THE EFFECTS OF CHRONIC PSYCHOSTIMULANT EXPOSURE ON MORPHINE-INDUCED ANTINOCICEPTION AND TOLERANCE

BY

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MICHELLE CATHERINE CYR WIDOLFF find it satisfactory and recommend that it be accepted.

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THE EFFECTS OF CHRONIC PSYCHOSTIMULANT EXPOSURE ON MORPHINE-INDUCED ANTINOCICEPTION AND TOLERANCE

Abstract

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Pre-weanling methylphenidate (MPH) exposure enhances systemic morphine-induced antinociception and tolerance in adult rats. This finding raises questions about the brain area(s) involved, the importance of developmental period during pretreatment, and whether other psychostimulants would produce similar effects. The purpose of the current studies was to address these unanswered questions. Four studies were conducted. Study 1 determined the potential involvement of the ventrolateral periaqueductal gray (vPAG) in MPH’s long term enhancement of morphine-induced antinociception and tolerance. It appears that the vPAG is not exclusively involved, since the results observed in the previously mentioned systemic study were not replicated when morphine was microinjected into the vPAG. Study 2 helped determine methamphetamine (METH) doses for subsequent studies that were high enough to produce an acute behavioral effect similar to our MPH dose, but different from saline controls. Study 3 assessed the effects of chronic periadolescent MPH and METH exposure on adult morphine-
induced antinociception and tolerance. Both age at pretreatment and psychostimulant compound used may mediate chronic MPH-induced enhancement of morphine-induced antinociception and tolerance, since all animals showed similar acute morphine-induced antinociception and only METH 3 mg/kg pretreated animals showed enhanced tolerance. Study 4 investigated whether morphine-induced antinociception and tolerance were altered following chronic adult exposure to MPH and METH. Different from pre-weanling and periadolescent animals, adult exposure to METH and MPH led to an acute reduction in morphine antinociceptive potency. In summary, the results demonstrate that (1) a brain area besides the vPAG must be involved in the enhancement of morphine-induced antinociception and tolerance observed following chronic pre-weanling MPH exposure, (2) the developmental period during which chronic psychostimulant exposure occurs impacts the degree and direction of effects on later morphine-induced antinociception and tolerance, and (3) different psychostimulant compounds may produce differing effects on morphine tolerance when exposure occurs during the periadolescent period of ontogeny.
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Dedication

From rockstar to neuroscientist my parents always encouraged me to follow my dreams. For their unending love and support I dedicate this dissertation to my Mom and Dad. Additionally, for his patience and love during my time as a graduate student, I also dedicate this dissertation to my husband, Matt.
CHAPTER ONE:
GENERAL INTRODUCTION

Attention-Deficit/Hyperactivity Disorder (ADHD) is the most diagnosed childhood psychiatric disorder, with estimated prevalence rates within the United States ranging from 3-7% of all school-age children (Diagnostic and statistical manual of mental disorders, 1994). Over 90% of these children are prescribed psychostimulants such as methylphenidate (MPH) (Kimko, Cross, & Abernethy, 1999). Moreover, continued treatment into adulthood and adult diagnosis of ADHD has become more common with prevalence rates in adults estimated to be 1-4% (de Graaf et al., 2008; Fayyad et al., 2007; Kessler et al., 2006). As with children, a large percentage of ADHD diagnosed adults are prescribed psychostimulants, with an average 0.8% of all adults currently undergoing pharmacological treatment (Castle, Aubert, Verbrugge, Khalid, & Epstein, 2007). Although MPH reduces ADHD symptomatology, little is known about the long term consequences of exposure to this compound. That said, research in rodents suggests that MPH treatment leads to a variety of long term behavioral alterations, including changes in drug reward (For review see Carlezon & Konradi, 2004) and drug-induced antinociception (Cyr & Morgan, 2009). These findings raise concerns about the long term safety of MPH treatment (Kollins, MacDonald, & Rush, 2001). This concern has been amplified in the past decade due to the increasing occurrence of MPH use in preschool-aged children (Vitiello, 2001; Zito et al., 2000) and children who do not meet the criteria for ADHD diagnosis (Marshall, 2000). Thus, a better understanding of the long term consequences of psychostimulant exposure is needed.
Effects of Acute Psychostimulant Administration

In addition to psychostimulant use in clinical populations, psychostimulants are frequently abused illicitly. In particular cocaine and amphetamines are regularly abused by humans (Substance Abuse and Mental Health Services Administration, 2007) and abuse of MPH is fairly common among high school and college students (Bogle & Smith, 2009). The common illicit abuse of psychostimulants is thought to result from their ability to directly activate the mesolimbic dopamine (DA) reward system. This system consists of DA projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) (See Figure 1 for circuitry diagram). Within the circuitry, psychostimulant administration results in excitation of excitatory DA neurons and inhibition of inhibitory γ-Aminobutyric acid (GABA) neurons, resulting in increased DA transmission from the VTA to the NAcc. Additionally, acute cocaine, amphetamine, methamphetamine (METH), and MPH administration alter many other molecular processes within the mesolimbic circuitry. For example, acute cocaine, amphetamine, and MPH induce transcription factor genes such as c-fos, FosB, and zif268 in rodents (Howell & Kimmel, 2008; Yano & Steiner, 2007) and increase expression of the neuropeptide substance P (Yano & Steiner, 2007). These acute alterations in brain chemistry if repeatedly induced can lead to long term changes in neurotransmission (Pierce & Kalivas, 1997).
Figure 1. Circuitry diagram of the mesolimbic dopamine reward pathway. Drug and natural reward increase DA transmission from the VTA to the NAcc. Psychostimulant drugs in particular, tend to increase this DA transmission via indirect activation of excitatory $D_1$ DA receptors on DA neurons and inhibitory $D_2$ DA receptors on GABA neurons.

*Effects of Chronic Psychostimulant Administration*

Human psychostimulant use tends to extend for years. Such long term use, whether prescribed as medication or used illegally, increases the likelihood of lasting adaptive changes. For example, animal studies have shown that chronic exposure to psychostimulants causes enduring changes to brain transcription factors and second messenger molecules (Carlezon &
Konradi, 2004; Crawford, McDougall, Meier, Collins, & Watson, 1998; Yano & Steiner, 2007) which correspond to alterations in DA and GABA transmission (See Figure 2) (Pierce & Kalivas, 1997). Specifically, 28 days following 14 days exposure to MPH, expression of the immediate early genes, c-fos and zif268 appear blunted in response to acute MPH and cocaine challenge (Yano & Steiner, 2007). Moreover, after chronic MPH exposure protein kinase A and DA-stimulated adenyl cyclase were decreased (Crawford, et al., 1998). Conversely, basal ΔFosB was increased following chronic pretreatment with cocaine and MPH (Howell & Kimmel, 2008). It follows that these molecular changes could result in behavioral alterations as well. For example, overexpression of the early immediate gene, ΔFosB, has been shown to impact a number of psychostimulant- and opiate-induced phenomena including dependence, addiction, sensitization, and tolerance (See Nestler, 2008 for review).

Figure 2. Changes in the VTA associated with chronic psychostimulant administration. There is a long term increase in DA transmission and a decrease in GABA transmission in the VTA following repeated injections of psychostimulants (From Pierce & Kalivas, 1997).
Given that the illicit abuse of psychostimulants is thought to result from their ability to directly activate the mesolimbic DA reward system, the majority of behavioral research to date has focused on chronic psychostimulant-induced changes in drug reward and addiction. The DA reward system is closely associated with the reinforcing properties of drugs, the rewarding properties of drugs (Ritz, Lamb, Goldberg, & Kuhar, 1987), and the expression of drug-induced behavioral sensitization (McNamara, Davidson, & Schenk, 1993). Typically drug reinforcement is measured by self-administration, which assesses the amount or frequency of drug infusions a rat is willing to respond for on an operant task. Alternately, drug reward is most often measured via conditioned place preference. This behavioral paradigm relies on the principals of Pavlovian conditioning and calculates the amount of time a rat spends in a drug paired compartment compared to a saline paired compartment. Lastly, behavioral sensitization is a phenomenon characterized by a progressive and persistent enhancement of drug-induced behavioral effects, such as locomotor activation (Robinson & Berridge, 1993, 2000). This phenomenon has been implicated in the shift from recreational to pathological abuse of locomotor activating drugs (Robinson & Berridge, 1993) and there is evidence to support the mediation of psychostimulant-induced locomotor behavioral sensitization by the mesolimbic DA system (Pierce & Kalivas, 1997). Repeated exposure to MPH, cocaine, and amphetamines produce locomotor behavioral sensitization (Dafny & Yang, 2006; Martin-Iverson & Burger, 1995; Vezina, 2004), increase psychostimulant self-administration (Brandon, Marinelli, Baker, & White, 2001; Schenk & Partridge, 1997; Vezina, 2004), and increase psychostimulant place preference (Lett, 1989; Meririnne, Kankaanpaa, & Seppala, 2001). These findings indicate that previous chronic exposure to MPH, cocaine, and amphetamines can lead to a subsequent increase in psychostimulant addiction potential (Kollins, et al., 2001; Robinson & Berridge, 1993, 2000).
The frequent use of psychostimulants such as MPH to treat ADHD in children, raises questions about the long term consequences of MPH use early in life (Kollins, et al., 2001). Unfortunately, designing developmental rodent studies that can be compared to ontogenic stage in humans is difficult due to the differences in developmental timelines and lifespan duration between the two species. In general, however, researchers have come to agree that in rats the first 50 days of life correspond to infancy through adolescence in humans (See Figure 3 for a diagram of rat and human developmental periods) (For review see Andersen, 2003).

Past research in rodents demonstrates that chronic MPH treatment leads to alterations in later psychostimulant responsiveness (Achat-Mendes, Andersen, & Itzhak, 2003; Brandon, et al., 2001; Crawford et al., 2007). Sometimes these alterations are consistent regardless of what age MPH exposure occurred, but often these changes in drug responsiveness are dependent upon the age of the rat during MPH pretreatment. For example, exposing rats to MPH during the pre-weanling (postnatal day [PD] 10 - 19) (Crawford et al., 2011), periadolescent (PD 35 - 46)
(Brandon, et al., 2001), and adult (PD > 55) (Schenk & Izenwasser, 2002) stages of ontogeny enhanced later cocaine self-administration. In contrast, pre-weanling MPH exposure had no effect on later cocaine place preference (Crawford, et al., 2011), but MPH exposure during the preadolescent period (PD 20 - PD 35) reduced cocaine place preference (Andersen, Arvanitogiannis, Pliakas, LeBlanc, & Carlezon, 2002; Carlezon, Mague, & Andersen, 2003). These findings may imply the occurrence of drug-induced permanent changes in neural reward pathways (Andersen, et al., 2002; Crawford, et al., 2007; Moll, Hause, Ruther, Rothenberger, & Huether, 2001), which could lead to an enhanced or reduced predisposition for drug seeking behavior and addiction in later life (Schenk & Izenwasser, 2002; Yang, Swann, & Dafny, 2003). Consequently, increasing concern has arisen in the public and scientific communities regarding the safety of MPH treatment and its relationship to drug abuse (Kollins, et al., 2001; Murray & Kollins, 2000).

Although psychostimulant drugs act by increasing DA transmission, the long term effects of psychostimulants appear to extend to other neurotransmitter systems as well. For example, recent research indicates that chronic MPH administration during the pre-weanling period of ontogeny alters later responsiveness to the µ-opioid receptor agonist, morphine (Crawford, et al., 2007; Cyr & Morgan, 2009; Halladay et al., 2009). MPH has been found to change morphine responsiveness across two very different behavioral phenomena: reward (Crawford, et al., 2007) and antinociception (Cyr & Morgan, 2009; Halladay, et al., 2009). Specifically, pre-weanling rats that received 5 mg/kg of MPH during development showed an enhanced morphine conditioned place preference as adults compared to saline pretreated controls (Crawford, et al., 2007). Additionally, MPH exposure during this time in development has been shown to
potentiate the acute antinociceptive effects of morphine on the hot plate thermal pain assay (Cyr & Morgan, 2009; Halladay et al., 2009). Rats pretreated with MPH showed a leftward shift in the acute morphine dose response curve compared to saline pretreated controls, indicating enhanced sensitivity to morphine as adults following MPH exposure during pre-weanling development (Cyr & Morgan, 2009). Moreover, this enhanced acute response to morphine-induced antinociception was followed by enhanced tolerance to morphine (Cyr & Morgan, 2009).

Since the period of ontogeny during which MPH exposure occurs sometimes dictates the direction of later psychostimulant response, it follows that age at the time of MPH exposure could affect later morphine-induced antinociception and tolerance as well. To date, the impact of ontogenic stage on psychostimulant-induced alterations in responsiveness to the antinociceptive effects of morphine has not been examined. Because psychostimulant use can occur at differing points across the lifespan, it is important to determine whether the developmental period during which psychostimulant exposure occurs modulates later responsiveness to opioid-induced antinociception and tolerance. If so, it would be important for prescribing physicians to know across what ages psychostimulant use took place in order to effectively treat acute and chronic pain. Therefore, one aim of this dissertation is to determine if age at the time of psychostimulant exposure influences the long term changes in morphine antinociceptive potency previously reported. In particular, the stages spanning periadolescence and adulthood will be examined (Chapters 4 and 5).
Psychostimulant Activity at the Dopamine Transporter

As a class of drugs, psychostimulants are broadly defined as drugs that stimulate the central and peripheral nervous systems. The most commonly used psychostimulants; cocaine, amphetamine, METH, and MPH are potent indirect DA receptor agonists acting at the dopamine transporter (DAT). That said, not all psychostimulants work the same way at the DAT. For example, MPH and cocaine are DAT inhibitors that increase DA in the extracellular space by binding to DAT and subsequently preventing DA from being cleared from the synapse (Schenk & Izenwasser, 2002). Differentially, METH and amphetamine are competitive analog substrates that not only compete for DAT binding, but also increase DAT mediated DA efflux (Kahlig & Galli, 2003).

Because different psychostimulants can work differently at DAT, it is possible that not all psychostimulants will produce similar changes in morphine responsiveness. Given that abuse of METH is common in adults and adolescents (Substance Abuse and Mental Health Services Administration, 2007), it is important to determine whether psychostimulant-induced enhancement of morphine-induced antinociception and tolerance is a characteristic of all dopaminergic psychostimulants regardless of different DAT mechanisms. Thus, a purpose of this dissertation is to determine whether different psychostimulants induce similar changes in morphine antinociception and tolerance. Specifically, the effects of chronic METH during periadolescence and adulthood will be compared to MPH and saline pretreated animals (Chapters 4 and 5).
Pain Modulation

MPH induced changes in pain modulation are particularly interesting because morphine antinociception is not directly related to the DA reward system circuitry activated by psychostimulants. Psychostimulants, however, have been shown to produce antinociception independent of opioid administration (Altier & Stewart, 1999; Lin, Morrow, Kirtsty-Roy, Terry, & Casey, 1989; Shyu, Kiritsty-Roy, Morrow, & Casey, 1992) and simultaneous administration of opioids and psychostimulants has been used in pain therapy for many years. One of the early combinations adopted by physicians was the Brompton mixture (Melzack, Mount, & Gordon, 1979; Melzack, Ofiesh, & Mount, 1976). A standard Brompton mixture contains a variable amount of morphine, cocaine (20 mg), ethyl alcohol (2.5 mL), syrup BP (5mL), and chloroform water. Additional, research in this area has indicated that combination treatment with morphine and amphetamine, cocaine, or MPH can potentiate the analgesic effects of morphine in humans, but questions still remain about the nature of these interactions (For review see Dalal & Melzack, 1998).

Although concomitant psychostimulant/morphine administration has been shown to produce analgesia, how chronic developmental MPH exposure alters the antinociceptive effects of opioids following a prolonged washout period remains unknown. Determining the brain area(s) involved in MPH’s enhancement of morphine antinociception and tolerance would move the field a step closer to elucidating the underlying mechanisms of these effects. The involvement of the periaqueductal gray (PAG) seems likely since it is crucially involved in morphine antinociception and tolerance (Morgan, Fossum, Levine, & Ingram, 2006; Morgan, Fossum, Stalding, & King, 2006). Therefore, the possibility that the PAG is involved in the
enhanced acute morphine-induced antinociception and tolerance observed following MPH pre-weanling exposure warrants examination. It is the final goal of this dissertation to determine whether pre-weanling MPH exposure also enhances adult morphine-induced antinociception and tolerance when morphine is microinjected directly into the ventro-lateral PAG (vPAG) (Chapter 2).

Summary of Studies

Psychostimulants are used and/or abused by humans across the lifespan. Previous work in our lab indicates that chronic pre-weanling exposure to a psychostimulant, MPH, enhances morphine-induced antinociception and tolerance in adult rats. Considering the popular use of this drug to treat ADHD in children, adolescents, and adults, additional research in this area is crucial. Addressing unanswered questions in the literature pertaining to the impact of pretreatment age and psychostimulant compound used is of particular importance. Moreover, determining the brain area(s) involved would help elucidate the underlying mechanisms driving these effects. To those ends the studies conducted within this dissertation seek to determine 1) if the effects of chronic MPH and METH pretreatment during periadolescence (Chapter 4) and adulthood (Chapter 5) alter later morphine-induced antinociception and tolerance and 2) if the PAG is involved in pre-weanling MPH-induced enhancement of morphine potency and tolerance in adulthood (Chapter 2). Additionally, locomotion will be assessed in Chapter 3 to determine METH doses that produce an acute behavioral effect similar to our MPH dose.
CHAPTER TWO:
EFFECTS OF PRE-WEANLING MPH EXPOSURE ON ADULT ANTINOCICEPTION
AND TOLERANCE FOLLOWING MORPHINE MICROINJECTION INTO THE PAG

ABBREVIATED TITLE: PRE-WEANLING PAG MICROINJECTION STUDY
Introduction

MPH pretreatment has been shown to enhance both the rewarding and acute antinociceptive properties of morphine (Crawford, et al., 2007; Cyr & Morgan, 2009; Halladay et al., 2009), but it is unclear how MPH pretreatment is changing morphine responsiveness. It is possible that the PAG DA network is involved since it has been shown to be integral in both the rewarding (Flores, Galan-Rodriguez, Ramiro-Fuentes, & Fernandez-Espejo, 2006; Olmstead & Franklin, 1997) and acute antinociceptive properties of opiates (Flores, El Banoua, Galan-Rodriguez, & Fernandez-Espejo, 2004). Specifically, 6-hydroxydopamine (6-OHDA)-induced lesions of the PAG region (Olmstead & Franklin, 1997) and infusions of dopaminergic D₂ antagonists into the PAG, (Flores, et al., 2006) abolished morphine and heroin place preference respectively. Similarly, 6-OHDA-induced lesions of large PAG neurons and intra-PAG microinjection of a DA D₁ antagonist attenuated systemic opioid-induced antinociception (Flores, et al., 2004).

Under normal conditions, tonic GABA release within the PAG inhibits firing of projection neurons to the rostral ventral medulla (RVM) and spinal cord. This results in normal behavioral responding to nociceptive stimuli. When opioid drugs such as morphine are administered they inhibit GABA’s inhibition of output neurons by binding to presynaptic μ-opioid receptors located on GABA terminals. This results in disinhibition of PAG and RVM projection neurons and consequently antinociception (Christie, Connor, Vaughan, Ingram, & Bagley, 2000; Vaughan & Christie, 1997; Vaughan, Ingram, Connor, & Christie, 1997).

Similar to μ-opioid receptors, DA D₂ receptors have been shown to presynaptically inhibit GABA release in the ventro-lateral orbital cortex (VLO) (Sheng, Qu, Huo, Du, & Tang,
and PAG (Meyer, Morgan, Kozell, & Ingram, 2009). Additionally, the analgesic capacity of cocaine and amphetamine are predicted in part by their impact on D$_2$ receptor binding (Wood, 2008). It follows that morphine and psychostimulants may produce antinociception through a shared ability to inhibit GABA inhibition. A study by Meyer, Morgan, Kozell, & Ingram (2009) provides additional support for this possibility since a DA D$_2$ receptor antagonist, but not a DA D$_1$ receptor antagonist attenuated apomorphine-induced antinociception when both compounds were microinjected into the PAG.

Stimulation of DA D$_1$-like and D$_2$-like receptors respectively increase and decrease adenylyl cyclase activity (For review see Jaber, Robinson, Missale, & Caron, 1996). Moreover, DA-stimulated adenyly cyclase activity in the dorsal striatum is decreased following chronic MPH exposure (Crawford, et al., 1998). Thus, it is possible that chronic MPH causes sensitization of DA D$_2$-like receptors and/or desensitization of DA D$_1$-like receptors. If similar changes to DA receptor function occur in the PAG following chronic MPH administration, changes to DA D$_1$-like and/or D$_2$-like receptors within the PAG may be responsible for the enhanced acute morphine-induced antinociception observed following chronic pre-weanling MPH exposure.

Acute administration of morphine causes µ-opioid receptor activation and repeated morphine administration leads to morphine tolerance. Moreover, both antinociceptive potency and µ-opioid receptor desensitization have been suggested to influence the expression of tolerance to the antinociceptive effects of morphine (Ingram, Macey, Fossum, & Morgan, 2008). It follows that increased acute responsiveness to morphine should lead to greater desensitization of opioid neurons during the induction of tolerance, so it is not surprising that exposure to pre-
weanling MPH enhanced morphine tolerance. The PAG has been shown to be an important site in the development of tolerance to morphine, since repeated microinjection of morphine into the PAG produces tolerance (Jacquet & Lajtha, 1976; Morgan, Clayton, & Boyer-Quick, 2005; Siuciak & Advokat, 1987; Tortorici, Robbins, & Morgan, 1999), and selective blockade of opioid receptors in the PAG attenuates tolerance to the antinociceptive effect of systemically administered morphine (Lane, Patel, & Morgan, 2005). Consequently, the enhanced expression of morphine tolerance observed following MPH pre-weanling pretreatment could be mediated by the PAG. It was therefore the purpose of Study1 to test the hypothesis that the vPAG contributes to the enhanced morphine-induced antinociception and tolerance observed following pre-weanling MPH treatment. Specifically, it was hypothesized that pre-weanling rats pretreated with MPH would show enhanced acute antinociception and tolerance in response to intra-vPAG morphine as adults.

Methods

Subjects

Subjects were 34 male Sprague-Dawley rats, born and raised at Washington State University, Vancouver. Rats were housed with their littermates and dam until weaned (PD 25). Beginning on PD 25 rats were housed in groups of 4. Following cannula implantation surgery on PD 55 rats were housed in individual cages. The colony room was kept under a reverse 12 hr light/dark cycle and maintained at 22 - 24°C. Rats were given food and water ad libitum throughout the duration of the experiment except during testing. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of the
International Association for the Study of Pain. This experiment was approved by the Animal Care and Use Committee at Washington State University.

**Drugs**

MPH hydrochloride (Sigma-Aldrich, St. Louis, USA) was dissolved in saline and injected intraperitoneally (i.p.) at a dose of 5 mg/kg and volume of 5 ml/kg. Morphine sulfate (a gift from NIDA) was dissolved in saline and injected into the vPAG at a volume of 0.4 µl.

**Pretreatment**

Starting at PD 10, rats were randomly assigned to one of two pretreatment groups and received daily i.p. injections of saline or MPH (5 mg/kg). These daily injections continued for 10 consecutive days at which time rats were left undisturbed except for weekly tail coloring and colony maintenance until PD 55. The dose of 5 mg/kg of MPH was chosen because it was found to alter morphine responsiveness in adulthood after pre-weanling MPH exposure (Cyr & Morgan, 2009).

**Guide Implantation Procedure**

On PD 55 rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and implanted with a guide cannula (9 mm long; 23 gauge) aimed at the vPAG (AP: +1.7 mm, ML: ±0.6 mm, DV: -5.0 mm from lambda) using stereotaxic techniques. The guide cannula was attached to two screws in the skull by dental cement. At the end of the surgery a stylet was inserted to plug the guide cannula. The rat was maintained under a heating lamp until awake, and then moved to a cage of its own.
Microinjections

Morphine was administered through a 31-gauge injection cannula inserted into and extending 2 mm beyond the tip of the guide cannula. Rats received a sham injection prior to the experiment, in which an injector was inserted into the guide cannula but no drug was administered. This procedure reduces confounds resulting from mechanical stimulation of neurons on the test day. Testing with drug administration began at PD 60. Microinjections were administered in a volume of 0.4 µl and a rate of 0.1 µl/10 s while the rat was gently restrained by hand. The injection cannula remained in place an additional 20 seconds (s) to minimize backflow of the drug up the cannula track. Following injections, the stylet was replaced and the rat was returned to its home cage.

Nociceptive Assessment

Nociception was assessed using the hot plate test. The hot plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5°C plate. The rat was removed from the hot plate if no response occurred within 50 s.

Experiments

Two experiments were carried out. Both experiments used a third log cumulative dose procedure in which increasing cumulative doses of morphine were microinjected into the vPAG every 20 minutes (1.0, 1.2, 2.4, 5.4, and 12 µg/0.4 µl) resulting in third log doses of 1.0, 2.2, 4.6, 10.0, and 22 µg/0.4µl. Nociception was assessed using the hot plate test 15 min after each microinjection. Our previous research has shown that these cumulative injections produce dose-dependent increases in antinociception and clear shifts in these dose response curves as a result of the development of tolerance or administration of receptor antagonists (Morgan, Fossum,
Levine, & Ingram, 2006; Morgan, Fossum, Stalding, & King, 2006). Experiment 1 assessed the effects of pre-weanling MPH treatment on acute morphine antinociception at 60 days of age (analogous to adulthood in humans). Following Experiment 1, on PD 61, rats were match paired based on hot plate latency after the fourth morphine microinjection on the acute test day and further divided into tolerance treatment groups. Half of the animals in each pretreatment group (MPH or saline) were microinjected with either saline or morphine (5µg/0.4 µl), twice daily for two consecutive days from PD 61 - 62. Experiment 2 was conducted on PD 63 and assessed the effects of pre-weanling MPH treatment on morphine tolerance.

**Histology**

Rats were exposed to a lethal dose of halothane following testing. The brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (100 µm) and the location of the injection site was identified (Paxinos & Watson, 2005).

**Data Analysis**

Baseline nociceptive hot plate latency was analyzed using a one way ANOVA with pretreatment as the between group factor. Following the induction of tolerance, baseline hot plate latency was analyzed using a 2 x 2 ANOVA with pretreatment and tolerance treatment as the between group factors. Morphine dose response curves and the half maximal antinociceptive effect (D$_{50}$) were calculated for all groups using non-linear regression (GraphPad Prism 5 software) (See Appendix A for more information). The lower limit for calculating D$_{50}$ values was set at the mean baseline hot plate response. The upper limit was set at the mean response produced by the highest dose of morphine (22 µg/0.4 µl). Changes in D$_{50}$’s were assessed using ANOVA (GraphPad Prism 5 software). Significance for all statistical analyses was set at $p < 0.05$. 
Results

Experiment 1: Acute Antinociception

Results are only reported for animals with verified cannula placements within the vPAG. All other animals were excluded from the analyses. Baseline hot plate latencies differed between pretreatment groups (F(1, 32) = 5.109, p < 0.05; Figure 4). Specifically, saline pretreated animals had higher baseline latencies compared to MPH pretreated animals.

Microinjection of morphine into the vPAG caused a dose dependent increase in antinociception, but pre-weanling MPH pretreatment did not alter acute morphine antinociception following morphine microinjection into the vPAG (F(1, 161) = 0.0090, p = 0.9245; Figure 5; Table 1). Because nociceptive baselines differed according to pretreatment condition, an additional analysis was conducted following data conversion to percent of the maximal possible effect (% MPE). Although the % MPE calculations were not significant, this analysis showed a tendency for an enhanced antinociceptive effect in MPH pretreated rats (F(1, 161) = 3.526, p = 0.0623; See Appendix B for % MPE graph).
Figure 4. Pre-weanling MPH pretreatment reduced acute nociceptive baselines on the hot plate test when measured on PD 60 (n = 16 - 18 per group). * denotes statistical difference $p < 0.05$
Figure 5. Pre-weanling MPH pretreatment did not alter acute morphine-induced antinociception following morphine microinjection into the PAG on PD 60.

Experiment 2: Morphine Tolerance

Results were reported for animals with verified cannula placements within the vPAG. All other animals were excluded from the analyses. Baseline hot plate latencies did not differ between pretreatment (F (1, 31) = 0.12, p = 0.73) or tolerance treatment (F (1, 31) = 0.64, p = 0.43) groups following the induction of tolerance (See Figure 6).
Saline and MPH pretreated rats treated with morphine for 2 days showed a rightward shift in the morphine dose response curve compared to rats treated with saline for 2 days (F (1, 76) = 10.66, p = 0.0016; F (1,66) = 11.33, p = 0.0013; See Table 1 and Figure 7). These rightward shifts indicate the development of tolerance to morphine. Previous MPH exposure did not alter intra-vPAG morphine-induced tolerance in adult animals (F (1, 76) = 1.373, p = 0.229). Because nociceptive baselines differed according to pretreatment condition an additional analysis was conducted following data conversion to % MPE. The results did not differ from the original analysis (F (1,76) = 2.252, p = 0.1379; See Appendix B for % MPE graph).

![PRE-WEANLING PRETREATMENT Baselines Following Induction of Tolerance](image)

**Figure 6.** Baseline hot plate latencies did not differ between pre-weanling pretreatment or tolerance treatment groups on PD 63 following the induction of tolerance (n = 6 - 8 per group).
Figure 7. Tolerant rats in both pretreatment groups showed a rightward shift in the morphine dose response curve compared to similarly pretreated tolerance controls. Previous MPH exposure did not alter intra-vPAG morphine-induced tolerance.
Table 1. Comparison of morphine D<sub>50</sub> values on acute and tolerance test days.

<table>
<thead>
<tr>
<th></th>
<th>Pre-weanlings</th>
<th>Tolerance</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Acute 95% CI’s</td>
<td>Saline 95% CI’s</td>
</tr>
<tr>
<td>SAL</td>
<td>4.5 3.8 - 5.3</td>
<td>5.3 3.8 - 6.4</td>
</tr>
<tr>
<td>MPH</td>
<td>4.5 3.6 - 5.5</td>
<td>3.7 1.7 - 5.0</td>
</tr>
</tbody>
</table>

Discussion

Pre-weanling exposure to MPH has been shown to enhance systemic morphine-induced antinociception and tolerance in adult rats (Cyr & Morgan, 2009; Halladay, et al., 2009). Results from the current study indicate that this effect is not exclusively mediated by the vPAG, since pre-weanling MPH administration did not alter adult morphine-induced antinociception and tolerance when morphine was microinjected directly into the vPAG.

Acute nociceptive baseline hot plate latencies differed between MPH and saline pretreated animals. Specifically, rats exposed to MPH had lower baseline scores than saline pretreated controls. Although this finding indicates a possible alteration in basal nociceptive hot plate responding following pre-weanling MPH exposure, results of previous research suggest that pre-weanling MPH exposure does not change baseline responding on the hot plate (Cyr & Morgan, 2009; Halladay, et al., 2009). In the current study rats underwent cannula implantation surgeries and pentobarbital was used as a general anesthetic, but in previous studies no surgery or exposure to pentobarbital occurred. Chronic exposure to cocaine enhances later pentobarbital-
induced sedation (Ma et al., 2008) demonstrating the ability of chronic psychostimulant exposure to alter later responsiveness to pentobarbital. It is possible that in MPH pretreated animals, exposure to pentobarbital during surgery somehow altered baseline hot plate response latencies. Alternatively, the stress and nociception induced by the surgical procedure could also account for the reduction in hot plate response times in MPH exposed animals.

Although acute baseline scores differed significantly between pretreatment groups, the difference was small and consequently did not impact subsequent assessment of morphine-induced antinociception and tolerance. That said pre-weanling MPH exposure had no effect on intra-vPAG morphine-induced antinociception and tolerance in adulthood. This finding suggests that the vPAG is not exclusively involved in the previously reported enhancement of morphine-induced antinociception and tolerance produced by pre-weanling MPH exposure (Cyr & Morgan, 2009; Halladay, et al., 2009). Since microinjection of morphine directly into the vPAG would not change the activity of neurons projecting into the PAG, it is still possible that PAG mediated inhibition of the descending pain pathway is involved in the enhanced morphine-induced antinociception and tolerance observed following chronic pre-weanling MPH exposure. Alternatively, it may be that pre-weanling exposure to MPH alters adult neurotransmission in a number of brain areas including the PAG and simultaneous morphine activation of circuitry within these areas is necessary to change morphine-induced antinociceptive responding. Thus, the involvement of the PAG cannot be completely ruled out.

More research in this area is needed to determine what brain areas are involved in pre-weanling MPH-induced changes in adult morphine antinociceptive responsiveness. Given the involvement of mesolimbic dopaminergic mechanisms in modulation of nociception (Altier &
Stewart, 1998; Burkey, Carstens, & Jasmin, 1999; Coffeen et al., 2008; Koyanagi, Himukashi, Mukaida, Shichino, & Fukuda, 2008; Magnusson & Fisher, 2000; Saade, Atweh, Bahuth, & Jabbur, 1997; Sotres-Bayon, Torres-Lopez, Lopez-Avila, del Angel, & Pellicer, 2001; Taylor, Joshi, & Uppal, 2003) and the activation of this circuitry by MPH, VTA involvement is probable. That said, there are numerous ways in which the VTA could be mediating these effects. For example, dopamine projections from the VTA to the VLO have been described (Brown et al., 1979; Emson & Koob, 1978; Groenewegen & Uylings, 2000; Oades et al., 1987; Tzschentke, 2001) and recent research indicates that GABA_A-containing afferents from VLO project to the PAG (Huo, et al., 2009). It is possible then that sensitization of DA receptors within the VTA would lead to enhanced DA transmission in the VLO, which would reduce D_2-mediated GABA transmission in the VLO, and consequently increased firing of VLO projection neurons synapsing within the PAG (See Figure 8 for schematic). This proposed circuitry warrants investigation, since VLO modulation of nociception is mediated by the PAG (Zhang, Tang, Yuan, & Jia, 1997a, 1997b) and microinjection of DA D_2 agonists into the VLO produced antinociception that was attenuated and potentiated by GABA_A receptor agonists and antagonists respectively.
Pharmacological treatment of ADHD with MPH is increasing in preschool aged children (Vitiello, 2001; Zito, et al., 2000) and the use of morphine to treat pain has been common clinical practice for years. If rodent studies extrapolate to humans, the ability of pre-weanling MPH to alter adult morphine antinociceptive potency could have vast implications on clinical pain management. Consequently, further research that seeks to identify the brain areas and the underlying mechanisms involved in MPH-induced long term changes in morphine-induced antinociception and tolerance is needed.
CHAPTER THREE:
EFFECTS OF ACUTE PERIADOLESCENT AND ADULT PSYCHOSTIMULANT EXPOSURE ON LOCOMOTOR ACTIVATION

ABBREVIATED TITLE: PERIADOLESCENT AND ADULT LOCOMOTOR STUDY
Introduction

The MPH dose (5 mg/kg) selected for all of the dissertation studies was chosen based on past research (Crawford, et al., 2007; Cyr & Morgan, 2009; Halladay, et al., 2009), but appropriate METH doses needed to be determined. Acute psychostimulant administration induces robust locomotor activation in adult (Dafny & Yang, 2006; Martin-Iverson & Burger, 1995; Vezina, 2004) and periadolescent rats (Laviola, Adriani, Terranova, & Gerra, 1999; McPherson & Lawrence, 2006). Consequently, this behavioral assessment seemed an adequate measure for determining comparable behavioral output based on psychostimulant-induced neuronal activation. It was therefore the purpose of this study to determine appropriate METH doses for subsequent studies (Chapters 4 and 5). Specifically, doses that produce acute periadolescent and adult locomotor activation similar to our MPH dose, but different from saline controls were sought. METH doses of 1 and 3 mg/kg were investigated for use in the subsequent studies (Chapters 4 and 5) because they have been shown to consistently produce behavioral locomotor sensitization in rats (Bevins & Peterson, 2004; Hall, Stanis, Marquez Avila, & Gulley, 2008; Shuto et al., 2006). It was hypothesized that MPH (5 mg/kg) and METH (1 and 3 mg/kg) treated animals would display enhanced locomotor activity compared to saline treated animals.

Methods

Subjects

Subjects were 31 periadolescent and 33 adult male Sprague-Dawley rats, purchased from Harlan (Livermore, CA). Rats were group housed with littermates throughout the experiments. The colony room was kept under a reverse 12 L: 12 D cycle and maintained at 22 - 24°C. Rats
were given continuous access to food and water throughout the experiment except during testing. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This experiment was approved by the Animal Care and Use Committee at Washington State University.

**Drugs**

MPH and METH (Sigma Aldrich, St. Louis, USA) were dissolved in saline and injected i.p. at a volume of 1 ml/kg for adult rats and 2 ml/kg for adolescent rats.

**Equipment**

Locomotor activity chambers measuring 47 cm x 25.4 cm and equipped with Photobeam Activity System data collection software (San Diego Instruments, San Diego, CA) were used. Each chamber contained 7 photobeams that ran horizontally along the base of the chamber. Locomotor activity was assessed by comparing the total number of beam breaks in one hour.

**Procedure**

Two experiments were conducted. Experiment 1 assessed locomotor activity in adult animals at 55 days of age following saline, MPH (5 mg/kg), or METH (1 & 3 mg/kg) administration. Experiment 2 assessed locomotor activity in periadolescent animals at 35 days of age following saline, MPH (5 mg/kg) or METH (1 & 3 mg/kg) administration (For developmental timelines see Andersen, 2003). In both experiments rats were divided into 1 of 4 drug groups and received an injection of MPH (5 mg/kg), METH (1 or 3 mg/kg), or saline immediately before being placed in a locomotor chamber. Immediately following injection, locomotor activity was assessed in 5 minute bins for 60 minutes.
Data Analysis

Locomotor activity was analyzed using a 4 x 2 ANOVA with drug group and age as the two factors. A 4 x 12 repeated measures ANOVA was calculated to determine differences in psychostimulant-induced locomotor activity over time. Post hoc analyses were conducted using Tukey HSD tests. Significance for all statistical analyses was set at $p < 0.05$.

Results

Rats treated with a psychostimulant were significantly more active than saline treated rats as indicated by an overall effect of drug treatment on locomotor activity ($F (3, 64) = 29.52, p < 0.001$). Tukey HSD post hoc comparisons of the three drug groups indicate that MPH and METH (1 and 3 mg/kg) treated animals had significantly more beam breaks than saline treated controls ($p < 0.001$; See Figure 9). Although there were variations in the time courses for locomotor activation between MPH and METH (1 and 3 mg/kg) in periadolescent ($F (3, 297) = 8.15, p < 0.0001$) and adult rats ($F (3, 319) = 7.38, p < 0.0001$), post hoc comparisons revealed no difference in overall activity ($p > 0.05$). In periadolescent rats MPH treated animals displayed more locomotion from 5 - 15 minutes following injection compared to METH 1 mg/kg ($p < 0.01; p < 0.001$) and METH 3 mg/kg ($p < 0.05; p < 0.01$) treated rats. In adult rats MPH treated animals displayed more locomotion from 5 - 10 minutes ($p < 0.01$) and less locomotion from 50 - 60 minutes ($p < 0.01; p < 0.001$) following injection compared to METH 3 mg/kg treated rats. Overall locomotor activity differed as a function of age, with adult rats displaying significantly more locomotion than periadolescents ($F (1, 64) = 4.71, p < 0.05$). No drug x age interaction was found ($F (3, 64) = 1.58, p > 0.05$) (See Table 2 for summary).
Because differences in baseline locomotion could have confounded the original analyses, the data were transformed and analyzed as a change from saline treated controls. The appropriate group mean of the saline animals was subtracted from each psychostimulant animal in both age groups and comparisons were made using a 2 x 3 ANOVA. Results from this analysis indicated that psychostimulant-induced locomotion was greater in periadolescent compared to adult animals (F (1, 42) = 10.535, p < 0.01). There was no difference in overall locomotion between the psychostimulant treated rats (F (1, 42) = 0.549, p > 0.05) and no drug x age interaction was found (F (2, 42) = 1.014, p > 0.05).
Figure 9. Locomotor activity following i.p. injection of saline, MPH (5 mg/kg), or METH (1 or 3 mg/kg) (n = 7 - 8 per group). Rats administered MPH and METH were more active than rats that received saline. MPH and METH (1 and 3 mg/kg) treatment caused a similar increase in locomotor activity. Overall adult rats (PD 55) were more active than periadolescent rats (PD 35). * denotes statistical difference from METH 3 mg/kg ** denotes statistical difference from METH 1 and 3 mg/kg
Table 2. Number of beam breaks in one hour.

<table>
<thead>
<tr>
<th></th>
<th>Periadolescents</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>1178 ± 158</td>
<td>1949 ± 154</td>
</tr>
<tr>
<td>MPH</td>
<td>3118 ± 222 *</td>
<td>2975 ± 284 *</td>
</tr>
<tr>
<td>METH 1</td>
<td>3028 ± 226 *</td>
<td>3430 ± 212 *</td>
</tr>
<tr>
<td>METH 3</td>
<td>3124 ± 167 *</td>
<td>3397 ± 208 *</td>
</tr>
</tbody>
</table>

* denotes statistical significance from saline $p < 0.05$

**Discussion**

Acute administration of MPH (5 mg/kg) and METH (1 and 3 mg/kg) resulted in similar levels of overall locomotor activation. Moreover, all psychostimulant pretreated animals displayed significantly more locomotion compared to saline controls. In periadolescent animals, the analysis of locomotor activity across time indicates that the peak effect of MPH occurs slightly earlier than METH (1 and 3 mg/kg). This is also the case in adult animals but only when MPH is compared to METH 3 mg/kg. Despite apparent differences in peak activation, overall locomotor activity within the psychostimulant groups was comparable indicating similar overall neuronal activation. Therefore, doses of 1 and 3 mg/kg of METH were used in the subsequent studies.

In a recent study, investigators found that MPH-induced, but not amphetamine-induced acute locomotion was greater in early adolescent compared to adult animals (Walker et al., 2010). Although the current data show no drug by age interaction, the increase in activity caused by MPH and METH appeared to be greater in periadolescent compared to adult rats. It is possible that the differences in age between the rats in the current study (PD 35) and the study by Walker et al. (2010) (PD 28) may account for the conflicting amphetamine results.
CHAPTER FOUR:

EFFECTS OF PERIADOLESCENT MPH AND METH EXPOSURE ON
ADULT MORPHINE-INDUCED ANTINOCICEPTION AND TOLERANCE

ABBREVIATED TITLE: PERIADOLESCENT SYSTEMIC STUDY
Introduction

A growing body of research focuses on the effects of MPH exposure during different times in ontogeny. Pretreatment with MPH has been shown to change the behavioral output of rats responding on a self-administration paradigm. Specifically, in adult rats, pretreatment of low doses of MPH were discovered to facilitate increased cocaine self-administration (Brandon, et al., 2001) and pretreatment of high doses of MPH were found to increase the rate at which cocaine self-administration behavior is acquired (Schenk & Izenwasser, 2002). Additionally, repeated exposure to MPH during both adulthood and the pre-weanling period produces behavioral sensitization (Crawford, et al., 1998; Dafny & Yang, 2006; Gaytan, al-Rahim, Swann, & Dafny, 1997; McDougall, Collins, Karper, Watson, & Crawford, 1999).

While some effects do not appear to differ according to age of MPH exposure, other behavioral responses differ based on the stage of development in which MPH pretreatment occurs. Researchers have consistently found that early MPH exposure alters responsiveness to cocaine, but the direction of these effects seems to be dependent upon the age of the rat during MPH pretreatment. For example, exposing rats to MPH during the periadolescent period (PD 35 - 44) appears to increase drug responsiveness (Brandon, et al., 2001), while MPH exposure during the earlier preadolescent period (PD 20 - 34) reduces responsiveness to later psychostimulant administration (Andersen, et al., 2002; Carlezon, et al., 2003).

Pre-weanling pretreatment with MPH led to enhanced morphine-induced antinociception and tolerance in adulthood, but it is unknown whether MPH pretreatment during periadolescence produces the same effect. Children are prescribed MPH during adolescence so determining the
effect of exposure during this developmental period is clinically relevant. Moreover, given that abuse of another psychostimulant, METH, is common in human adolescents (Substance Abuse and Mental Health Services Administration, 2007) research investigating the effects of periadolescent METH exposure on later morphine responsiveness is also necessary. Therefore it was the purpose of the current study to determine the long term effects of periadolescent MPH and METH pretreatment on morphine-induced antinociception and tolerance in adult rats. Given that periadolescent pretreatment with MPH has been show to increase drug responsiveness (Brandon, et al., 2001), it is hypothesized that morphine-induced antinociception and tolerance will be enhanced in adulthood following periadolescent exposure to both MPH and METH.

Methods

Subjects

Subjects were 71 periadolescent male Sprague-Dawley rats purchased from Harlan (Livermore, CA). Rats were group housed with littermates throughout the experiments. The colony room was kept under a reverse 12 L: 12 D cycle and maintained at 22 - 24°C. Rats were given continuous access to food and water throughout the experiment except during testing. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This experiment was approved by the Animal Care and Use Committee at Washington State University.
Drugs

MPH hydrochloride and METH (Sigma Aldrich, St. Louis, USA) were dissolved in saline and injected i.p. at a volume of 2 ml/kg. Morphine sulfate (a gift from NIDA) was dissolved in saline and injected subcutaneously (s.c.) at a volume of 1 ml/kg.

Pretreatment

Starting at PD 35, 71 rats were randomly assigned to one of four pretreatment groups and received daily i.p. injections of saline, MPH (5 mg/kg), or METH (1 or 3 mg/kg). These daily injections continued for 10 consecutive days at which time rats were left undisturbed except for weekly tail coloring and colony maintenance until PD 60 (For developmental timelines see Andersen, 2003). The dose of 5 mg/kg of MPH was chosen because it was found to alter morphine responsiveness in adulthood after pre-weanling MPH exposure (Cyr & Morgan, 2009). The doses of 1 and 3 mg/kg of METH were chosen based on Chapter 3 of this dissertation.

Nociceptive Assessment

Nociception was assessed using the hot plate test. The hot plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5°C plate. The rat was removed from the hot plate if no response occurred within 50 s.

Experiments

Two experiments were carried out. Both experiments used a quarter log cumulative dose procedure in which increasing cumulative doses of morphine were injected s.c. every 20 minutes (1.8, 1.4, 2.4, 4.4, and 8 mg/kg) resulting in quarter log doses of 1.8, 3.2, 5.6, 10.0, and 18 mg/kg. Nociception was assessed using the hot plate test 15 min after each injection. Experiment 1 assessed the effects of peradolescent MPH and METH treatment on acute morphine-induced
antinociception at 60 days of age (analogous to adulthood in humans). Following Experiment 1, on PD 61, rats were match paired based on hot plate latency after the fourth morphine injection on the acute test day and further divided into tolerance treatment groups. Half of the animals in each pretreatment group (MPH 5 mg/kg, METH 1 mg/kg, METH 3 mg/kg, or saline) were injected with either saline or morphine (5 mg/kg, s.c.), twice daily for two consecutive days from PD 61 - 62. Experiment 2 was conducted on PD 63 and assessed the effects of periadolescent MPH and METH treatment on morphine antinociception following the induction of tolerance.

Data Analysis

Baseline nociceptive hot plate latency was analyzed using a one way ANOVA with pretreatment as the between group factor. Following the induction of tolerance, baseline hot plate latency was analyzed using a 2 x 4 ANOVA with pretreatment and tolerance treatment as the between group factors. Post hoc comparisons for baseline assessments were conducted using Tukey HSD tests. Morphine dose response curves and the D50 values were calculated for all groups using non-linear regression (GraphPad Prism 5 software) (See Appendix B for more information). The lower limit for calculating D50 values was set at the mean baseline hot plate response. The upper limit was set at the mean response produced by the highest dose of morphine (18 mg/kg). Changes in D50’s were assessed using ANOVA (GraphPad Prism 5 software). Because multiple post hoc comparisons with statistical testing can lead to a higher chance of Type 1 error, significance for post hoc comparisons of D50 values was adjusted and set at \( p < 0.01 \). Significance for all other statistical analyses was set at \( p < 0.05 \).
Results

Experiment 1: Acute Antinociception

Baseline hot plate latencies for rats pretreated with saline, MPH, and METH (1 or 3 mg/kg) as periadolescents did not differ on PD 60 (F (3, 64) = 1.74, p = 0.17; See Figure 10).

Morphine dose dependently produced antinociception in all three pretreatment groups (Figure 11). Periadolescent pretreatment altered acute morphine-induced antinociception in adulthood, (F (3, 347) = 2.912, p = 0.0345), but psychostimulant pretreated animals did not differ from saline controls as evidenced by examination of the 99% confidence intervals (Table 3).

When comparisons of the 99% confidence intervals between the psychostimulant pretreatment groups were made it was discovered that the difference found in acute morphine-induced antinociceptive potency was between MPH and METH 1 mg/kg pretreated animals. Specifically, MPH pretreated animals were more sensitive to acute morphine-induced antinociception compared to METH 1 mg/kg pretreated animals.
Figure 10. Periadolescent pretreatment with saline, MPH, or METH (1 and 3 mg/kg) from PD 35 - 44 did not alter acute nociceptive baseline latencies on the hot plate test when measured on PD 60 (n = 16 - 19 per group).
Figure 11. Adult morphine antinociceptive potency following periadolescent pretreatment with saline, MPH, or METH (1 and 3 mg/kg). Animals pretreated with psychostimulants from PD 35 - 44 did not differ from saline controls on morphine-induced antinociception when measured by the hot plate test on PD 60.
Experiment 2: Morphine Tolerance

Neither periadolescent psychostimulant pretreatment (\(F(3, 64) = 0.20, p = 0.90\)), nor tolerance treatment (\(F(1, 64) = 2.84, p = 0.10\)) altered baseline responding following the induction of tolerance (See Figure 12).

Saline, MPH, and METH (1 and 3 mg/kg) pretreated rats treated with morphine for 2 days showed a reduction in morphine potency compared to rats treated with saline for 2 days (\(F(1, 82) = 11.49, p = 0.00015\); \(F(1, 86) = 7.657, p = 0.0069\); \(F(1, 77) = 7.708, p = 0.0069\); \(F(1, 71) = 57.51, p < 0.0001\); Table 3; Figure 13). The magnitude of the rightward shifts in tolerant animals varied according to periadolescent pretreatment. This difference is evident whether raw data (\(F(3, 157) = 2.678, p = 0.049\)) or \% MPE is analyzed (\(F(3,157) = 3.658, p = 0.0138\)). Analysis of 99\% confidence intervals indicate that this difference is caused by periadolescent animals pretreated with METH 3 mg/kg showing greater tolerance to morphine as adults compared to MPH and saline pretreated rats (See Table 3). Additionally, METH 1 mg/kg pretreated animals showed greater tolerance to morphine compared to MPH pretreated animals. This latter finding is confounded because METH 1 mg/kg pretreated animals showed less acute antinociception compared to MPH pretreated rats. More specifically, if similar tolerance was induced in these 2 groups it would be expected that METH 1 mg/kg pretreated rats would also show less antinociception after the induction of tolerance.

Another potential confound is the development of tolerance in the tolerance controls caused by morphine administration during the acute antinociception test. Therefore, additional analyses were conducted to assess this possibility. Saline, MPH, and METH (1 and 3 mg/kg) pretreated rats treated with saline for 2 days displayed a significant rightward shift in the
morphine dose response curve compared to rats in the same pretreatment group on the acute test day (F (1, 126) = 14.27, p = 0.0002; F (1, 121) = 20.39, p < 0.0001; F (1, 116) = 9.07, p = 0.0032; F (1, 121) = 9.351, p = 0.0027). Morphine-induced antinociception in the saline tolerance treatment groups did not differ between the four periadolescent pretreatment conditions (Raw data: F (3, 167) = 2.38, p = 0.0715; % MPE: F (1, 167) = 0.7402, p = 0.5295). Since the rightward shift did not vary according to pretreatment, and because METH 3 mg/kg pretreated animals did not differ acutely from any of the other pretreatment groups, the original assessment that METH 3 mg/kg pretreated animals showed greater tolerance to morphine as adults compared to MPH and saline pretreated rats remains valid.

Figure 12. Baseline hot plate latencies did not differ between periadolescent pretreatment or tolerance treatment groups on PD 63 following the induction of tolerance (n = 8 - 9 per group).
Figure 13. Adult morphine tolerance following periadolescent pretreatment. (A) Saline pretreated rats in the tolerance control condition showed a rightward shift in the morphine dose response curve. A further shift in the dose response curve occurred following repeated exposure to morphine. (B) MPH pretreated rats in the tolerance control condition showed a rightward shift in the morphine dose response curve. A further shift in the dose response curve occurred following repeated exposure to morphine. (C) METH 1 mg/kg pretreated in the tolerance control condition showed a rightward shift in the morphine dose response curve. A further shift in the dose response curve occurred following repeated exposure to morphine. (D) METH 3 mg/kg pretreated rats in the tolerance control condition showed a rightward shift in the morphine dose response curve. A further shift in the dose response curve occurred following repeated exposure to morphine.
Table 3. Comparison of morphine D$_{50}$ values on acute and tolerance test days.

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<th>99% CI’s</th>
<th>Tolerance</th>
<th>99% CI’s</th>
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<td></td>
<td></td>
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<td>6.2</td>
<td>5.0 - 7.3</td>
<td>8.4 *</td>
<td>7.0 - 9.7</td>
<td>11.1 #</td>
<td>9.2 - 13.0</td>
</tr>
<tr>
<td>MPH</td>
<td>5.4</td>
<td>4.3 - 6.4</td>
<td>8.0 *</td>
<td>6.9 - 9.1</td>
<td>10.1 #</td>
<td>8.5 - 11.8</td>
</tr>
<tr>
<td>METH 1</td>
<td>7.2</td>
<td>6.1 - 8.3</td>
<td>8.9 *</td>
<td>7.4 - 10.4</td>
<td>12.6 #</td>
<td>10.1 - 15.1</td>
</tr>
<tr>
<td>METH 3</td>
<td>6.4</td>
<td>5.4 - 7.4</td>
<td>7.7 *</td>
<td>6.3 - 8.1</td>
<td>14.3 †#</td>
<td>12.4 - 16.1</td>
</tr>
</tbody>
</table>

‡ denotes statistical significance from saline pretreated rats * denotes statistical significance from the acute test day # denotes statistical significance from tolerance controls

Discussion

Rats pretreated with MPH and METH during the periadolescent period of ontogeny showed no change in acute morphine-induced antinociception when compared to saline pretreated controls. Following the induction of tolerance, however, rats exposed to METH (3 mg/kg) showed enhanced morphine tolerance compared to saline pretreated controls. This last finding could indicate that periadolescent psychostimulant-induced enhancement of adult morphine tolerance is compound and dose dependent. It is also possible that this result could be an artifact of sampling error. That said, the occurrence of a sampling error in this case seems doubtful, since animals were match paired for distribution into tolerance treatment groups based on hot plate latencies following the fourth morphine injection on the acute test day.
Previous research in our lab found that pre-weanling pretreatment with 5 mg/kg of MPH enhanced acute morphine-induced antinociception and tolerance in adulthood (Cyr & Morgan, 2009). This effect was not replicated in the current study when chronic MPH treatment was administered during the periadolescent period. In both studies hot plate assessments began at PD 60, so the amount of elapsed time between pretreatment and testing was shorter (2 weeks) in the present study. It is possible that this discrepancy in the amount of time between cessation of MPH treatment and morphine testing could explain the differences observed between the previous pre-weanling and current periadolescent studies. This is unlikely, however, because a 2 week withdrawal period following periadolescent MPH exposure was sufficient to produce a robust enhancement of cocaine-induced locomotion and cocaine self-administration (Brandon, et al., 2001). Consequently, it is probable that differences in the development and function of DA systems between these two ontogenic stages account for the divergence in results. More specifically, as the DA system develops during early ontogeny psychostimulant-induced neuronal activation could vary. It follows, that long term changes induced by chronic administration of these drugs would vary depending on the pharmacodynamic action of the agonist and the developmental stage during which exposure occurred (See Chapter 6 for further discussion).

Animals that received saline during the induction of tolerance displayed a rightward shift in the dose response curve compared to the acute test day. This expression of tolerance in tolerance control rats was not expected and could have occurred because behavioral or pharmacological tolerance was induced on the acute test day. Behavioral tolerance is a phenomenon characterized by a progressive increase in sensitivity to a behavioral test following
repeated exposure to the testing apparatus (Lane & Morgan, 2005; Milne, Gamble, & Holford, 1989). For example, repeated nociceptive testing on the hot plate following systemic saline or morphine injection has been shown to decrease paw lick latency (Lane & Morgan, 2005). Moreover, Lane & Morgan (2005) found that repeated testing induced morphine tolerance in saline pretreated animals and enhanced morphine tolerance in morphine pretreated rats. It follows, that the 6 hot plate exposures rats received during the acute test day could have sensitized hot plate responding and induced behavioral tolerance. Alternatively, pharmacological tolerance to morphine could also explain the rightward shift observed in rats that received saline during the induction of tolerance. Recent research in our lab indicates that a single exposure to morphine is sufficient to produce a significant rightward shift in the morphine does response curve 18 hours later (personal communication). Thus, morphine administration on the acute test day could have led to a reduction in the antinociceptive potency of morphine on the tolerance test day.

Previous research has indicated that MPH pretreatment during the pre-weanling period enhances systemically administered morphine-induced antinociception and tolerance in adult rats (Cyr & Morgan, 2009). It makes sense that greater $\mu$-opioid receptor activation acutely would lead to greater desensitization of these receptors with repeated administration of morphine. Thus, the ability of pre-weanling pretreatment with MPH to enhance both morphine-induced antinociception and tolerance is not surprising. It follows, that typical acute $\mu$-opioid receptor activation by morphine would lead to normal levels of tolerance. Consistent with this, periadolescent pretreatment with MPH and METH 1 mg/kg had no effect on acute morphine-induced antinociception or tolerance in adult rats. In contrast, METH 3 mg/kg pretreated animals
displayed no change in morphine-induced antinociception acutely, but showed enhanced
tolerance to morphine. The ability of periadolescent METH 3 mg/kg pretreatment to enhance
morphine tolerance, but not acute morphine antinociception raises questions. Mechanistic
theories about opioid-induced antinociceptive tolerance are plentiful in the scientific literature,
but a consensus has not been reached. As a result, how repeated administration of opioids
produce tolerance to opioid-induced antinociception remains undetermined. That said, μ-opioid
receptor phosphorylation, desensitization, downregulation, and internalization are molecular
events that have been implicated (For review see Borgland, 2001). It is possible that chronic
periadolescent METH 3 mg/kg exposure is causing long term changes in the brain which impact
one of these molecular events without concomitantly changing responsiveness to the acute
effects of morphine.

The fact that only periadolescent METH 3 mg/kg pretreatment produced an enhancement
of morphine tolerance in adult animals indicates that this finding is drug and/or dose dependent.
Specifically, since MPH and METH act differently at the DAT, differences in the
pharmacodynamic activity of these drugs may be important to the long term psychostimulant-
induced changes in morphine tolerance observed in the present study (See Chapter 6 for further
discussion). Additionally, because chronic periadolescent exposure to METH 1 mg/kg had no
effect on adult morphine tolerance, it is likely that the dose of psychostimulant used during
pretreatment is important. MPH and METH (1 and 3 mg/kg) produced comparable increases in
locomotor activity acutely in periadolescent rats, indicating similar DAT mediated neuronal
activation. Although acute activation did not appear to differ, periadolescent sensitization to the
locomotor activating effects of psychostimulants has been shown to occur following repeated
psychostimulant treatment (Laviola, Adriani, Terranova, & Gerra, 1999; McPherson & Lawrence, 2006). It follows that daily exposure to METH 3 mg/kg across the dosing period (PD 35 – 44) could have led to enhanced neuronal activation during repeated administration that would not have been witnessed behaviorally on the first injection day.

Tolerance to the analgesic effects of morphine is a potential problem in clinical chronic pain populations (For review see Nicholson, 2003) and the use of psychostimulants occurs regularly in human adolescents (Substance Abuse and Mental Health Services Administration, 2007). Fortunately, the majority of the findings in the present study indicate that psychostimulant exposure during the periadolescent period does not alter later tolerance to morphine. The exception to this is that periadolescent METH 3 mg/kg exposure enhanced morphine tolerance. Consequently, it still may be important for prescribing clinicians to know about past METH use when determining appropriate pharmacological treatment for the management of chronic pain.
CHAPTER FIVE:

EFFECTS OF ADULT MPH AND METH EXPOSURE ON ADULT MORPHINE-INDUCED ANTI-NOCICEPTION AND TOLERANCE

ABBREVIATED TITLE: ADULT SYSTEMIC STUDY
Introduction

Illicit use of both psychostimulants and opioids is common. Taken together with the wide usage of both drug classes clinically, it is reasonable to assume that a significant number of adults use psychostimulants at one point and opioids at a later point. Very little research has been done examining the effects of adult psychostimulant exposure on opioid-induced antinociception. In the one study found, researchers determined that daily injections of cocaine (30 mg/kg) for 3 days in rats did not alter morphine-induced antinociception on the hot plate test (Lutfy & Maidment, 2002). This result was observed when rats were tested both 24 hours and 7 days after the last cocaine injection. Tolerance to morphine was not assessed.

The disparities between the study conducted by Lutfy & Maidment (2002) and the pre-weanling studies where antinociception was enhanced (Cyr & Morgan, 2009; Halladay, et al., 2009) might be due to differences in the age of the rats during which psychostimulant exposure occurred. Specifically, since pre-weanling MPH exposure enhanced adult morphine-induced antinociception and adult cocaine exposure did not, the period of ontogeny wherein psychostimulant exposure takes place may be of crucial importance. This hypothesis is supported by the fact that periadolescent MPH exposure had no effect on adult morphine-induced antinociception and tolerance (Chapter 4) and pre-weanling MPH exposure enhanced morphine induced antinociception and tolerance. It is possible however that the duration of psychostimulant exposure, the length of the washout period, and/or the psychostimulant compound used may be responsible for the lack of effect in adult cocaine pretreated rats. It is therefore the purpose of this study to examine the effects of 10 day adult METH and MPH exposure on morphine-induced antinociception and tolerance. Given that psychostimulants tend
to cause prolonged changes in opioid-induced antinociception when administered during development only (Cyr & Morgan, 2009; Halladay, et al., 2008; Chapter 4), it is hypothesized that pretreatment with METH and MPH will not alter acute morphine-induced antinociception or tolerance.

**Methods**

*Subjects*

Subjects were 73 adult male Sprague-Dawley rats, purchased from Harlan (Livermore, CA). Rats were group housed with littermates throughout the experiments. The colony room was kept under a reverse 12 L: 12 D cycle and maintained at 22 - 24°C. Rats were given continuous access to food and water throughout the experiment except during testing. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This experiment was approved by the Animal Care and Use Committee at Washington State University.

*Drugs*

MPH hydrochloride and METH (Sigma Aldrich, St. Louis, USA) were dissolved in saline and injected i.p. at a volume of 1 ml/kg. Morphine sulfate (a gift from NIDA) was dissolved in saline and injected s.c. at a volume of 1 ml/kg.

*Pretreatment*

Starting at PD 55 rats were randomly assigned to one of four pretreatment groups and received daily i.p. injections of saline, MPH (5 mg/kg), or METH (1 or 3 mg/kg). These daily injections continued for 10 consecutive days at which time rats were left undisturbed except for
weekly tail coloring and colony maintenance until PD 80. The dose of 5 mg/kg of MPH was chosen because it was found to alter morphine responsiveness in adulthood after pre-weanling MPH exposure (Cyr & Morgan, 2009). The doses of 1 and 3 mg/kg of METH were chosen based on Chapter 3 of this dissertation.

**Nociceptive Assessment**

Nociception was assessed using the hot plate test. The hot plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5°C plate. The rat was removed from the hot plate if no response occurred within 50 s.

**Experiments**

The procedure was identical to Chapter 4 except that adult, not periadolescent rats were pretreated with MPH or METH. Specifically, two experiments were carried out. Both experiments used a quarter log cumulative dose procedure in which increasing cumulative doses of morphine were injected s.c. every 20 minutes (1.8, 1.4, 2.4, 4.4, and 8 mg/kg) resulting in quarter log doses of 1.8, 3.2, 5.6, 10.0, and 18 mg/kg. Nociception was assessed using the hot plate test 15 min after each injection. Experiment 1 assessed the effects of adult (PD 55 - 64) MPH and METH treatment on acute morphine antinociception at 80 days of age (analogous to adulthood in humans). Following Experiment 1, on PD 81, rats were match paired based on hot plate latency after the fourth morphine injection on the acute test day and were further divided into tolerance treatment groups. Half of the animals in each pretreatment group (MPH 5 mg/kg, METH 1 mg/kg, METH 3 mg/kg, or saline) were injected with either saline or morphine (5 mg/kg, s.c.) twice daily for two consecutive days from PD 81 - 82. Experiment 2 was conducted
on PD 83 and assessed the effects of adult MPH and METH pretreatment on morphine-induced antinociception following the induction of tolerance.

Data Analysis

Baseline nociceptive hot plate latency was analyzed using a one way ANOVA with pretreatment as the between group factor. Following the induction of tolerance baseline hot plate latency was analyzed using a 2 x 4 ANOVA with pretreatment and tolerance treatment as the between group factors. Morphine dose response curves and the D<sub>50</sub> values were calculated for all groups using non-linear regression (GraphPad Prism 5 software) (See Appendix C for more information). The lower limit for calculating D<sub>50</sub> values was set at the mean baseline hot plate response. The upper limit was set at the mean response produced by the highest dose of morphine (18 mg/kg). Changes in D<sub>50</sub>’s were assessed using ANOVA (GraphPad Prism 5 software). Because multiple post hoc comparisons with statistical testing can lead to a higher chance of Type 1 error, significance for post hoc comparisons of D<sub>50</sub> values was adjusted and set at p < 0.01. Significance for all other statistical analyses was set at p < 0.05.

Results

Experiment 1: Acute Antinociception

Acute nociceptive baseline hot plate latencies differed according to pretreatment group (F (3, 65) = 2.81, p < 0.05; See Figure 14). Post hoc analysis indicated that the difference in acute baseline responding existed between MPH and METH 1 mg/kg animals (p = 0.039). Adult pretreatment with MPH, METH 1 mg/kg, and METH 3 mg/kg did not alter baseline hot plate latency when compared to saline pretreated controls (p = 0.19, p = 0.82, and p = 0.89).
Morphine administration produced a dose-dependent antinociception in all groups (Figure 15). Rats pretreated with MPH, METH 1 mg/kg, and METH 3 mg/kg during adulthood were less sensitive to the acute antinociceptive effects of morphine as indicated by a significant rightward shift in the morphine dose response curve compared to saline pretreated controls, (F (3, 357) = 12.40, p < 0.0001; Table 4). Because nociceptive baselines differed according to pretreatment condition additional analysis was conducted following data conversion to % MPE. The results did not differ from the original analysis (F (3, 357) = 12.68, p < 0.0001). The D₅₀’s for acute morphine antinociception in all three of the psychostimulant pretreated groups were outside the 99% confidence interval for morphine antinociception in the saline pretreated animals.
Figure 14. Compared to saline, adult pretreatment with MPH or METH (1 and 3mg/kg) from PD 55 - 64 had no effect on baseline responding (n = 17 - 22 per group). MPH pretreated animals had higher baseline latencies compared to METH 1 mg/kg pretreated animals. * denotes statistical significance $p < 0.05$
Figure 15. Adult morphine antinociceptive potency following adult pretreatment with saline, MPH, METH 1 mg/kg, or METH 3 mg/kg. Pretreatment with psychostimulants from PD 55 - 64 reduced morphine-induced antinociception compared to saline pretreated controls when measured by the hot plate test on PD 80.
Experiment 2: Morphine Tolerance

In adult pretreated animals, psychostimulant pretreatment ($F (3, 71) = 0.34, p = 0.80$), and tolerance treatment ($F (3, 71) = 0.16, p = 0.70$) did not affect baseline responding following the induction of tolerance (See Figure 16).

MPH and METH 1 mg/kg pretreated rats treated with morphine for 2 days showed a reduction in morphine potency compared to similarly pretreated rats treated with saline for 2 days ($F (1, 82) = 25.99, p < 0.0001; F (1, 82) = 7.008, p = 0.0097$). Saline and METH 3 mg/kg pretreated rats did not show a significant difference ($F (1, 112) = 0.6815, p = 0.4108; F (1, 81) = 0.6330, p = 0.4286$). Between pretreatment group analysis of animals treated with twice daily morphine for two days suggests that pretreatment had no effect of morphine tolerance (Raw data: $F (3, 177) = 2.296, p = 0.0794$; % MPE: $F (3, 177) = 1.990, p = 0.1174$). That is, morphine potency was comparable following repeated morphine injections whether rats were pretreated with a psychostimulant or not. Given, the contradictory nature of these findings, and the finding that no tolerance was evident in saline pretreated rats, a closer examination of the data is required.

Similar to the periadolescent pretreated animals in Chapter 4, rats in the tolerance control groups displayed a rightward shift in the morphine dose response curve compared to the acute test day. Additional within pretreatment group analyses confirmed that compared to the acute test day, saline and METH (1 and 3 mg/kg) pretreated animals showed a reduction in morphine-induced antinociception following 2 days of twice daily saline injections ($F (1, 162) = 51.37, p < 0.0001; F (1, 127) = 8.012, p = 0.0054; F (1, 126) = 58.26, p < 0.0001$; Table 4; Figure 17). MPH pretreated animals did not show a difference in morphine potency from the acute test day.
following exposure to saline for 2 days ($F(1, 122) = 2.156, p = 0.1446$). These within pretreatment group findings indicate that saline and METH 3 mg/kg pretreated rats had already developed maximum tolerance following exposure to morphine on the acute test day.

Further analysis indicated that morphine-induced antinociception in the saline tolerance treatment groups differed between pretreatment groups (Raw data: $F(3, 177) = 5.73, p = 0.0009$; % MPE: $F(3, 177) = 10.96, p < 0.0001$). Specifically, morphine potency was less in METH 3 mg/kg pretreated animals than MPH and saline pretreated animals in the same tolerance control condition as evidenced by a lack of overlap in the 99% confidence intervals. However, because METH 3 mg/kg pretreated animals were acutely less sensitive to morphine than saline pretreated rats, comparisons between these 2 groups are not meaningful. Moreover, because MPH and METH (1 and 3 mg/kg) pretreated animals displayed less morphine antinociception on the acute test day compared to saline pretreated rats, the between group analysis of animals that received morphine during the induction of tolerance only indicates a lack of difference in the relative potency of morphine on PD 83. Therefore, concluding that the magnitude of change in morphine potency from the acute test day does not differ between groups could be incorrect. Specifically, because psychostimulant pretreated animals showed significantly less morphine-induced antinociception compared to saline controls on the acute test day, the lack of a significant between group effect on the tolerance test day suggests that the magnitude of change in morphine potency caused by repeated morphine administration was less in psychostimulant pretreated animals.

Given that acute antinociception differed and rats in the tolerance control condition show variable shifts in morphine potency, the magnitude of tolerance between the pretreatment
conditions could not be compared. In an attempt to address this issue individual $D_{50}$ values were calculated so the change in log units between the acute and tolerance test days could be examined with a 4 x 2 ANOVA using pretreatment and tolerance treatment as the 2 factors. The results from this analysis indicated that regardless of pretreatment a greater shift occurred in animals treated with morphine compared to animals treated with saline following the induction of tolerance $F (1, 63) = 5.87, p = 0.0183$). Comparison between pretreatment groups was just shy of statistical significance ($F (3, 63) = 2.22, 0.094$). This lack of significance is probably caused by a reduction in statistical power (caused by a reduction in sample sizes) and the high degree of variability between pretreatment groups. Comparison of morphine potency before and after the induction of tolerance reveals a trend that suggests the change in $D_{50}$ varies with drug treatment. MPH pretreated animals showed a 152% $D_{50}$ shift, followed by METH 3 mg/kg pretreated animals with a 155% $D_{50}$ shift. METH 1 mg/kg pretreated animals showed a 189% $D_{50}$ shift and saline pretreated animals displayed the most robust tolerance with a 204% $D_{50}$ shift.
Figure 16. Baseline hot plate latencies did not differ between adult pretreatment or tolerance treatment groups on PD 83 following the induction of tolerance (n = 8 - 12 per group).
Figure 17. Adult morphine tolerance following adult pretreatment. (A) Saline pretreated rats in the tolerance control condition showed a rightward shift in the morphine dose response. A further shift in the dose response curve did not occur following repeated exposure to morphine. (B) MPH pretreated rats in the tolerance control condition did not express a change in morphine antinociceptive potency. Following repeated morphine exposure a rightward shift in the dose response curve occurred. (C) METH 1 mg/kg pretreated animals in the tolerance control condition displayed a rightward shift in the morphine dose response curve. A further rightward shift occurred following repeated exposure to morphine. (D) METH 3 mg/kg pretreated rats in the tolerance control condition showed a rightward shift in the morphine dose response curve. Following repeated morphine exposure a further shift in the dose response curve did not occur.
Table 4. Comparison of morphine $D_{50}$ values on acute and tolerance test days.

<table>
<thead>
<tr>
<th></th>
<th>Acute</th>
<th>99 % CI’s</th>
<th>Saline</th>
<th>99% CI’s</th>
<th>Morphine</th>
<th>99% CI’s</th>
<th>% Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>5.0</td>
<td>4.2 - 5.8</td>
<td>9.0 *</td>
<td>7.7 - 10.3</td>
<td>10.2</td>
<td>8.2 - 12.2</td>
<td>204%</td>
</tr>
<tr>
<td>MPH</td>
<td>7.5 †</td>
<td>6.4 - 8.5</td>
<td>8.1</td>
<td>6.7 - 9.7</td>
<td>11.4 #</td>
<td>9.0 - 13.8</td>
<td>152%</td>
</tr>
<tr>
<td>METH 1</td>
<td>7.3 †</td>
<td>6.3 - 8.3</td>
<td>9.6 *</td>
<td>7.5 - 11.6</td>
<td>13.8 #</td>
<td>10.8 - 16.9</td>
<td>189%</td>
</tr>
<tr>
<td>METH 3</td>
<td>7.8 †</td>
<td>6.6 - 9.4</td>
<td>10.6 †</td>
<td>9.1 - 12.2</td>
<td>12.1</td>
<td>8.9 - 15.3</td>
<td>155%</td>
</tr>
</tbody>
</table>

% Shifts were calculated as $D_{50}$ morphine ÷ $D_{50}$ acute x 100, within each pretreatment group. † denotes statistical significance from saline pretreated rats * denotes statistical significance from the acute test day # denotes statistical significance from tolerance controls.

Discussion

Pre-weanling pretreatment with MPH leads to an enhancement of morphine-induced antinociception and tolerance in adult rats (Cyr & Morgan, 2009). Results from the current study indicate that the opposite may occur when chronic psychostimulant exposure takes place during adulthood. Specifically, adult psychostimulant pretreated animals (MPH, METH 1 mg/kg, and METH 3 mg/kg) displayed a reduction in acute morphine antinociceptive potency. Overall, rats exposed to morphine during the induction of tolerance showed similar morphine-induced antinociception across all 4 pretreatment groups. However, since psychostimulant pretreated animals showed significantly less morphine-induced antinociception compared to saline controls on the acute test day, the lack of a significant between group effect on the tolerance test day.
suggests that the magnitude of change in morphine potency caused by repeated morphine administration was less in psychostimulant pretreated animals.

The observed reduction of morphine-induced antinociception following chronic adult MPH and METH exposure was not expected because past research has shown that daily administration of a similar psychostimulant, cocaine (30 mg/kg), has no effect on morphine antinociceptive potency (Lutfy & Maidment, 2002). As mentioned previously METH and cocaine act differently at the DAT. Moreover, although cocaine and MPH are both DAT inhibitors, unlike cocaine, MPH has no affinity for the serotonin transporter (SERT). Consequently, it is possible that the divergent results obtained by Lutfy & Maidment (2002) are related to differences in cocaine’s pharmacodynamic activity. Alternatively, compared to Lutfy & Maidment (2002) the duration of psychostimulant exposure was much longer in the current study, so it may be that psychostimulant-induced changes in morphine responsiveness only occur when dosing continues for a prolonged period. Moreover, the effects of cocaine withdrawal may have confounded the results of Lutfy & Maidment (2002) since rats were tested only 24 hrs and 7 days following cessation of psychostimulant exposure.

In addition to the observed reduction in acute morphine antinociceptive potency, limited tolerance was evident in MPH and METH (1 and 3 mg/kg) pretreated rats. A similar, but opposite, effect was observed following pre-weanling MPH exposure (Cyr & Morgan, 2009). Because activation of the µ-opioid receptor is the main mechanism of action by which morphine exerts antinociceptive effects, it makes sense that enhanced or reduced morphine antinociceptive potency would lead to respective increased and decreased µ-opioid receptor desensitization during the induction of tolerance. In line with this, past research indicates that opioid
desensitization plays an important role in the development of tolerance to opioids (For reviews see Borgland, 2001; Kieffer & Evans, 2002; Connor, Osborne, & Christie, 2004). Moreover, electrophysiological data collected from the brain slices of rats made tolerant to the antinociceptive effects of morphine indicates that morphine-induced tolerance is associated with both an increase in the potency of opioids acting on PAG µ-opioid receptors and subsequent enhanced µ-opioid receptor desensitization (Ingram, Macey, Fossum, & Morgan, 2008). Taken together, these findings imply that acute morphine-induced antinociceptive potency is positively correlated with the degree of tolerance expressed following repeated morphine administration.

Following acute morphine antinociceptive testing, saline and METH (1 and 3 mg/kg) pretreated animals that received saline during the induction of tolerance showed robust tolerance on PD 83. This expression of tolerance in tolerance treatment control groups is consistent with the findings of Chapter 4. As mentioned previously, behavioral (repeated testing on the hot plate) or pharmacological (previous morphine exposure) tolerance could be driving this effect. Surprisingly, saline and METH 3 mg/kg pretreated animals did not show further development of tolerance following 2 days of twice daily morphine administration. The lack of a further shift in the morphine dose response curve following 4 additional morphine exposures implies that saline and METH 3 mg/kg animals developed maximal tolerance as a result of morphine antinociceptive testing on the acute test day. This effect was unexpected and suggests that chronic pretreatment with saline and METH 3 mg/kg may decrease the number of morphine exposures needed to induce maximal tolerance.

Treatment of adult ADHD with psychostimulants has become common practice (Castle, et al., 2007; de Graaf, et al., 2008; Fayyad, et al., 2007; Kessler, et al., 2006) and illicit use of
METH and MPH by adults continues to be widespread (Bogle & Smith, 2009; Substance Abuse and Mental Health Services Administration, 2007). If these animal studies extrapolate to humans, prior METH and MPH exposure during adulthood may reduce acute morphine-induced analgesia. Consequently, information about past adult psychostimulant use would benefit doctors and dentists who prescribe opioid medications to treat acute and postoperative pain.
CHAPTER SIX:
GENERAL DISCUSSION

The studies in this dissertation demonstrate that chronic pretreatment with psychostimulants produces long-lasting effects on morphine-induced antinociception and tolerance. These effects varied based on the psychostimulant used for pretreatment and the age at which pretreatment occurred. Specifically, rats pretreated with MPH and METH (1 and 3 mg/kg) during the periadolescent period of ontogeny showed no change in acute morphine-induced antinociception, but rats exposed to METH (3 mg/kg) displayed enhanced morphine tolerance compared to saline pretreated controls (Chapter 4). Differentially, MPH and METH (1 and 3 mg/kg) pretreatment during adulthood led to a reduction in morphine antinociceptive potency acutely (Chapter 5). Moreover, it looks as though adult pretreatment with MPH and METH (1 and 3 mg/kg) also reduced morphine tolerance. Taken together these results indicate that developmental period and compound administered during chronic psychostimulant exposure are important determinates of the direction and magnitude of change in later morphine-induced antinociception and tolerance. These changes, however, do not seem to be exclusively mediated by the vPAG (Chapter 2).

Experimental Design Issues

There are both advantages and disadvantages to the use of within subjects experimental designs. They are useful because more data can be collected using fewer animals. Additionally, the impact of individual differences is reduced, since in a very general sense each animal is
compared to itself. However, as evidenced by the results reported in Chapters 4 and 5 of this dissertation, previous drug exposure and repeated testing can cause changes in subsequent behavioral responding making data interpretation difficult. This difficulty in gleaning meaningful information from data can be exacerbated when a mixed model (repeated measures and between subjects) experimental design is employed. This type of problem was encountered in Chapter 5 of this dissertation. Specifically, because behavioral responding on the acute test day differed according to the between subjects variable, a meaningful statistical comparison of the shift in morphine potency between subjects on the tolerance test day could not be conducted.

It is also important to note that longitudinal developmental studies have several inherent disadvantages. They are extremely labor intensive and costly since animals have to be treated, monitored, housed, and fed over long periods of time. Even when the strictest of care is taken to eliminate possible confounds, it is virtually impossible to conclusively compare the effects of experimental manipulation between subjects in different developmental stages. For example, handling from PD 1 - 19 has been shown to reduce both baseline nociceptive responding and morphine-induced antinociception (Fernández, Alberti, Kitchen, & Viveros, 1999). Moreover, this handling-induced change in baseline nociceptive responding has been shown to persist into adulthood (Stephan, Helfritz, Pabst, & von Horsten, 2002). It follows, that if handling during alternate ontological stages produces differing long term effects on basal nociception and morphine-induced antinociception, comparisons of the effects of manipulations done during different developmental periods would be confounded.
Chronic MPH exposure has been shown to cause alterations in later psychostimulant responsiveness (Achat-Mendes, et al., 2003; Brandon, et al., 2001; Crawford, et al., 2007). Sometimes these alterations are consistent regardless of what age MPH exposure occurred, but often these changes in drug responsiveness are dependent upon the age of the rat during MPH pretreatment. For example, exposing rats to MPH during the pre-weanling (Crawford, et al., 2011), periadolescent (Brandon, et al., 2001), and adult (Schenk & Izenwasser, 2002) stages of ontogeny enhanced later cocaine self-administration regardless of developmental pretreatment period. In contrast, pre-weanling MPH exposure had no effect on later cocaine place preference (Crawford, et al., 2011), but MPH exposure during the preadolescent period reduced cocaine place preference (Andersen, et al., 2002; Carlezon, et al., 2003). It is not surprising then that the developmental stage during which MPH exposure occurs could affect later morphine responsiveness as well. Moreover, as the DA system develops during early ontogeny responsiveness to DA agonists could fluctuate and subsequently long term changes induced by chronic administration of these drugs would vary depending on when exposure occurred.

Previous research in our lab found that pre-weanling pretreatment with 5 mg/kg of MPH enhanced acute morphine-induced antinociception and tolerance in adulthood. This effect was not replicated in Chapter 4 when chronic MPH treatment was administered during the periadolescent period. The lack of enhancement of acute adult morphine-induced antinociception and tolerance in periadolescent MPH pretreated rats suggests that there may be a critical period for enhancement that requires MPH exposure earlier in life.
Adult animals pretreated with both MPH and METH (1 and 3 mg/kg) showed a very different behavioral profile than pre-weanling and periadolescent pretreated animals. Unlike pre-weanling and periadolescent pretreated rats, adult pretreated animals displayed a reduction in acute morphine antinociceptive potency and a possible reduction in morphine tolerance (Chapter 4). The reason for this difference is not known, but changes in DA function across development may be involved.

*Dopamine Systems During Development*

During development an overproduction of synapses and receptors occurs from infancy to pubertal onset and is followed by pruning to adult levels during the transition from adolescence to adulthood (Huttenlocher, 1979). Specifically, a peak in striatal, accumbal, and cortical DA receptor density seems to occur at around PD 40 before reducing to adult levels (Andersen, Rutstein, Benzo, Hostetter, & Teicher, 1997; Levant, Zarcone, Davis, Ozias, & Fowler, 2010). It is possible that these developmental changes in DA receptor numbers could be responsible for the differences in behavioral outcomes observed between rats pretreated during the pre-weanling, periadolescent, and adult periods of ontogeny (Chapters 4 and 5). For example, if DA receptor densities are higher during early development, psychostimulant administration during early development would lead to activation of more DA receptors relative to adult psychostimulant administration. Consequently, these differences in activation could produce variable long term alterations in DA receptor sensitivity. Of course, other age related changes such as variation in the function and distribution of DA receptor subtypes may also play a role.
It is well established that DA receptors modulate inhibitory GABA signaling in multiple brain areas (Meyer, et al., 2009; Michaeli & Yaka, 2010; Sheng, et al., 2009; Tseng & O'Donnell, 2007a, 2007b). Consequently, GABA transmission may be involved in the long term changes observed in morphine antinociceptive potency following chronic psychostimulant exposure. In support of this hypothesis, Kroener & Lavin (2010) found that of D2 receptor-mediated GABA inhibition was abolished in animals that received 7 days of cocaine during adulthood followed by a 2 week washout period. Consequently, in cocaine sensitized rats D1 receptor-dependent increases in GABAergic synaptic transmission dominated. Adult chronic psychostimulant-induced elimination of D2-mediated inhibition of GABA could explain the reported reduction in morphine-induced antinociception observed in MPH and METH pretreated animals (Chapter 5). Specifically, since morphine produces antinociception by inhibiting GABA neurotransmission via activation of μ-opioid receptors located on GABA terminals, increases in GABA signaling would reduce morphine-induced antinociception.

Recent research has also indicated developmental differences in DA mediation of GABA signaling. Specifically, striatal D2-like receptor-mediated presynaptic inhibition of GABA decreases from PD 12 - 60 (Momiyama, 2002) and activation of D2 receptors can excite GABA neurons, only after puberty (Tseng & O'Donnell, 2007b). Taken together these results imply that, during early development D2 receptor activation inhibits GABA signaling exclusively, but following puberty can either facilitate or inhibit GABA neurotransmission. It is possible then that chronic psychostimulant-induced D2 receptor activation could exert differing long term effects on GABA transmission depending on the age at which psychostimulant exposure occurred.
Even though animals pretreated with MPH during periadolescence showed no change in morphine responsiveness in adulthood, periadolescent animals chronically exposed to 3 mg/kg of METH showed an enhancement in morphine tolerance as adults (Chapter 4). This could suggest a compound dependent effect when pretreatment occurs during periadolescence. Although both MPH and METH increase DA content in the synapse by actions at the DAT they do so in different ways. Specifically, MPH binds to DAT and subsequently prevents DA from being cleared from the synapse whereas METH binds to DAT causing it to act in reverse and transport free DA out of the nerve terminal. These differences in pharmacodynamic activity between METH and MPH might explain why only periadolescent METH (3 mg/kg) pretreatment enhanced later morphine tolerance (Chapter 4).

Unlike DAT inhibitors (MPH), the ability of DAT releasers (METH) to enhance DA neurotransmission is not related to the presence of basal DA in the synapse. That said, if basal levels of DA efflux differ across development, it would make sense that the amount of DA signaling and DA receptor activation facilitated by DAT inhibitors (MPH) would vary according to ontogenic stage. In support of this, it has been shown that in the striatum periadolescent animals display lower DA release when compared to adult animals (Stamford, 1989). Moreover, DAT inhibitors (MPH) but not DAT releasers (METH) induce much greater dopamine efflux in PD 28 relative to PD 70 rats (Walker, et al., 2010). Differences in acute behavior have also been observed. In particular, Walker, et al. (2010) found that psychostimulant-induced acute locomotion was greater in preadolescent compared to adult animals when the psychostimulant blocked DAT, but not when it facilitated DA release from DAT. These finding suggests that
during development, the way a psychostimulant acts at DAT to increase DA neurotransmission may be crucially important to acute and long term behavioral outcomes.

In Chapter 3 acute psychostimulant-induced locomotion was greater in periadolescent rats regardless of the psychostimulant compound administered. Specifically, compared to MPH (5 mg/kg) treated animals METH (1 and 3 mg/kg) treated rats showed similar levels of overall locomotor activation in both periadolescence and adulthood. This finding indicates that while the acute locomotor activating effects of DAT inhibitors (MPH) and DAT releasers (METH) are greater in periadolescent compared to adult animals, they do not differ according to pharmacodynamic activity at the DAT. That said, differences in how a psychostimulant works at the DAT during periadolescence could still be important to later morphine antinociceptive potency because the acute locomotor activating effects of these drugs do not seem to be related to the magnitude (Chapter 5) or direction (Chapter 4 and 5) of later morphine responsiveness.

**Relationship Between Tolerance and Acute Antinociception**

Acute administration of morphine causes µ-opioid receptor activation and repeated morphine administration leads to morphine tolerance. Both antinociceptive potency and µ-opioid receptor densensitization have been suggested to influence the expression of tolerance to the antinoceceptive effects of morphine (Ingram, Macey, Fossum, & Morgan, 2008). It follows that greater acute antinociception would lead to enhanced tolerance and less acute antinociception would lead to decreased tolerance. It is not surprising then that exposure to MPH and METH (1 and 3 mg/kg) during adulthood reduced acute morphine-induced antinociception and seemed to also reduce morphine tolerance (Chapter 5). A similar, but opposite relationship was evident in
pre-weanling rats treated chronically with MPH, since MPH pretreated rats showed enhanced morphine-induced antinociception and tolerance (Cyr & Morgan, 2009). Moreover, compared to saline pretreated animals, rats pretreated with MPH and METH 1 mg/kg during periadolescence showed no change in antinociception or tolerance (Chapter 4). Similarly, pre-weanling MPH pretreatment did not alter intra-vPAG-induced antinociception or tolerance (Chapter 2). Only periadolescent rats pretreated with METH 3 mg/kg do not seem to follow this pattern. Thus, most of the data suggest that acute morphine antinociceptive potency is positively correlated with the expression of tolerance to morphine.

Possible Brain Areas Responsible for the Alterations in Morphine Antinociceptive Responsiveness Following Psychostimulant Exposure

While it is obvious that chronic MPH and METH exposure have enduring effects on morphine responsiveness, the underlying mechanisms and brain areas responsible are unknown. PAG involvement seems possible given that it receives input from the cortex, limbic forebrain, and diencephalon and projects to the rostral ventral medulla (RVM) which relays the message to the dorsal horn of the spinal cord. Within the PAG, GABA tonically inhibits projection neurons to the RVM. Under certain conditions such as fear (Baptista, Bussadori, Nunes-de-Souza, & Canto-de-Souza, 2009) and opioid administration (Vaughan & Christie, 1997) respective GABA firing within the limbic forebrain and PAG is prevented. This disruption in tonic GABA release leads to antinociception. The vPAG also contains dopaminergic neurons (Hokfelt, Johansson, Fuxe, Goldstein, & Park, 1976) and microinjection of DA agonists into the vPAG produces antinociception (Meyer et al., 2009). However, the results of the studies conducted in Chapter 2
suggests that vPAG DA circuitry is not exclusively involved in the enhanced adult morphine-induced antinociception and tolerance observed following chronic pre-weanling MPH exposure. That said, the possibility that the PAG is involved cannot be ruled out, since multiple brain areas could be co-dependently driving the psychostimulant-induced changes in morphine responsiveness seen across development.

Because the effects of chronic psychostimulant exposure on later morphine antinociception and tolerance appear to differ based on the age of the rat during psychostimulant exposure, it makes sense that relevant circuitry that develops to maturity between PD 10 - 60 would be involved. The descending pain pathway is one such system. Specifically, prior to PD 21 the pathways from the PAG to the RVM and the RVM to the spinal cord are immature and weak relative to adult strength (Bardoni, 2009) (Figure18). This relative weakness has been demonstrated behaviorally since PAG activation does not produce antinociception until PD 21 (Fitzgerald, 2005). Because morphine microinjection into the vPAG could leave the signaling of neurons projecting to the PAG unaffected, the descending pain pathway may still be involved. In particular, changes in neurotransmission in brain areas that project to the PAG, such as the VLO and VTA, warrant investigation. It is also possible that the underlying mechanisms of MPH-induced alterations in morphine antinociceptive potency are exerting effects downstream of the PAG. More specifically, it appears as if prior to PD 21 RVM afferent projections produce a net excitatory effect on dorsal horn neurons that contribute to nociception, whereas following PD 21 RVM afferent projections inhibit dorsal horn neurons, resulting in antinociception (Hathway, Koch, Low, & Fitzgerald, 2009). These findings indicate that the balance of excitatory and inhibitory influence of descending RVM fibers changes during early development, first acting in
a facilitatory manner and then shifting to exert predominantly inhibitory control over the spinal pain circuits (Bardoni, 2009). It is possible that downstream antinociceptive signaling in the RVM is being altered as a function of psychostimulant exposure and the results of the alterations are developmentally dependent upon the balance of excitatory and inhibitory signaling from RVM to the spinal cord. Upstream limbic and cortical areas that are involved in morphine reward and have DA modulation of GABAergic inhibition could be concomitantly or alternately involved as well.

Figure 18. Prior to PD 21 pain modulating pathways projecting from the PAG to the RVM and from the RVM to spinal cord system are weak relative to rats greater than 28 days old. (From Bardoni, 2009).
Clinical Implications and Future Directions

The studies contained within this dissertation indicate that chronic psychostimulant exposure during periadolescence and adulthood changes later morphine antinociceptive responsiveness. Furthermore, previous work has shown that MPH pretreatment during the pre-weanling period of ontogeny enhances subsequent morphine-induced antinociception and tolerance (Cyr & Morgan, 2009). Although the direction and magnitude of these effects seem to be dependent on the age at which exposure occurs, these changes in drug responsiveness persist long after psychostimulant treatment has ceased.

Opioid addiction and tolerance can be obstacles in effective long term pain management and as such are frequently discussed among scientists, clinicians, and the general public alike. If the previous and current findings presented in this dissertation extrapolate to humans it could be important for physicians to know about prior psychostimulant use when prescribing opioid drugs for pain relief. Moreover, given the popularity of MPH use clinically and METH abuse illicitly, future research in this area that seeks to elucidate the mechanisms and brain areas involved in chronic psychostimulant-induced changes in antinociceptive potency is necessary. Additional developmental studies are also warranted. In particular, the effects of preadolescent psychostimulant exposure on later morphine antinociceptive potency should be investigated. Finally, because the development of tolerance to morphine could be altered in chronic pain states determining the effect of psychostimulant exposure on morphine tolerance during chronic pain is also needed.
REFERENCES


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APPENDIX A

Study 1: Diagram of Experimental Design and Planned Analyses

Indicates between pretreatment group D50 comparisons using ANOVA.

Indicates between tolerance treatment group D50 comparisons using ANOVA. Two analyses were conducted and each analysis only included animals that received the same pretreatment drug.

Indicates further division into tolerance treatment groups.
Appendix B. Compared to saline pretreated animals, pre-weanling pretreatment with MPH did not alter intra-vPAG morphine-induced antinociception or tolerance.
APPENDIX C

Study 3: Diagram of Experimental Design and Planned Analyses

Indicates between pretreatment group D_{50} comparisons using ANOVA. If a significant F value was obtained between pretreatment group differences were determined using the 99% CI’s.

Indicates between tolerance treatment group D_{50} comparisons using ANOVA. Four analyses were conducted and each analysis only included animals that received the same pretreatment drug.

Indicates further division into tolerance treatment groups.
APPENDIX D

Study 4: Diagram of Experimental Design and Planned Analyses

Indicates between pretreatment group D_{50} comparisons using ANOVA. If a significant F value was obtained between pretreatment group differences were determined using the 99% CI’s.

- - - - - Indicates between tolerance treatment group D_{50} comparisons using ANOVA. Four analyses were conducted and each analysis only included animals that received the same pretreatment drug.

- - - - - Indicates further division into tolerance treatment groups.