it takes for the drug to reach its peak level in the periphery (Kuczenski and Segal., 2002). When MPH is administered orally, it is absorbed through the intestinal tract, has a half-life of about 1 hour, and peaks in the blood stream at approximately 60-90 minutes (Gerasmiov et al., 2000; Kuczenski and Segal 2002; Stewart & Badiani 1993) (Garland 1998; kuczenski and Segal 1991;2001; Solanto 1998, 2000). However, following intravenous (IV) injection or intraperitoneal (IP) administration, peak levels of MPH in the periphery are reached at approximately 8-20 minutes and 15-28 minutes, respectively (Gerasmiov 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/pleasure response and adverse effects, such as a sensitization response.

Behavioral Sensitization and Tolerance with MPH Use

MPH has been shown to cause different behavioral responses even when subjects are given the same dose of the drug. Some studies report that MPH elicits behavioral sensitization, whereas other studies report the same dose of the drug can also result in behavioral tolerance (Barron et al., 2004; Eckerman et al., 1991; Gaytan et al., 1996, 2000; Yang et al., 2003; 2006, 2007). The aim of the latter study was to first determine if there are differences in neuronal responses in individuals, particularly with respect to neuronal firing patterns in the caudate nucleus (CN), and whether these differences are responsible for the conflicting reports on behavioral responses with chronic MPH exposure. We hypothesized that animals will exhibit different overall neuronal responses depending on whether that animal exhibits behavioral sensitization or behavioral tolerance. Furthermore, we specifically 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 phenomenon in animals that represent an enduring response to medications even after the discontinuation of drug use (Laasko et al., 2002), and are used as a model for drug craving and dependence (Kalivas et al., 1998; Robinson and Berridge, 1993; Wolf, 1998). 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), and 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 neural changes that result in sustained augmented behavioral responses despite cessation of psychostimulant use (Pierce and Kalivas, 1997). Thus, equal or increased behavioral activity is observed when animals are given a re-challenge administration of drugs

following a period of time when the drug is no longer in their system. It is generally 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), etc.

The Role of the Claudate Nucleus in Responses to MPH

The CN is part of the basal ganglia and receives input from different cortical areas, including the thalamus and substantia nigra, which ascend to the globus pallidus (Carpenter 1976; Kande et al., 2000). The main efferent projections come from the globus pallidus and project to the thalamus via the ansa lenticularis and lenticular fasciculus. From the thalamus projections extend to the cortex via the paliotegmental neurons which continue to cover several subcortical areas (Carpenter, 1976). The CN contains both a direct excitatory pathway and an indirect inhibitory pathway, which are modulated by the activation of CN medium spiny neurons (MSN) that express D1 and D2 dopamine neurons. The CN is stimulated by the cortex to release the neurotransmitter gamma amino-butyric acid (GABA), which will project to the globus pallidus external (GPe) via the indirect pathway. However, using the direct pathway, GABA will project to the globus pallidus internal (GPi). GPe inhibition leads to suppression of the subthalamic nucleus, resulting in disinhibition of the GPi and ultimately, inhibition of motion through GPi's suppressive action on the thalamus. GPi inhibition (through the direct pathway) causes disinhibition of the thalamus, thereby allowing motion (Carpenter, 1976; Kreitzer and Malenka, 2008; Zhang et al., 2004)(Fig. 1). This gives rise to the hypothesis that the dominant role of the CN is to regulate motor performance (Zhang et al., 2004).

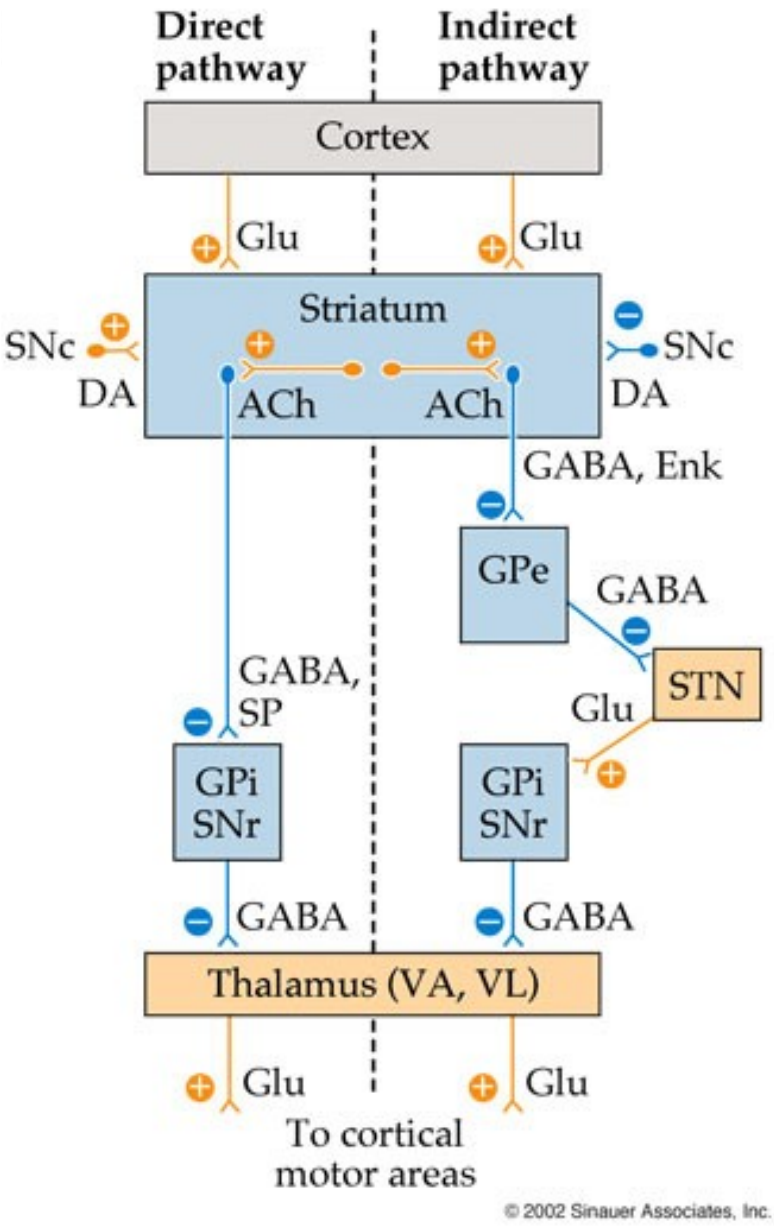

Figure 1 shows the direct (excitatory) and indirect (inhibitory) pathway of the CN. The direct pathway receives projections from the globus pallidus internal and substantia nigra reticular via the thalamus and other cortical areas. The indirect pathway receives projections from the globus pallidus external via the subthalamic nucleus and thalamus.
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The CN has been reported to be structurally altered by acute and repeated administration of MPH. These alterations include the regulation of the transcription factors CREB and ΔFos B, both implemented in drug dependence (Chao and Nestler, 2004; Nestler 2004, 2008). The CN direct pathway contains an over abundance of D1 DA receptors which control extracellular signal-kinase (ERK). ΔFos B gene expression is increased by the upregulation of ERK which has been reported to induce long-term neuroadaptions in the brain (Shi and McGinty, 2010 and Zhang et al., 2004). ΔFos 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). Thus we hypothesized that animals expressing behavioral sensitization will also show an increase in extracellular neuronal responses following chronic drug exposure.

MSNs of the indirect pathway coexpress an abundance of D2-like DA receptors which exert inhibitory effects (Kreitzer and Malenka, 2008; Zhang et al., 2004). Using D3-like DA receptor (subset of D2 receptors) mutant mice Zhang et al. (2004) exhibited an increase in ERK activation, thus increasing sensitivity to the drug.. Previous studies in the NAc report that CREB activation via psychostimulants creates a homeostatic negative feedback adaption, inhibiting sensitivity to future drug administration (Chao and Nestler, 2004 and Nestler, 2004). Therefore I hypothesized for those animals expressing behavioral tolerance will exhibit decreased neuronal firing patterns after chronic MPH exposure.

Previous studies reported that the ablation of the CN by nonspecific electrolytic lesion and specific ablation of the DA system by 6-OHDA that destroyed the nerve endings of the DA system (Claussen et al., 2012) found that the electrolytic lesion

animals maintained their response to chronic MPH exposure, however, the 6-OHDA lesion group did not respond to the acute and chronic MPH administration. Owing to the response following 6-OHDA lesion and no response to the electrolytic lesion we know that the push pull mechanism described earlier involves DA transmission (Claussen et al., 2012). Therefore, an upset of the balance between the direct and indirect pathways is mediated by the DA system and moreover the DA system will mediate the responses observed during the current study.

Previous neurophysiological studies targeting the reward circuit independent of the animals behavioral activity show that 2.5 mg/kg MPH modulates the neuronal activity of the CNS sites; NAc, PFC, CN following both acute and chronic MPH exposure (Chong et al., 2012; Claussen and Dafny, 2012; Salek et al., 2012)

In this study, we endeavored to compare neuronal responses and behavioral responses to acute and chronic MPH use and determine whether there are any relationships that can be made between these responses. We hypothesized that animals exposed to the same dose of MPH will exhibit different behavioral responses; some exhibiting behavioral sensitization and others behavioral tolerance. Also, we hypothesized that when these animals are separated based on their behavioral responses of sensitized or tolerance, their neuronal responses will be statistically significantly different. 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 (Gately et al., 1999). We believe that finding a correlation between the neuronal and behavioral properties of animals in response to acute and chronic MPH 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 MPH exposure.

Methods

2. Experimental Procedure

2.1 Subjects

Forty-seven (47) adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were obtained.

> The animals weighed 150-175 grams upon arrival and were housed in single Plexiglas cages inside a sound-attenuated animal facility room for acclimation. The rats homecage used during the acclimation was also used as their test cage throughout experiment. The room was maintained on a 12-h light/dark cycle (lights on 06:00), at an ambient temperature of 21 ± 20^0C and at a humidity of 58-62%. Rats were supplied food and water ad libitum for the entire duration of the study. All experiments were 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.
>
> Chong et al., 2012

2.2 Drug

Methylphenidate hydrochloride (MPH) was obtained from Mallinckrodt Inc. (St. Louis, MO, USA). A study from 289 patients treated with MPH reported that the range of doses ingested by these patients was from 0.06 to 29.3 mg/kg with the majority of patients being treated with 1.0 to 3.0 mg/kg MPH. Approximately 2 to 3 mg/kg (i.p.) MPH (White and Yadao 2000) in rodents achieved plasma levels similar to those achieved in clinical use (Bowman and Kuhn, 1996; Brandon and Steiner, 2003; Gerasimov et al., 2000). MPH doses between 0.5 to 3.5 mg/kg, i.p., were reported to promote peak plasma concentration within the typical clinical range (Kuczenski and Segal, 2002). In rodents, a MPH dose below 5.0 mg/kg, i.p., is considered a low dose and is comparable to doses in clinical use. The range of 5 to 10 mg/kg MPH is considered moderate dosage and

above 10 mg/kg as a high dosage (Bowman and Kuhn, 1996; Brandon and Steiner, 2003; Faraj et al., 1974; Kollins et al., 2001; Rush et al., 2001; Sagvolden and Sergeant, 1998; Santosh and Taylor, 2000; Solanto, 1998, 2000; Spear et al., 1983; Stewart and Badiani, 1993). Previous work using MPH doses from 0.1 to 40 mg/kg i.p. showed that doses of 0.6, 2.5 and 10.0 mg/kg MPH administered intraperitoneal (i.p.) elicited behavioral sensitization or tolerance (Askenasy et al., 2007; Dafny and Yang, 2006; Gaytan et al., 1997b; 2000; Yang et al., 2006a; 2007), therefore these were the three doses chosen (0.6, 2.5 and 10.0mg/kg). The MPH was dissolved in 0.9% saline (NaCl) solution and the dose was calculated as free base. All injections were given between 08:00am and 9:00am and equalized to 0.8 ml with saline so that all injections volumes were the same for all animals.

2.3 Electrode Implantation

On the day of surgery rats were weighed and anesthetized with 50 mg/kg i.p. pentobarbital. The top of the rat's head was shaved to expose the skin and coated with a thin layer of 2% Lidocaine Hydrochloride Jelly (Akorn,Inc.). The animal was then placed in a stereotaxic instrument. An inch incision was made with muscle and connective tissue removed to expose the skull. A single hole for the reference electrode was drilled above the frontal sinus and two bilateral 0.6mm diameter holes were drilled over the CN, all in accordance to the coordinates derived from Paxinos and Watson (1986) rat brain atlas (1.0mm anterior from bregma, 3.0mm lateral). Prior to electrode placement 6 anchor screws were put in vacant areas of the skull to secure the skull cap with dental acrylic cement. Two twisted Nickel-Chromium, Diamel coated; 60 micron diameter wire electrodes (fully insulated except at tips) were secured each to a 1cm copper connector pin made prior to surgery. A reference electrode was placed in the frontal sinus and the two twisted recording electrodes were implanted each in the CN as follows: One twisted electrode (i.e., two electrodes together) was inserted into the drilled hole at an initial depth of 4mm. Unit activity was monitored during placement of electrodes by using a Grass emitter Hi Z Probe connected to a Grass P511 series pre-amplifier. Electrodes were fixed to the skull only when spike activity exhibited at least a 3:1 signal to noise ratio in both electrodes. If the activity did not match the 3:1 spike to noise ratio criteria, the electrode was moved down in approximate increments of 10 microns until a depth of 5.0mm until they displayed a proper signal to noise ratio of neuronal activity. Once a sufficient signal was obtained, the electrode was fixed in the skull with Webglue, cyanoacrylate surgical adhesive (Webster Veterinary). The secondary twisted electrode was implanted using identical procedures in the other hemisphere (Chong et al., 2012; Claussen and Dafny, 2012; Dafny, 1982;

> Dafny et al., 1983; Dafny and Terkel, 1990; Yang et al., 2006a; 2006b; 2006d; 2007). The electrode connector pins were inserted into Amphenol plugs which were positioned on the skull and secured to the skull with dental acrylic cement. Rats were allowed to recover from the surgical procedure for approximately 4 to 7 days. During this recovery period, every day for 2 hours, the rat, with his home cage, was placed in the experimental behavioral apparatus and connected to the wireless (telemetric) head stage transmitter (Triangle BioSystems, Inc; Durham, NC, USA) for daily acclimation to the recording systems.
>
> Chong et al., 2012

On the first day of recording the animals weighed between 200 and 220grams, postnatal days P62-65.

2.4 Experimental protocol

Animals were randomly assigned to four groups; saline, 0.6, 2.5 or 10.0 mg/kg MPH groups. The experimental protocol was adapted from previous experiments (Chong et al., 2012; Claussen and Dafny 2012, Salek 2012). Experimentation began 4 to 7 days post-surgery and lasted for 10 days. On experimental day 1 (ED1) prior to the start of the recording session, animals were again allowed to acclimate to the recording system for 20-30 minutes. During this time, the recording parameters were organized in order to properly record the neuronal activity and save the files. Immediately post saline (0.8 ml of 0.9%) injection, a 60 min baseline of neuronal and behavioral activity was recorded simultaneously. This was followed by a second injection of saline or 0.6, 2.5 or 10.0 mg/kg MPH injection and recordings were resumed immediately after injection for another 60 minutes. From ED2 to ED6 rats were injected once daily with the same MPH concentration and at the same time as they have been injected at ED1. All injections were done in their home cages (which were also their test cages), ED7 to ED9 were washout days in which no injections were given. On ED10, identical experimental protocol as ED1 was followed (Table 1); neuronal and behavioral baseline activity was recorded for 60 min following a saline injection, as well as an additional 60 min neuronal and behavioral recording after a saline or MPH rechallenge injection (table 1).

Table 1: Experimental Protocol

	Experimental Days:	Day 1 Acute (initial)	Day 2 to 6 Maintenance	Day 7 to 9 Washout	Day 10 Rechallenge
Treatment:	**Saline** **N=10**	saline / saline	saline	Washout	saline / saline
	0.6 mg/kg **N=12**	saline / 0.6 mg/kg	0.6 mg/kg	Washout	saline / 0.6 mg/kg
	2.5 mg.kg **N=12**	saline / 2.5 mg/kg	2.5 mg/kg	Washout	saline / 2.5 mg/kg
	10.0 mg/kg **N=12**	saline / 10.0 mg/kg	10.0 mg/kg	Washout	saline / 10.0 g/kg

2.5 Behavioral Recording System

Locomotor activity was recorded using an open field computerized animal activity system (Opto-M3, Columbus Instruments, Columbus, OH). The open field system comprised of a clear acrylic cage with infrared beam sensors that run 40 cm in length, by 20 cm in width with 16 by 8 infrared beams respectively, and their sensors set 5 cm above the floor of the cage. Movement across any of the infrared beams results in a beam break and was subsequently recorded as total counts (TC) of locomotion. TC's were compiled and downloaded to a PC in 10 minute bin increments and were evaluated from 60 min post injection for both the saline baseline and MPH administration on ED1 and ED10. The Opto-M3 software was used to count the number of stereotypic movements (NOS) was calculated manually by subtracting the horizontal activity from the total counts of locomotion. The objective of the behavioral recoding was to distinguish animals that expressed behavioral sensitization following repeated MPH exposure from animals that expressed behavioral tolerance. This grouping would be used as the basis for the neurophysiological data analyzing.

2.6 Behavioral Analysis

The locomotor activity recorded on ED1 and ED10 was summed into 10 min bins for 60 min (i.e.6 bins/hr). The TC and NOS were then analyzed for each individual rat using a paired t test with significance set at $P<0.05$. Three comparisons were made: (1) ED1 baseline compared to ED1 post MPH administration to determine the acute effect of the drug (Table 1); (2) ED10 baseline compared to ED1 baseline to evaluate whether the six daily MPH exposures and the three washout days elicits changes on ED 10 baseline compared to ED1 baseline activity (3) ED10 MPH chronic effect compared to ED1 MPH acute effect to determine if behavioral sensitization or tolerance was expressed (Table1).

Based on the third comparison, the animals were divided into two groups of either behaviorally sensitized or no change/tolerant. As a group the rats were then analyzed (for all three comparisons) using an ANOVA with repeated measures with adjustments for correlation among measurements. Post ad hoc comparisons were used to estimate changes between days within groups.

2.7 Electrophysiology Data Acquisition

> On the experimental recording day, the rat was placed with his home cage in a Faraday testing box to reduce noise during signal transmission. The wireless Triangle Bio Systems (Durham, NC, USA) head stage was connected to the electrode pins of the skull cap. The Triangle BioSystem head stage sent neuronal activity signals through a receiver that connects to a Cambridge Electronic Design (CED) analog –to- digital converter (Micro1401-3; Cambridge, England) which then collected and stored the recorded data on a PC. Spike 2.7 software (CED) was used off line to sort for identical spike amplitude and waveforms by examining single spike activity exhibiting similar amplitude and wave form patterns before and after MPH administration for ED1 and ED10 (See section 2.7.2 on spike sorting for more details) to produce a sequential frequency histogram and to calculate the firing rate in spikes per second. Approximately one to two spikes (units) were analyzed per electrode.
>
> Chong et al., 2012

2.8 Spike sorting

> For spike sorting Spike2 version 7 software (Cambridge Electronics Design-CED) was used. The analog recording (sampling rates up to 200 kHz) was captured by the program and processed using low and high pass filters (0.3-3 kHz). Two window levels were set, one for positive-going spikes and one for negative-going spikes. Spikes with peak amplitudes that were triggered by the window were used to create templates. One 1000 waveform data points was used to define a spike. The spikes were extracted when the input signal enters an amplitude window. Spikes with a peak amplitude outside these limits were rejected. The algorithm that was used to capture a spike allowed the extraction of templates that provide high-dimensional reference points that can be used to perform accurate spike sorting, despite the influence of noise, spurious threshold crossing and waveform overlap. All temporally displaced templates were compared with the selected spike event to find the best fitting template that yields the minimum residue variance. Secondly, a template matching procedure is then performed; when the distance between the template and waveform exceeds some threshold (80%) the waveforms were rejected. That means that the spike sorting accuracy in the reconstructed data is about 95%. All these parameters of spike sorting for each electrode were sorted and used for the activity recorded in experimental day 1 (ED1) and in ED10 i.e., we use identical criteria to sort spikes

in ED1 and ED10 to ensure the spike pattern captured on ED1 is the same as ED10.

Claussen et al., 2012

2.9 Electrophysiological Data Analysis

The sorted neuronal activity obtained from the fixed template matching system was converted by the spike 2 version 7 software (CED) into their firing rates (spikes per second) for the baseline control recording and for the activity following MPH administration. These firing rates were exported into a spread sheet format displaying the rat's number, experimental day, MPH dose and recording channel (to distinguish hemisphere). Firing rates were evaluated for normality assumptions to determine parametric or non-parametric methods to evaluate differences before and after MPH treatments. The firing rates were determined to not hold normality assumptions, so we assessed differences in mean firing rates by using the critical ratio (CR) test.

Chong et al., 2012

This test was used to determine whether acute and chronic MPH treatments altered CN unit activity (C.R.=E-C/$\sqrt{E+C}$ ± l.96=P<0.05) when comparing the effect of the initial (acute) MPH exposure, C – represents the activity following saline and E – the activity post MPH injection; when comparing the effect of six daily MPH exposures and three washout days on ED10 baseline C – represents the ED1 baseline, and E – represents the ED10 baseline activity; when comparing the effects of MPH rechallenge at ED10 to ED1; E represents the effects of MPH at ED10, and C represents the ED1 neuronal activity post MPH injection. In addition changes of each unit activity induced by the treatment was considered statistically significant if the firing rate after drug treatment differed by at least 2 standard error (S.E.) from the mean. (Claussen and Dafny, 2012; Dafny, 1980; 1982; Salek et al., 2012).

Multiple approaches were used to further analyze the neuronal activity based on the behavioral responses. First a natural log odds ratio was utilized to determine the likelihood of one group of animals to show an increase in neuronal activity compared to the other group of animals (comparing the neuronal population recorded from

behaviorally sensitized group to those recorded from animals exhibiting tolerance). This was done for all three comparisons; the initial (acute) MPH injection, the baseline neuronal activity at ED10 compared to baseline at ED1 and the rechallenge MPH administration at ED10 compared to ED1 initial MPH injection. 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 higher likelihood of increased neuronal activity in one group compared to the other group of animals, conversely a number smaller than 1 represents a less likely increase in neuronal activity.

A log linear model was used next to control for dose when comparing the overall activity (acute, baseline and chronic) between the two groups (behaviorally tolerant and sensitized) to determine if there was a significant difference between dose behavior and firing patterns for each group (0.6, 2.5 and 10.0 mg/kg). P-values of <0.05 obtained from the log linear model was considered as significant.

2.10 Histological verification of electrode placement

> At the end of the experimental protocol, rats were deeply anesthetized with sodium pentobarbital. The rat's brain was transcardially perfused with 10% formalin solution containing 3% potassium ferrocyanide. A 2 mA DC current was passed through the electrode connector pin for 40 seconds to produce a small lesion. The brain was then excised and stored in 10% formalin for subsequent histological processing. Placements of the electrodes were verified in 60 micron thick coronal sections that were stained with cresylviolet. Coordinate position of the electrode tips were established by matching equivalent locations of the lesion and the prussian blue spot by using the Rat Brain Atlas by Paxinos and Watson (1986) (Fig. 2).
>
> Chong et al., 2012

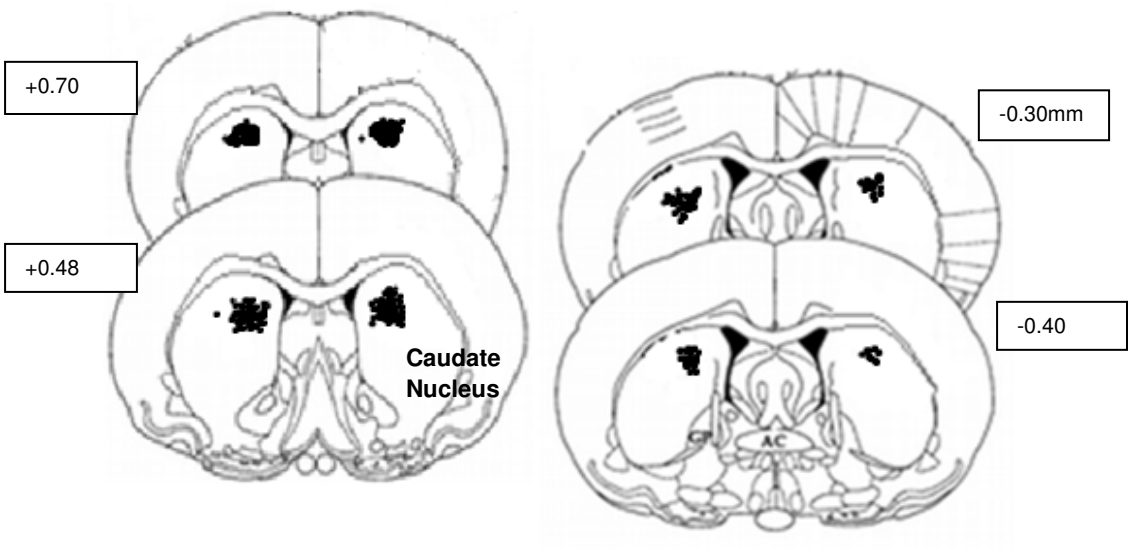


Figure 2 reconstructs the histologically verified electrode tip placement in the caudate nucleus (CN). The black dots on the rat atlas plates (Paxinos and Watson, 1986) represent the location of the CN recording electrodes in serial coronal sections. The number on the top right corner of each section represents the anterior/posterior distance (mm) from bregma. ***Rights for reuse of figure obtained through Elsevier.**

3 Results
3.1 Overall

3.1.1 Overall Behavioral

Forty-seven (47) rats that met the histological verification of electrode location placement and the neurophysiological requirements and exhibited similar spike amplitude and pattern at ED1 and ED10 were included in the study (saline N=10; 0.6 mg/kg N = 12; 2.5 mg/kg N = 13; 10.0 mg/kg N = 12 animals respectively). Each animal's Total counts (TC) of activity were evaluated separately using a paired t-test to determine whether they exhibited behavioral sensitization or tolerance. This was done by comparing their locomotor activity after MPH administration at ED10 to the effect of the initial MPH exposure on ED1 (Table 1). The number of stereotypic movements (NOS) was analyzed as a second measure to verify that animals exhibiting tolerance or no change were not in fact exhibiting an increased stereotyped behavior; a form of behavioral sensitization. The NOS results show that all animals expressing behavioral tolerance also exhibited statistically ($p<0.05$) significant decreases in stereotypic movement (data not shown). The control group showed no effect on behavioral activity following acute or multiple injections of saline (data not shown). This lack of change in behavioral activity following saline injection shows that our recording procedures have no effect on the rats locomotor activity.

3.2 Overall Saline

Sixty CN units were recorded from 10 rats injected with saline only. In general these CN units exhibited similar neuronal firing activity at ED1 following the second saline injection compared to the initial saline injection. Their baseline activity at ED10 following six daily saline injections compared to the activity at ED1 showed that the units exhibited similar neuronal activity at ED1 and at ED10. This observation in the saline control group

provides evidence that daily handling and injection volume did not modulate the CN units neuronal firing rates.

3.3 Results (0.6mg/kg)

3.3.1 Behavioral Results (0.6mg/kg)

As a group, the 0.6mg/kg MPH dose had no effect on acute and chronic behavioral response (Fig. 3A). This lack of response as a group is due to some individual animals increasing locomotion and others decreasing their locomotion, thus as a group they cancel each other out. However, when each animal was analyzed individually and grouped based on their response (sensitized or tolerant) to chronic MPH; four animals treated with 0.6 mg/kg MPH exhibited significant [$F_{1,6}=5.98, P=.03$] behavioral sensitization (Fig. 3B), the eight animals that individually exhibited no change (tolerance) in activity also showed no significant [$F_{1,14}=4.6, P=0.2$] (Fig. 3C) changes in activity as a group. Thus, the 0.6mg/kg MPH gave two responses; significantly sensitized and non significant tolerance. These differences were further evaluated by their neuronal responses to elucidate an intrinsic difference as previously hypothesized.

3.3.2 CN Neuronal (0.6 mg/kg, Table 2A)

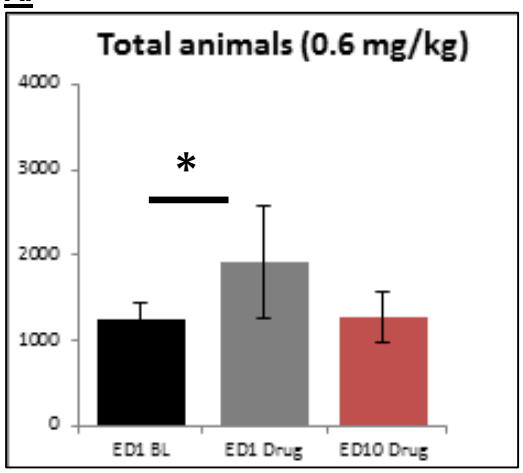
A total of 101 units were recorded from the CN following acute and chronic 0.6 mg/kg MPH administration. 80 of the units responded to acute 0.6 mg/kg MPH administration, the majority 48/80 of the CN MPH responsive units showed an increase in their neuronal activity while 32 CN units exhibited attenuation of their neuronal activity (Table 2A, under acute). When ED10 baseline neuronal activity was compared to ED1 baseline neuronal activity, all CN units exhibited significant ($p<0.05$) changes, with the majority 70/101 exhibiting a decrease in baseline neuronal activity (Table 2A, under baseline). MPH rechallenge at ED10 elicited in all CN units significant ($p<0.05$) changes in their neuronal

firing rates compared to ED1 initial MPH, with the majority of the CN units, 65/101, exhibiting an attenuation of activity (Fig. 4B) and 36/101 exhibited increased neuronal firing rates (Table 2A, under rechallenge; Fig. 4A). The balance of increased/decreased neuronal firing patterns underlies the respective lack of behavioral response to 0.6mg/kg MPH.

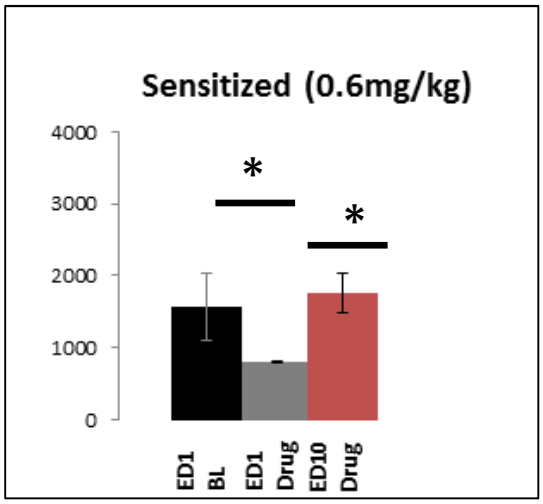
3.3.3 CN neuronal based on behavior (0.6mg/kg, Table 2B and 2C)

Table 2B and 2C summarizes the CN unit responses to 0.6mg/kg MPH based on their behavioral responses to chronic MPH (comparing the effect of MPH at ED10 to ED1). The natural log odds ratio shows that for the acute MPH exposure to 0.6 mg/kg, the animals expressing behavioral tolerance (Table 2C) were more likely, (ln .046) to show an increase in neuronal activity. This increase in neuronal responses for the behaviorally tolerant animals explains why those same animals showed a higher increase in behavioral activity at ED1 compared to those exhibiting sensitization. For the baseline neuronal activities at ED10 compared to ED1 and at ED10 rechallenge MPH administration the odd ratio showed that the behaviorally sensitized (Table 2B) were likely to show increased neuronal activity with a score of ln1.0, ln 0.322. Therefore as previously postulated the animals exhibiting behavioral sensitization did in fact show an increase in their neuronal firing patterns. However when neuronal responses were statistically compared for those exhibiting behavioral sensitization to those neuronal responses for those exhibiting behavioral tolerance, no significant differences [df 2; x^2:3.08, p=0.2] were observed. The CN is a motor structure, 0.6mg/kg MPH is considered a low dose therefore the lack of statistical difference between the two groups could potentially be due to 0.6 mg/kg having minimal effect on the CN.

3A.



3B.



3C.

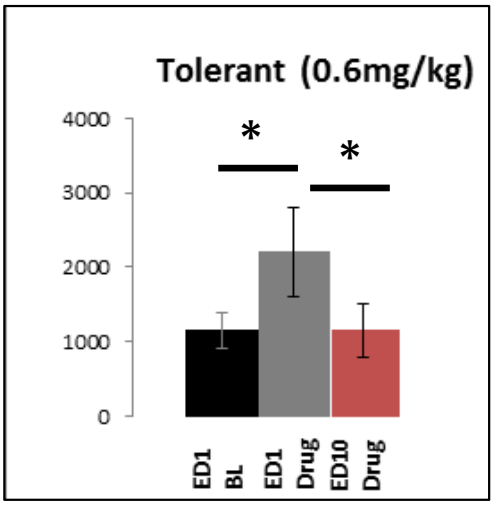


Figure 3: 3A the top histogram summarizes the overall behavioral (N=12) response to acute and chronic 0.6mg/kg MPH. The bottom histogram separates the animals based on their individual response of either behaviorally sensitized, (N=4) (3B) or behaviorally tolerant (N=8) (3C). * represents significant ($p<0.05$) differences. ED = experimental day; v= compared to; BL =baseline activity; MPH = methylphenidate.

Table 2

.

	All animals			Animals Exhibiting Behavioral Sensitization			Animals Exhibiting Behavioral tolerance		
	A) 0.6 mg/kg total (N=101)			B) 0.6mg/kg (N=32)			C) 0.6mg/kg (N=69)		
	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)
increase ↑	48	31	36	19	15	12	29	16	21
decrease ↓	32	70	65	13	17	20	19	53	48
No change ≠	21	0	0	0	0	0	21	0	0

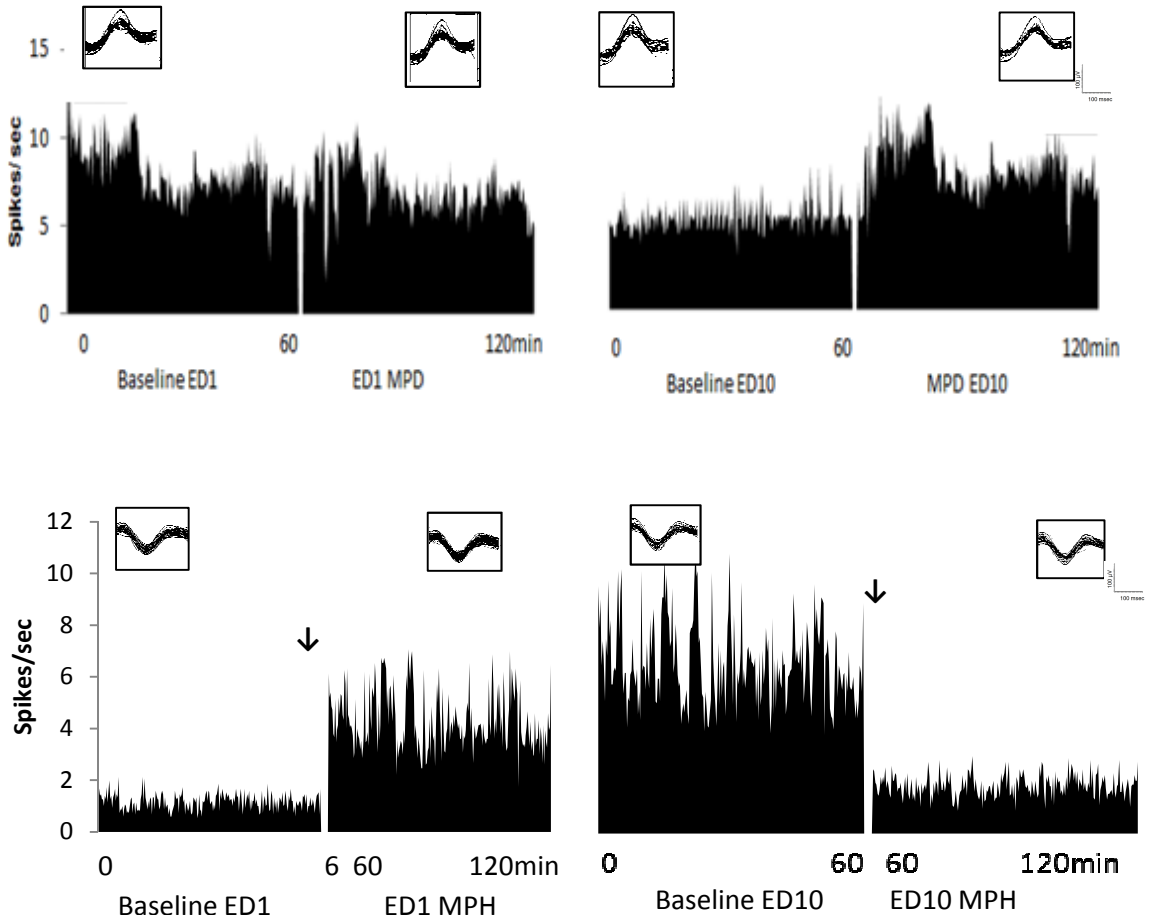


Figure 4A: The figure shows a representative firing rate histogram of a CN units exposure to 0.6mg/kg MPH. To the left shows the baseline activity at experimental day (ED) 1 baseline, followed by initial MPH exposure. To the right the first histogram shows the baseline firing activity of the CN unit at ED10, followed by MPD rechallenge activity at ED10. In the upper corners are 20 super imposed spikes during the control, initial MPH exposure at ED1 and control and rechallenge MPH exposure at ED10. The spikes show that identical spike

Figure 4B shows a representative histogram of a CN units exposure to 0.6mg/kg MPH. To the left shows the baseline activity at experimental day (ED) 1 baseline, followed by ED1 drug initial exposure. To the right the histogram shows the baseline firing activity of the CN unit at ED10 baseline, followed by decrease in firing at the rechallenge activity on ED10.

3.4 Results (2.5mg/kg)

3.4.1 Behavioral Results (2.5mg/kg)

The 2.5 mg/kg MPH group showed no significant change in activity when comparing the effect of the drug at ED10 MPH rechallenge to MPH given at ED1 (Fig. 5A). This lack of response is due to some individual animals increasing their activity and some individual animals decreasing their behavioral activity, so when grouped together their activity averages to an amount close to baseline. Analyzed individually, seven animals exhibited behavioral sensitization to 2.5mg/kg MPH, and significant [$F_{1,7}=4.74$, $P=0.01$] differences in behavioral activity at ED10 rechallenge compared to ED1 acute injection (Fig. 5B). This significant increase in behavioral activity is expected when you look at table 3B, under rechallenge, nearly all units responded majority of excitatory responses are observed. Five animals exhibited behavioral tolerance when analyzed individually however as a group they did not exhibit significant [$F_{1,8}=5.31$, $P=0.13$] differences in locomotor activity(Fig. 5C). Table 3C under baseline shows that the majority of responses are inhibitory, therefore when the rechallenge was administered less units responded with increased activity. This inhibition in neuronal firing rates lead to a reduced behavioral response compared to the day one drug. Similar to the 0.6mg/kg dose, 2.5mg/kg MPH gave two responses: significantly sensitized and not significantly tolerant. Furthermore as above mentioned these responses show a correlation to the respective neuronal responses, further detailed below.

3.4.2 CN Neuronal (2.5 mg/kg, Table 3A)

A total of 117 CN units were recorded following acute and chronic 2.5 mg/kg MPH administration (Table 3). 66/117 of the CN units responded with significant increases in neuronal activity ($p<0.05$) to acute 2.5 mg/kg MPH administration (Table 3A, under

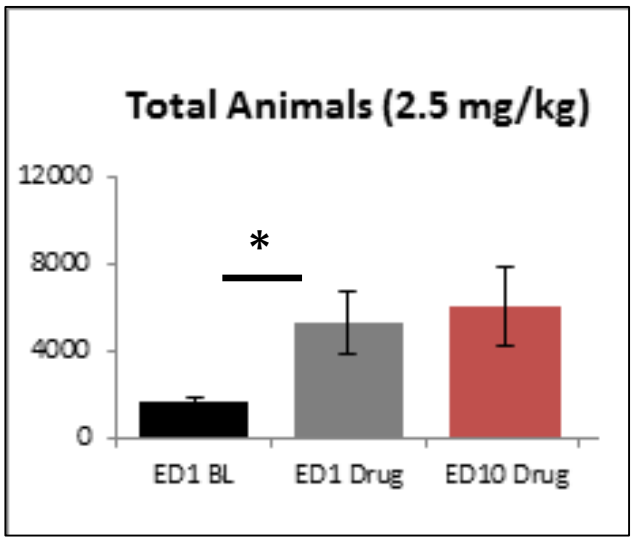
acute). This increase in neuronal responses elucidates why a significant increase in behavioral activity was seen (Table 3A). ED10 baseline neuronal activity compared to ED1 baseline showed that 116/117 CN units exhibited significant ($p<0.05$) change in their baseline neuronal firing patterns, with the majority, 78/116, decreasing their neuronal firing rate at ED10 compared to ED1 (Table 3A, under baseline; Fig. 6A). MPH rechallenge at ED10 resulted in 106 of CN units responding with significant ($p<0.05$) changes in their neuronal firing rate when compared to the effect of the acute 2.5 mg/kg administration. Of the 106 CN units responding to MPH, 88 exhibited an increase in their neuronal firing rates (Table 3A, under rechallenge; fig. 6B); whereas the other 18/106 decreased their neuronal firing rates.

3.4.3 CN neuronal based on behavior (2.5 mg/kg, Table 3B and 3C)

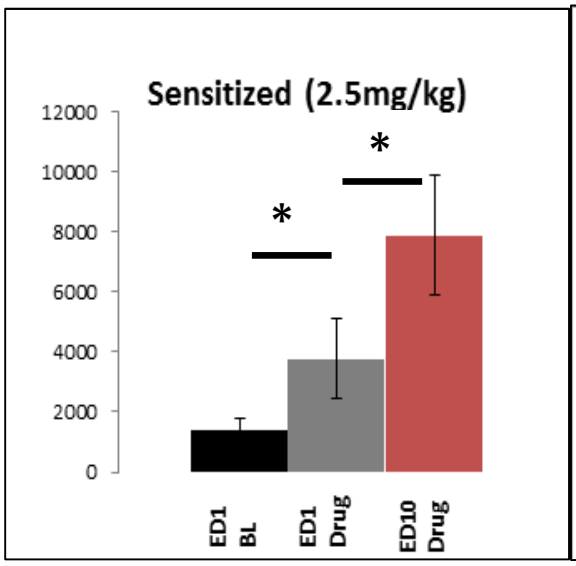
Table 3B and C summarizes the CN unit responding to 2.5mg/kg based on their behavioral responses to chronic MPH. The natural log odds ratio shows that for the acute exposure to 2.5mg/kg MPH the animals expressing behavioral tolerance were more likely, (ln 2.7) to show an increase in neuronal activity. This increase in neuronal activity for animals expressing behavioral tolerance is also seen in their acute behavioral activity. They show a higher increase in activity following initial drug exposure versus the sensitized animals. For the baseline neuronal activities at ED10 compared to ED1 the behaviorally sensitized were likely to show increased neuronal activity with a score of ln 1.47, while at ED10 rechallenge MPH administration the odd ratio showed that the behaviorally tolerant animals were more likely to show an increase in activity ln 0.362. When a statistical comparison was made from the neuronal responses of those exhibiting behavioral sensitization to the neuronal responses of those exhibiting behavioral tolerance, as hypothesized, a significant difference was observed [df 2: x^2:30.41, p=0.001]. This difference between the two groups implies that there are

intrinsic differences within animals that can cause them to respond differently to the same dose of MPH.

5A.



5B.



5C.

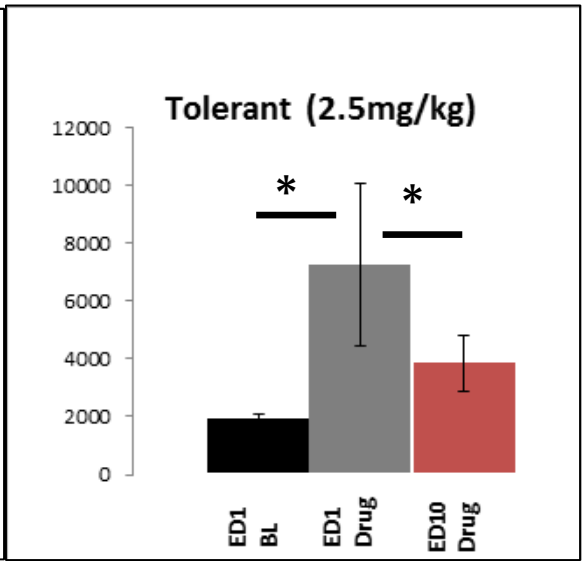


Figure 5A top histogram summarizes the overall (N=12) behavioral response to acute and chronic 2.5mg/kg MPH. The bottom histogram separates the animals based on their individual response of either behaviorally sensitized (N=7)(left 5B) or behaviorally tolerant (N=5)(right 5C). * represents significant ($p<0.05$) differences. ED = experimental day; v= compared to; BL =baseline activity; MPH = methylphenidate.

Table 3

	All animals			Animals Exhibiting Behavioral Sensitization			Animals Exhibiting Behavioral tolerance		
	A) 2.5 mg/kg total B) N=117			C) 2.5mg/kg D) N=66			C) 2.5 mg/kg N=51		
	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)
increase ↑	66	38	88	23	30	46	43	8	42
decrease ↓	31	78	18	28	35	16	3	43	10
No change ≠	20	1	11	15	0	9	5	1	2

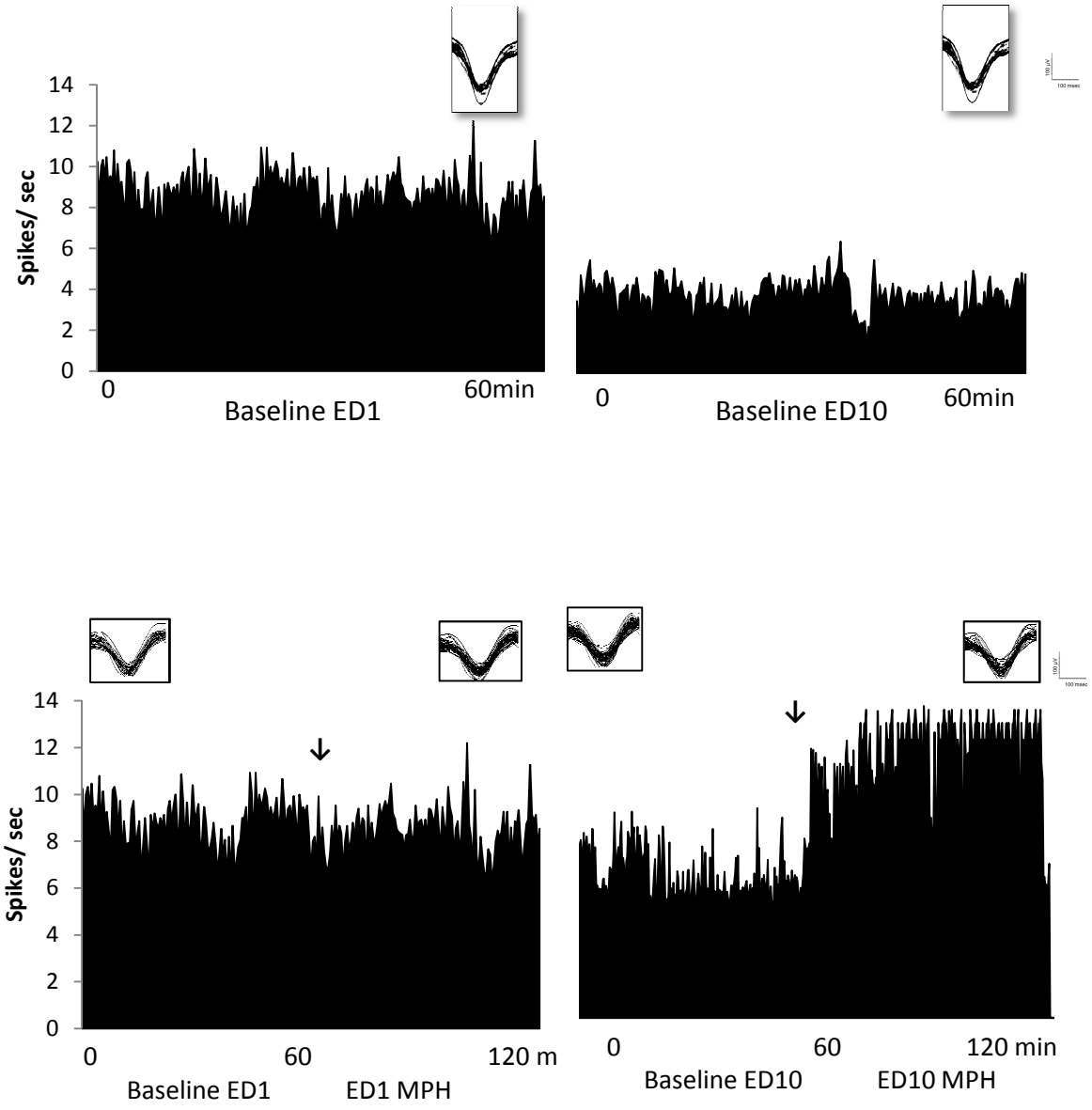


Figure 6A: The figure is a representative histogram showing the baseline activity expressed for the majority of CN units at ED1 (left side) with the baseline at ED10 (to the right). This CN unit showed significantly decreased neuronal firing rates compared to the initial baseline firing activity prior to 2.5 mg/kg MPD administration.
Figure 6B: The figure shows a representative histogram of CN firing rates that did not respond to initial MPH injection (to the left) however at ED10 rechallenge (histogram to right) the MPH exposure elicited an increased in this CN unit's neuronal firing response, exhibiting neurophysiological sensitization.

3.5 Results (10.0mg/kg)

3.5.1 Behavioral Results (10.0mg/kg)

10.0mg/kg MPH administration resulted in significant increased behavioral activity at ED1 initial exposure and following MPH rechallenge at ED10 (Fig. 7A). Analyzed individually, two animals exhibited behavioral sensitization, and when statistical evaluated, they did not exhibit significant [$F_{1,2}=18.53$, $P=0.9$] (Fig. 7B) differences, due to the small N value. Ten animals exhibited behavioral tolerance, and exhibited significant [$F_{1,18}=4.41$, $P=0.01$] attenuation post MPH rechallenge at ED10 when compared to MPH exposure at ED1 (Fig. 7C).

This data shows that the same dose of chronic 10.0mg/kg MPH in some animal's elicited behavioral sensitization and in others behavioral tolerance.

3.5.2 CN neuronal Results (10.0mg/kg, Table 4)

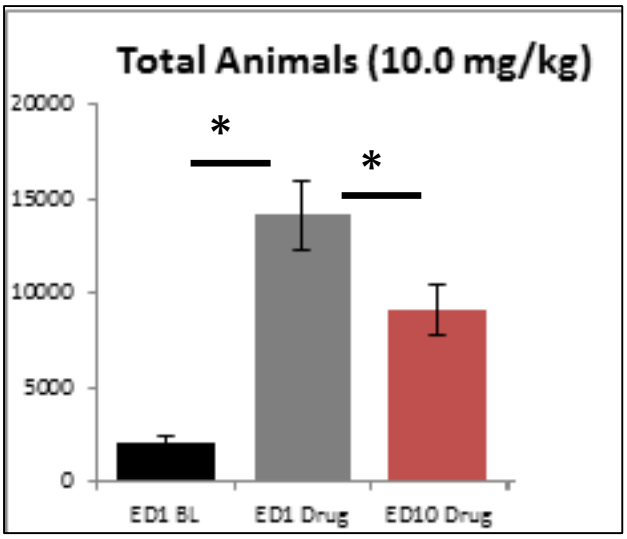
A total of 135 units were recorded from the CN following acute and chronic 10.0 mg/kg MPH (Table 4A). The majority of the CN units, 121/135 responded with significant ($p<0.05$) changes in neuronal activity following acute 10.0 mg/kg MPH administration (Table 4A, under acute). Of the 121 CN units responding to acute MPH, 90 exhibited significant ($p<0.05$) increase in their neuronal firing rate activity compared to their saline baseline activity. This overall increased neuronal firing pattern in the CN elucidates why the animals as a group showed significant increased locomotor activity. At ED10, all of the CN units exhibited significant ($p<0.05$) changes in their ED10 baseline neuronal firing compared to ED1 baseline (Table 4A, under baseline), with the majority of them, 101/135, exhibited decreases in their neuronal firing rates. All CN units responded with significant ($p<0.05$) changes in their firing rates to MPH rechallenge at ED10 when

compared to acute MPH administration, the majority of the CN units, (98/135), exhibiting increased neuronal activity (Table 4A, under rechallenge; Fig. 8)

3.5.3 CN neuronal based on behavior (10.0mg/kg, Table 4B and 4C)

Table 4B and C summarizes the CN unit responding to 10.0mg/kg based on their behavioral responses to chronic MPH. The natural log odds ratio shows that for 10.0m/kg the animals expressing behavioral sensitization were more likely, (ln 3, ln 1.7) to show an increase in neuronal activity during initial (acute) MPH exposure and at ED10 baseline compared to ED1 baseline. MPH rechallenge at ED10 administration showed that the behaviorally tolerant animals were more likely to show an increase in activity ln 0.32. Furthermore when the CN neuronal population of those exhibiting behavioral tolerance was compared to the neuronal activity of those exhibiting behavioral sensitization a significant [df 2: x^2:13.19; p=0.001) difference was observed. Accordingly, proving our hypothesis that there are intrinsic differences in responses to MPH exposure that lead to different behavioral responses.

7A.



7B. **7C.**

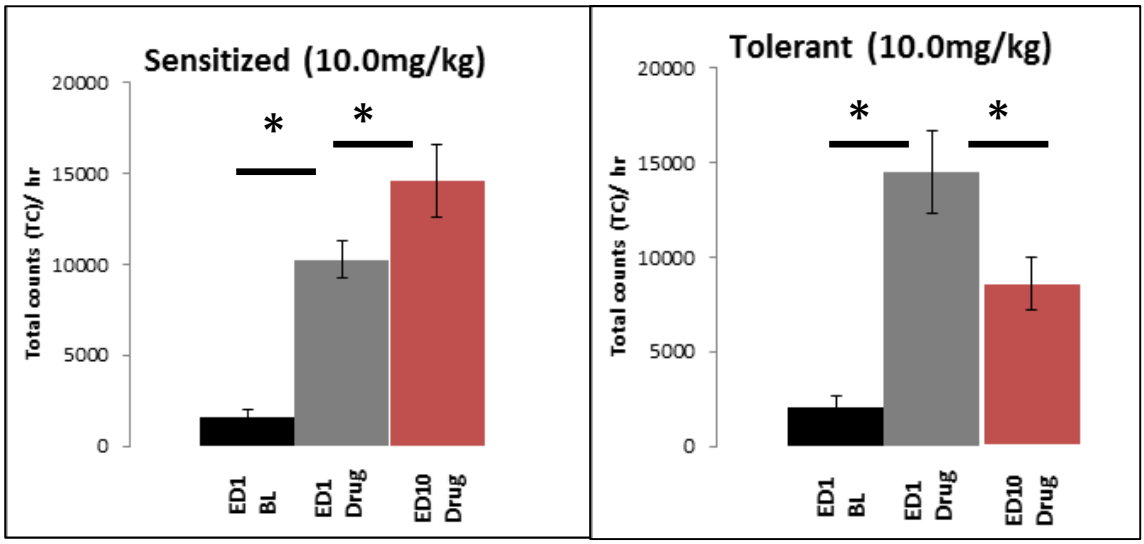


Figure 7: 7A top histogram summarizes the overall (N=12) behavioral response to acute and chronic 10.0mg/kg MPH. The bottom histogram separates the animals based on their individual response of either behaviorally sensitized (N=2) (left 7B) or behaviorally tolerant (N=10)(right 7C). * represents significant ($p<0.05$) differences. ED = experimental day; v= compared to; BL =baseline activity; MPH = methylphenidate.

Table 4:

	All animals			Animals Exhibiting Behavioral Sensitization			Animals Exhibiting Behavioral tolerance		
	A) 10.0mg/kg total (N=135)			B) 10.0mg/kg (N=22)			C) 10.0mg/kg (N=113)		
	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)	Acute ED1	Baseline (ED10 to ED1)	Rechallenge (ED10)
increase ↑	90	34	98	22	15	21	68	19	77
decrease ↓	31	101	37	0	12	10	31	89	27
No change ≠	14	0	0	0	0	0	14	0	0

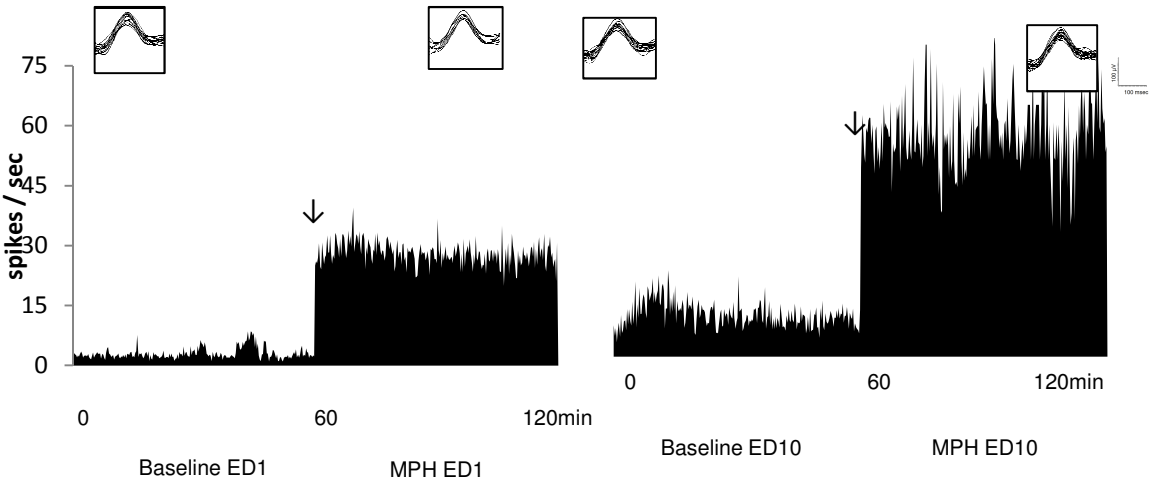


Figure 8: The figure shows a representative histogram of CN unit firing rate following saline and MPH (10.0 mg/kg) at experimental day 1 (ED1) and ED10. In the left side are the recordings of ED1 following saline and by MPH 10.0 mg/kg acute injection. The drug elicited an increase in neuronal firing rates. In the right part of the figure is the histogram of the baseline activity and the activity following 10.0 mg/kg MPH at ED10. The histogram shows that at ED10 the baseline activity was evaluated compared to ED1 baseline and following rechallenge of 10.0 mg/kg MPH exposure a further increase in firing patterns were observed compared to the activity obtained at ED1. This further increase in neuronal firing rate at ED10 can be interpreted as the unit is expressing neurophysiological sensitization.

5 Statistical comparisons

5.1 CN units based on behavior (0.6, 2.5 and 10.0mg/kg MPH) controlling for dose

Based on the separations of the neuronal activity for the animals behavioral response of either tolerant or sensitized, a log linear model was used to statistical compare the relationship between dose, behavior and firing patterns for each group (0.6, 2.5 and 10.0 mg/kg MPH) with significance set at ($P<0.05$). The results shows that the response pattern of CN units recorded from animals that exhibited behavioral sensitization to 0.6 mg/kg MPH dose were not significantly (df 2; X^2 :3.08; $p = 0.2$) different from the CN units firing patterns recorded from animals that exhibited behavioral tolerance. The response of CN units to MPH recorded from animals that exhibited behavioral sensitization following 2.5 and 10.0 mg/kg MPH were significantly (df 2: X^2 :30.41; $p = 0.001$; df 2: X^2 :13.19; $p = 0.001$) different from the CN units which were recorded from animals exhibiting behavioral sensitization. This statistical comparisons show the importance of evaluating the effect of chronic drug exposure on neurophysiological events based on animal behavioral response to repetitive drug exposure.

6 Discussion

Although MPH is a commonly prescribed drug therapy, its neurochemical mechanism is still unclear. Several studies have shown that MPH acts similarly to other psychostimulants, such as cocaine and methamphetamine (Gately et al., 1999, Patrick and Markowitz, 1997; Teo et al., 2003). Merchant et al., demonstrated that while most psychostimulants affect both the limbic and basal ganglia circuitry, MPH appears to act selectively on the basal ganglia circuit, including the caudate nucleus (CN). MPH is known to indirectly increase synaptic levels of dopamine (DA) by blocking DA re-uptake into the presynaptic terminal, thereby increasing the levels of extracellular DA in the synaptic cleft (Diaz et al., 2004; Kuczenski and Segal 1997; Nestler 2001; Volkow et al 1995). In addition to the neurochemical mechanism of MPH being unclear, it is also not known how MPH might influence behavioral responses, such as sensitization or tolerance. Previous studies have shown conflicting results as to whether certain doses of MPH cause behavioral sensitization or behavioral tolerance (Yang et al., 2006). We have postulated that MPH causes behavioral sensitization in some animals and behavioral tolerance in others, even when the animals have been administered the same dose of the drug. This, perhaps, may explain previous findings that yielded conflicting results. In our studies using electrophysiological methodologies, we demonstrated that acute and chronic MPH (2.5mg/kg) causes an increase in CN response patterns in some animals, while decreasing CN response patterns in others (Claussen and Dafny 2012). However, there are currently no studies that combine the simultaneously recorded behavioral activities and neuronal responses following MPH administration. We endeavored to compare behavioral activities to neuronal responses in order to aid in further understanding their relationship after either acute or chronic MPH exposure. We investigated this relationship using freely behaving rats as a model, as their behavioral

and neuronal responses to MPH are similar to those responses observed in humans. The results obtained from these investigations will aid in better understanding the mechanism(s) of how acute and chronic MPH exposure may cause behavioral sensitization or tolerance. We hypothesized that, following chronic MPH exposure, the CN neuronal population activity from animals exhibiting behavioral sensitization would respond differently than the CN units recorded from animals exhibiting behavioral tolerance. The first specific aim of this project was to individually analyze the animals' behavioral responses to acute and chronic MPH exposure in varying doses (0.6, 2.5 and 10.0mg/kg) to determine if the animals exhibited behavioral sensitization or behavioral tolerance. The second specific aim involved recording all of the CN neuronal responses with no relation to the animals' behavior. Finally, in the third specific aim we sorted the CN neuronal responses from animals into two groups: 1) those that exhibited behavioral sensitization and 2) those that exhibited behavioral tolerance. We then determined if there was a difference between these two groups' electrophysiological responses. To accomplish all of these aims, we simultaneously recorded the neuronal and behavioral activity of animals exposed to single and repetitive doses (0.6, 2.5 and 10.0mg/kg) of MPH.

Overall, we observed that MPH, in fact, caused behavioral sensitization in some animals and behavioral tolerance in others, even when these animals were administered the same dose of the drug. We also demonstrated that there were differences in CN neuronal responses to acute and chronic MPH exposure that were independent of their behavioral responses. Finally, we observed that these different responses were statistically significant in regard to the CN activity between animals that exhibited behavioral sensitization and those that exhibited behavioral tolerance when administered specific doses of MPH. Taken together, the results from this study support our

hypothesis that the behavioral responses following acute and chronic MPH exposure are related to neuronal activity in the CN.

Behavioral Responses to Varying Doses of MPH

Previous experiments have reported that both behavioral sensitization and tolerance can occur when an animal is given the same dose of MPH (Yang et al., 2006a; Yang et al., 2006b). Therefore, we investigated the behavioral responses, measured as locomotor activity, in all animals, initially as a group, and then individually to determine if individual responses were observed using the same dose of MPH. The saline control group showed no changes in locomotor activity. This suggests that animal handling for the duration of the study had no significant effects on their behavioral responses. We next investigated how acute exposure to MPH altered behavioral responses. Animals that were acutely exposed to MPH exhibited an increase in locomotor activity in a dose-dependent manner. We also examined how MPH elicited a chronic effect by re-challenging the animals at a later time after cessation of acute exposure. The behavioral responses observed on re-challenge were then compared to responses recorded during the first day of initial acute exposure to MPH. This comparison was performed for each dose of MPH (0.6, 2.5, and 10mg/kg) that was administered. Interestingly, upon re-challenge, some animals exhibited decreased locomotor activity, while others exhibited greatly increased locomotor activity. While there was a dichotomy in behavioral responses upon re-challenge, there were no significant differences in the combined locomotor activity for all of the animals re-challenged with MPH, as there was a cancelling out effect during statistical analyses. Based on this observation, animals were subsequently analyzed individually and then separated into two groups: behaviorally sensitized and behaviorally tolerant. When the animals were separated based on their behavioral responses, the animals exhibiting behavioral tolerance to chronic MPH showed both a larger and a dose-dependent

increase in response to the initial (acute) MPH exposure when compared to the behaviorally sensitized animals' responses (Fig. 3B&C, 5B&C, 7B&C). This was true for all doses administered. The opposite was seen following MPH re-challenge; the sensitized groups showed significant increases in locomotor activity upon re-challenge compared to their initial response, and the tolerant group showed significantly decreased locomotor activity. In conclusion, it is evident that MPH can elicit either behavioral sensitization or tolerance that is independent of the dose administered and that the varying levels of behavioral responses following acute MPH exposure may predict behavioral responses to chronic MPH exposure.

CN Neuronal Responses to Acute MPH Exposure

Saline administration (N=10) did not have an effect on CN neuronal activity, suggesting that the handling of the animals also had no effect. The 0.6, 2.5 and 10.0 mg/kg MPH exposures altered the majority of CN neuronal activity. Some CN units responded with increased neuronal activity, while others responded with attenuation of neuronal responses or were nonresponsive. The CN is comprised of approximately 90% medium spiny neurons (MSNs); there are two types of MSNs which express two different DA receptors: D1- and D2-like receptors. The D1 receptors act on the direct output pathway that connects to the thalamus and other cortical areas by way of the globus pallidus internal (GPi) and substantia nigra pars reticulars (SNPr) (Hummel & Unterwald, 2002). Acute MPH administration is believed to stimulate C-Fos mRNA expression in the direct pathway, which increases D1 receptor-mediated signal transduction and causes an overall excitatory response for all doses (Yano and Steiner 2005; Chase et al., 2005). Therefore, D1 receptors have an excitatory effect when activated. The indirect pathway is regulated by D2 receptors with connections that project to the globus pallidus external (GPe), which then project to the subthalamic nucleus and causes an overall inhibition of activity (Henry & White, 1995). Extracellular recordings from permanent electrodes

previously implanted into the CN are not able to identify the exact origin of the recordings or whether they are comprised of neurons expressing a majority of D1 or D2 DA receptors. Therefore, it has been postulated that when excitatory responses are observed, across all doses, that this is due to D1 receptor activity; whereas, when inhibitory responses are observed, D2 receptors are involved. It is known that the MSN are usually quiet and do not exhibit any spontaneous activity, therefore, when no response is observed, it may be because of a week stimulus that is unable to activate the cell.

CN Neuronal Responses to Chronic MPH Exposure

The saline control group showed no changes in neuronal firing patterns at experiment al 10 (ED10) when compared to their activity at ED1, confirming that handling of the animals and multiple injections had no effect on the CN neuronal responses. When the CN units were exposed to a rechallenge (chronic) MPH, following 6 days of administration and 3 days washout, we observed three types of neurophysiological sensitization and three types of neurophysiological tolerance. Neurophysiological sensitization was considered if the neuronal population at ED1 was increased compared to the ED1 baseline neuronal responses and then a further increase at ED10 compared to ED1 neuronal activity or similarly a decrease at ED1 and ED10, also when no changes were observed at ED1 drug compared to ED1 baseline neuronal activity followed by an increase at ED10 drug rechallenge when compared to ED1 drug neuronal responses. Neurophysiological tolerance was observed when neuronal responses at ED1 were increased following acute drug exposure followed by a decrease in neuronal firing rates at ED10 drug compared to ED1 drug or oppositely a decreased followed by increase, also when there were no response at ED1 drug however a decrease in neuronal firing was at ED10 compared to ED1 drug was observed. When the CN neuronal responses were analyzed independently of the

animals' locomotor behavior, the CN units responded initially to 0.6mg/kg MPH, with a majority showing an increase at ED1. However, at ED10, the majority of the CN neuronal responses elicited an opposite response by exhibiting a decrease in neuronal activity. An increase in neuronal activity following initial MPH exposure and then a decrease following chronic MPH exposure can be interpreted as neurophysiological tolerance. The 2.5 and 10.0 mg/kg MPH exposures elicited increases in CN neuronal activity and revealed an overall greater rise in activity at ED10 following re-challenge with MPH compared to their acute response at ED1. This increase in CN neuronal activity at ED1 followed by a further increase at ED10 re-challenge with MPH can be interpreted as neurophysiological sensitization. There were also CN units that exhibited a decrease in neuronal activity at ED1 followed by a further decrease in neuronal firing rates, thereby exhibiting neurophysiological sensitization. There were some units for all doses (0.6, 2.5 and 10.0mg/kg) that did not respond following initial MPH exposure but did elicit an increase in activity at ED10 re-challenge. Therefore, these CN units exhibited neurophysiological sensitization. Some CN units responded to initial MPH exposure at ED1 but were nonresponsive at ED10 re-challenge. These CN units were classified as expressing neurophysiological tolerance. Based on these experiments it is clear that there are different responses occurring at different days and across different doses. Therefore, as hypothesized we further separated the animals based on their behavioral response and compared the CN neuronal activity. First, however, I will attempt to explain the reasons for why there are differing results across doses and days.

Nikolaus et al. (2011) used *in vivo* experiments to measure the level of D2 inhibitory receptors in the striatum of both baboons and rats and reported that there was a decrease in the amount of D2 receptors when animals were exposed to increasing doses of MPH. Also, chronic MPH exposure has been shown to increase the number of ΔFosB positive MSN D1, ΔFosB is a transcription factor that is shown to increase in

concentration on the medium spiny neurons (MSN) that express D1 like Dopamine (DA) receptors (Nikolaus et al., 2011). The above reports could explain why the majority of CN units exhibited increased firing rates at ED10 when exposed to 10.0 mg/kg of MPH. For those animals administered 0.6 mg/kg of MPH, we found that the majority of CN units demonstrated either no change in activity or a decrease in neuronal activity, which contradicts what was found in previous studies. This type of activity may result from more D2 receptor availability, which would mediate inhibition.

The phenomenon of sensitization can be examined by looking at gene expression and neuropeptides levels (Alburges et al., 2011; Brandon and Steiner, 2003; Nikolaus et al., 2011; Yano and Steiner, 2005). The differential behavioral and neuronal responses following MPH exposure that we observed in this study may be explained by two possible mechanisms. One of these possible mechanisms is that chronic MPH ultimately increases the expression of ΔFosB leading to sensitization. The striatum is believed to be involved in psychostimulant induced gene regulation due to overstimulation of DA receptors (Yano and Steiner 2005). It has been demonstrated in some animals that chronic exposure to psychostimulants results in an increase in the density of dendrite spines in MSNs that express D1 DA receptors (Kim et al., 2009) as well as the transcription factor ΔFosB (Robison and Nestler, 2011). Psychostimulant induced overexpression of ΔFosB has been linked to the exhibition of a sensitized state in animal models (Kim et al., 2009).It has also been shown that repetitive exposure to psychostimulants results in super sensitivity of D1-like DA receptors in the nucleus accumbens (NAc), which leads to the expression of the sensitized response (Wolf, 2002; Wolf et al., 2003; 2004). This increase in the upregulation of ΔFosB, which causes units to express a sensitized state, explains the results of our current study. We found that when animals were exposed to either 2.5 or 10.0 mg/kg of MPH, the majority of CN neuronal units showed a greater increase in firing rates and therefore exhibit

neurophysiological sensitization. ΔFosB is generated by alternative splicing and lacks the two degron domains present in the full-length protein (Kelz et al., 1999). Without these two domains, previous studies have shown there is a four-fold increase in protein stability (Robinson and Nestler, 2011). In addition, using both *in vitro* and *in vivo* methodologies, studies have demonstrated that ΔFosB is phosphorylated, which further stabilizes the protein by approximately ten-fold (Kelz et al., 1999). This intrinsic protein stability provides a molecular mechanism by which psychostimulant changes in gene expression can persist long after the administration of MPH is ceased (Robinson and Nestler 2011). Another possible mechanism to explain the results of this study is that the alterations in the neuronal activity that we observed may be due to the structural plasticity of the neurophil (soma and dendrite spine morphology) in the brain reward circuitry as a result of repetitive MPH exposure (Dietz et al., 2009; Robinson and Kolb, 2004; Russo et al., 2010). Increased activation of D1-like DA receptors and increases in neurophil density may also result in neurophysiological sensitization. Further studies are needed to be performed to further dissect which mechanisms may be involved in MPH modulation of behavioral responses.

Previously, it has been shown that overexpression of cAMP- responsive element binding protein (CREB) occurs both in the D1 and D2-like DA receptors and decreases the rewarding effects of psychostimulants, thereby causing neurophysiological tolerance (Robinson and Nestler, 2011). Activation of D2-like DA receptors and decreases in neurophil density result in attenuated MPH-mediated effects that can be explained as neurophysiological tolerance. Moreover, Madsen et al. (2012) reported that mice deficient in striatal CREB1, a transcription factor that is known to initiate downstream genes and is specific to MSNs of the dorsal striatum and CREB-binding protein (CBP)-, created a heightened sensitivity to psychostimulants by causing a prolonged to them.

Comparison of CN neuronal activity in animals exhibiting behavioral sensitization to those expressing behavioral tolerance after chronic MPH exposure

The CN neuronal responses recorded following MPH exposure were evaluated based on the animals' behavioral responses to chronic MPH administration. Animals were then classified based on their behavioral response and grouped as either behaviorally sensitized or behaviorally tolerant. Finally, their neuronal activity was evaluated based on their separate behavioral responses. First, we applied statistical analyses to CN neuronal activities recorded from animals exhibiting behavioral sensitization and those exhibiting behavioral tolerance to determine if a significant difference was found in their responses to acute and chronic MPH .These statistical analyses were also performed to compare groups that were acutely and chronically exposed to MPH.

In the group of animals exposed to 0.6 mg/kg of MPH, there were no significant differences in the neuronal responses for those exhibiting behavioral sensitization compared to those exhibiting behavioral tolerance. Furthermore, when investigating neuronal responses to better understand behavioral responses, no significant correlations were observed. This is explained by the fact that the CN is a motor circuit, and 0.6 mg/kg of MPH is considered a low dose that does not modify the motor responses; therefore, this response was expected.

Significant differences were found in CN neuronal populations exposed to 2.5 mg/kg of MPH when results were separated based on the animals' behavioral response of either sensitization or tolerance. The CN neuronal responses to acute 2.5 mg/kg MPH exposure in animals exhibiting behavioral sensitization showed a combination of increases and decreases in neuronal activity, whereas the majority of those animals that exhibited behavioral tolerance showed an increase in their neuronal responses. This may be explained by the fact that the tolerant animals showed a heightened behavioral

response (Table 3C, Fig. 5C) to the initial (acute) drug exposure compared to sensitized animals (Table 3B, Fig. 5B). This same correlation phenomenon was seen when animals were re-challenged with 2.5 mg/kg MPH at day 10 after the initial administration of MPH had ceased. The behaviorally sensitized animals that were re-challenged with MPH exhibited more CN units that increased their firing rates, resulting in increased behavioral responses (Table 3B, Fig. 5B).The behaviorally tolerant animals showed a significant decrease in their baseline firing rate coupled with a mixture of neuronal response types (Table 3C, Fig. 5C).

The CN neuronal responses to 10.0 mg/kg MPH were also found to be statistically different when separated based on the animals' behavioral responses of sensitization or tolerance. When comparing the CN neuronal activity to the respective behavioral responses, similar results were observed as those seen when animals were administered the 2.5 mg/kg MPH dose. The CN neuronal responses to acute 10.0 mg/kg MPH, from animals exhibiting behavioral sensitization, exhibited a combination of increases and decreases, whereas the neuronal responses from the majority of those exhibiting behavioral tolerance were increased. This could be because the tolerant animals showed a heightened behavioral response (Table 4C, Fig. 7C) to the initial (acute) drug exposure compared to the sensitized animals (Table 4B, Fig. 7B), similar to what was observed at the lower dose of 2.5 mg/kg of MPH. This same correlation phenomenon was observed when animals were re-challenged at ED10 with 10.0 mg/kg MPH, as the behaviorally sensitized animals expressed more units that increased their firing rates, resulting in increased behavioral responses (Table 3B, Fig. 7B). The behaviorally tolerant animals showed a significant decrease in their baseline firing rates and a mixture of neuronal responses (Table 3C, Fig. 7C), which, again, is similar to what was observed when the animals received a lower dose of MPH.

In conclusion, experiments in which both neuronal and behavioral responses were examined using an MPH dose response protocol, it was found that MPH can elicit different locomotor and neuronal responses to acute and chronic MPH exposures. Also, as hypothesized, we observed that there is, in fact, a statistically significant difference in the firing patterns of CN neurons when animals are grouped based on their behavioral responses to higher doses (2.5 and 10.0 mg/kg) of MPH. Moreover, correlations were observed when we compared the neuronal responses to the behavioral responses. Understanding the differences in animals' reactions to chronic exposure of MPH, in terms of both the behavioral and neuronal aspects, following chronic MPH exposure is imperative in future research for analyzing the mechanism by which MPH acts on neuronal events based on behavioral responses. MPH acts predominately on the DA receptors, and activation of D1 and D2 DA receptors is known to elicit differing responses, further studies using specific DA antagonists are needed to investigate their specific roles in the behavioral and neuronal sensitization/tolerance responses.

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Vita

Catherine Claussen earned her Bachelor of Science in Psychology Major at the University of Houston – Clear Lake. After undergrads, she initially enrolled as a non-degree seeking student, she became a degree seeking student at the Graduate School of Biomedical Sciences (GSBS), University of Texas Health Science Center at Houston in August 2011. While a student at GSBS, she presented her findings at Society for Neuroscience (Washington DC, 2011; New Orleans 2012) and the 8th annual International Brain Research Organization (Florence, Italy 2011) and The Federation of European Neurosciences (Barcelona, Spain 2012).

Claussen3214@yahoo.com