Age-dependent effects of methylphenidate on locomotor sensitization and Netrin-1 receptor expression in the ventral tegmental area

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Abstract

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Repeated exposure to psychostimulants activates mesocorticolimbic dopamine (DA), resulting in an increased locomotor response to the same dose of drug, known as sensitization. Research has yet to establish whether locomotor sensitization differs based on the age when a stimulant treatment is administered. Netrin-1 is a guidance cue in the brain that has been implicated in stimulant-induced locomotor sensitization. Netrin-1 receptors, deleted in colorectal cancer (DCC) and UNC-5, are expressed throughout the mesocorticolimbic system, and repeated amphetamine (AMPH) treatment alters levels of these receptors in the ventral tegmental area (VTA) in an age-dependent manner. To investigate whether methylphenidate (MPH) treatment induces locomotor sensitization and changes in Netrin-1 receptor expression differentially across development, prepubescent and adult rats were administered MPH (2mg/kg) or saline twice daily, for 10 days. One or 4 weeks after treatment ended, rats were administered a dose of cocaine (COC; 10mg/kg) and sensitization was assessed. DCC and UNC-5 levels in the VTA were evaluated through Western blots. The results show that prepubescent rats tested 4 weeks after MPH treatment exhibit significant locomotor sensitization compared to saline controls. However, adults treated with MPH and tested 1 or 4 weeks after treatment, and prepubescent rats tested 1 week after treatment, did not exhibit sensitization. MPH treated rats from both age groups had no significant differences in DCC or UNC-5 expression in the VTA compared to saline controls. These findings indicate prepubescent MPH

treatment induces locomotor sensitization, a behaviour that does not require changes in Netrin-1 receptor expression.

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Stimulant drugs are highly addictive, and there are over 750 000 chronic stimulant users in Canada alone (Canadian Executive Counsel on Addiction, 2005). The negative effects of stimulant drug use have become increasingly apparent in our society; illegal drug use costs Canadian taxpayers over 8.2 billion dollars in prevention and treatment programs annually (Rehm et al., 2007). In order to better understand psychostimulant dependency and to develop prevention/treatment programs, research has addressed the behavioural and neurobiological effects of stimulant drugs, and models explaining the mechanism of action and long-term effects of stimulants are being developed.

Psychostimulant drugs are prescribed to children to treat certain disorders, such as attention-deficit hyperactivity disorder (ADHD). Although this disorder has a well-characterized behavioural profile, which includes poor sustained attention and an abnormally high activity level in inappropriate situations, the neurological underpinnings of ADHD are not fully understood (Williams, 2008). While stimulant drugs seem to mitigate these behavioural problems, the neurobiological mechanisms underlying this treatment are unclear (Novartis, 2006). The lack of information on the neurochemical mechanisms that underlie the therapeutic effects of stimulants, coupled with mounting evidence of their addictive effects in adults, has led to concern for children medicated with stimulants throughout childhood and adolescence. Furthermore, ADHD diagnoses have increased five fold in the Canada over the last 15 years; approximately 8% of Canadian youth are currently diagnosed with the disorder (Skounti, Philalithis, &

Galanakis, 2007). The increasing prevalence of ADHD has resulted in more children being exposed to stimulant drugs, adding urgency to the need for research examining the long-term effects of stimulant treatment during the juvenile period.

Because of ethical constraints surrounding drug testing in children, research on the effects of psychostimulants administered during development is predominantly rodent based. Interestingly, this research has established that prepubertal and pubertal rodents have different behavioural and neurobiological responses to stimulant drugs than adults (Andersen, Arvanitogiannis, Pliakas, LeBlanc, & Carlezon, 2002; Augustyniak, Kourrich, Rezazadeh, Stewart, & Arvanitogiannis, 2006; Tirelli, Laviola, & Adriani, 2003). However, the extent and implications of these developmentally-dependent differences in response to psychostimulants remains unknown. This thesis aims to contribute to this field by contrasting adult and prepubertal behavioural responses to stimulants, and further investigating the neurobiological underpinnings of these behavioural differences.

Behavioural effects of psychostimulants

Psychostimulants, including amphetamines (AMPH), cocaine (COC), and methylphenidate (MPH), are classified together because they have similar effects in the central nervous system, and elicit similar behavioural responses (Holman, 1994; Robinson & Berridge, 2003). One typical behavioural response to repeated administration of stimulant drugs is sensitization, which refers to a gradual increase in the strength of a drug response induced by multiple experiences with the same or similar drugs (Perugini & Vezina, 1994; Pierce & Kalivas, 1997; Tirelli et al., 2003). It has been established that

in adult rodents, repeated psychostimulant administration sensitizes both the incentive properties and the locomotor activating effects of these drugs (Di Chiara & Bassareo, 2007; Robinson & Berridge, 2001; Shimosato & Ohkuma, 2000). These two sensitized responses are considered relevant to each other because the neural substrates that mediate these behaviors overlap (Robinson & Berridge, 2001). Sensitization of these effects of stimulants in rodents is theorized to be a behavioural reflection of the neurobiological changes associated with addiction that are occurring in these animals (Robinson & Berridge, 2003, 2008). Thus, sensitization of the incentive properties and locomotor effects of psychostimulants are critical in animal models of stimulant dependency, as they provide observable measures of stimulant drug effects.

Psychostimulant drugs are highly reinforcing, and initial experiences with these drugs indicate their rewarding nature, resulting in incentive to acquire more drug (Di Chiara & Bassareo, 2007; Robinson & Berridge, 2003). As these incentive properties of stimulant drugs become sensitized, through multiple exposures to the drug, this creates a bias towards drug-associated stimuli, and a persistent drive to obtain and consume the drug (Robinson & Berridge, 2008). For example, once rats are trained to press a lever for COC, the number of lever presses required for each drug administration can be gradually increased, a procedure known as a progressive ratio schedule of reinforcement (McGregor, Baker, & Roberts, 1996; Liu, Morgan, & Roberts, 2007). The greatest number of lever presses that animals are willing to complete to obtain the drug is known as the break point. This point is dose-dependent, as higher doses of COC elicit greater effort to obtain this drug (McGregor et al., 1996). The increase in breakpoints demonstrates that incentive properties of COC have sensitized, as rats have increased the

effort they will expend to acquire drug. Sensitization of the incentive properties of psychostimulants is long lasting, and experience with one stimulant drug results in sensitization to others, known as cross-sensitization. This can be seen in work demonstrating that rats who receive repeated administrations of AMPH will acquire COC self-administration more readily, an effect that persists 45 days after AMPH administration is terminated (Valades & Schenk, 1994; Liu et al., 2007).

Another method of assessing sensitization of incentive properties of psychostimulants is through conditioned place preference (CPP). In CPP a primary reinforcer, such as a stimulant drug, is paired with specific contextual cues, which take on secondary reinforcing properties. In this protocol a rat receives a stimulant drug treatment in one environment and saline in another. On the test day the animal receives no drug, but is allowed to choose its preferred environment. It has been consistently shown that adult rats trained in CPP protocol prefer the drug paired to the saline paired environment (Bardo, Rowlett, & Harris, 1995; Calcagnetti, Keck, Quatrella, & Schechter, 1995). If adult rats are treated with either AMPH or COC prior to the CPP protocol, they will demonstrate a preference for the drug-paired environment in fewer CPP training sessions, and to lower doses of drug in CPP training sessions (Shippenberg & Heidbreder, 1995). This demonstrates that prior experience with psychostimulants sensitized incentive properties of these drugs, as animals more readily associate the drug-paired environment with the rewarding qualities of the drug.

Repeated stimulant administration also results in an enhancement of the locomotor activating effects of psychostimulants. Experiments that assess locomotor sensitization typically involve two phases. First, animals are repeatedly administered a

psychostimulant drug or saline at specific intervals (i.e. daily/every 3 days). Following this treatment, all animals are given a challenge injection of the drug and locomotor activity is recorded. This protocol results in a greater locomotor response in rats that had previously received repeated stimulant treatment when compared to animals that previously received saline treatment (Ferrario et al., 2005; Kuczenski & Segal, 2001; Yang, Swann, & Dafny, 2006). Sensitization of stimulant-induced locomotor activity can be split into two components: induction and expression. The induction of locomotor sensitization includes the transient sequence of cellular and molecular events resulting from repeated psychostimulant administration, which leads to enduring changes in neuronal functioning responsible for the expression of locomotor sensitization (Pierce & Kalivas, 1997). Although locomotor sensitization is seen during this induction phase, there is a marked time-dependency of this locomotor response, as prolonged withdrawal from these drugs (> 72hrs) prior to the challenge intensifies the expression of locomotor sensitization (Vanderschuren & Kalivas, 2000). Once this sensitized locomotor response is developed, it is expressed for months after the initial stimulant treatment, indicating that locomotor sensitization is long-lasting (Leith & Kuczenski, 1982).

Unique pubertal responses to psychostimulants, Although sensitization to psychostimulants is well established in adult animals, a different behavioural profile emerges when stimulant drugs are administered to rodents during the developmental period. Evidence indicates that prepubertal animals, aged 22-35 days, and pubertal animals, aged 35-50 days (Spear, 2000), do not show typical sensitized responses to the incentive properties of psychostimulants (Andersen et al., 2002; Augustyniak et al., 2006; Tirelli et al., 2003). Like adult animals, pubertal animals will readily self-administer both

COC and AMPH (Brandon, Marinelli, Baker, & White, 2001), a behaviour that is developed more readily in pubescent than adults at low drug doses (Shahbazi, Moffett, Williams, & Frantz, 2008). This indicates that psychostimulants are reinforcing in these juvenile rats, because they demonstrate incentive to acquire the drug. However, when a progressive ratio schedule is applied to COC self-administration, pubertal animals have significantly lower breakpoints than adult animals (Shahbazi et al., 2008). Thus, sensitization of incentive properties is attenuated in these young rats, as they exhibit significantly less effort to obtain drug than adult animals.

Results of CPP experiments with juvenile animals support self-administration findings suggesting that young rodents do not sensitize to the incentive properties of stimulant drugs as readily as adults. Prepubertal and pubertal animals show a preference for a drug-paired compartment over a saline-paired compartment, similar to adult rats (Brenhouse & Andersen, 2008; Tirelli et al., 2003). However, if a CPP protocol using COC is administered following a MPH pre-treatment, adult animals pre-treated with MPH and saline display a preference for the drug-paired compartment, while prepubertal animals pre-treated with MPH display a significant attenuation, abolition, or reversal of preference for the drug-paired compartment compared to saline controls (Andersen et al, 2002; Augustyniak et al., 2006). It is possible to elicit typical place preference behaviour in young animals treated with MPH, but only with high doses of COC (20mg/kg; Augustyniak et al., 2006). These findings provide further support for the theory that prepubertal rats show attenuated sensitization to the incentive properties of stimulant drugs.

Although juvenile rodents exhibit attenuated sensitization to the incentive properties of stimulant drugs, their locomotor response to these drugs remains unclear. It has been repeatedly reported that juvenile rodents demonstrate locomotor sensitization to AMPH, COC, and MPH (Adriani, Chiarotti, & Laviola, 1998; Ujike et al., 1994; Yang, Swann, & Dafny, 2003). Thus, initially it appeared that juvenile animals demonstrate the same sensitized locomotor response to stimulants as adult animals. However, research directly comparing the expression of locomotor sensitization in adults and prepubertal rodents indicates that young animals treated repeatedly with AMPH or COC express elevated locomotor sensitization to a challenge dose of COC when compared to adult animals (Adriani, Chiarotti, & Laviola, 1998; Laviola, Wood, Kuhn, Francis, & Spear, 1995). Additionally, unpublished data from this laboratory indicates that repeated exposure to MPH in prepubertal rats induces locomotor sensitization, while the same treatment in adult rats does not (Augustyniak, Lipscombe, & Arvanitogiannis, 2004). However, other studies have reported that pubertal animals repeatedly administered COC show attenuated locomotor sensitization, when compared to adults (Collins & Izenwasser, 2002; Frantz, O'Dell, & Parsons, 2007), which implies a juvenile attenuation of sensitization to the incentive and locomotor properties of stimulants. Age-dependent behavioural responses to stimulants are affected by the type of stimulant drug administered, drug dose, treatment regime, and age at the time of administration (Spear, 2000; Tirelli et al., 2003), which were not held constant between these experiments. Therefore, methodological differences between these experiments could explain these inconsistencies. Clearly further research is needed to determine whether locomotor sensitization to psychostimulant drugs is age-dependent.

Neurobiological effects of psychostimulant drugs

Psychostimulant drugs, including AMPH, COC and MPH, act on the mesocorticolimbic system to induce the behavioural profile associated with repeated stimulant use (Holman, 1994). The mesocorticolimbic system has cell bodies originating in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc), the caudate putamen (CPu), and the prefrontal cortex (PFC) (Di Chiara & Bassareo, 2007). COC and MPH increase availability of dopamine (DA) in this system by blocking binding sites on the DA transporter, DAT, so it is not able to remove DA from the synaptic cleft (Kahlig et al., 2006; Schiffer et al., 2006). In contrast, AMPH binds to DAT and is transported into the cell, inducing reverse transport of DA into the synaptic cleft where it can bind to receptors (Jones, Joseph, Barak, Caron, & Wightman, 1999; Sulzer et al., 1995). Through these mechanisms COC, MPH, and AMPH elevate DA in the VTA, NAc, CPu, and PFC, activating DA receptors in these regions (Ranaldi & Wise, 2001; Robinson & Berridge, 2001)

Repeated stimulant treatment effects lasting changes in DA activity in the mesocorticolimbic system that are involved in sensitization to the incentive properties and locomotor effects of these drugs (Di Chiara & Bassareo, 2007). Activation of dopaminergic neurons in the VTA is required for the induction of locomotor sensitization to AMPH (Vezina, 1996), and the basal activity level of VTA DA neurons, and DA release in the VTA in response to a COC challenge, is elevated during repeated psychostimulant treatment and early withdrawal (Kalivas & Duffy, 1988; Vanderschuren & Kalivas, 2000). However, these changes in the VTA are transient (Zhang, Loonam, Noailles, & Angulo, 2001), so they appear to be involved in short term behavioural

changes associated with stimulant drugs, but not enduring sensitization. In the NAc and CPU, DA levels are significantly increased by an acute dose of COC, and following prolonged withdrawal from a repeated stimulant treatment there significantly higher DA released in the NAc and CPu in response to a challenge dose of COC (Kalivas & Duffy, 1993). The shift from elevated DA activity in the VTA to the striatum during withdrawal is thought to be responsible for the longterm behavioural effects of psychostimulants, such as elevated locomotor sensitization in response to a challenge dose of drug following prolonged withdrawal (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). These changes in mesocorticolimbic circuitry in response to psychostimulant drugs have lasting effects on protein expression and gene regulation in this pathway, which have implications for behaviour. A discussion of this is beyond the scope of this thesis, although reviews on the subject are available (McGinty, Shi, Schwendt, Saylor, & Toda, 2008; Thomas, Kalivas, & Shaham, 2008; Ujike, Takaki, Kodama, & Kuroda, 2002).

Ontogenesis of the mesocorticolimbic DA system. Research has shown that the DA mesocorticolimbic pathway continues to develop into early adulthood (Spear, 2000). For example, DA synthesis in the NAc (Andersen, Dumont, & Teicher, 1997), and DA innervation to the PFC (Benes, Taylor, & Cunningham, 2000), both increase throughout the prepubertal and pubertal periods. Additionally, there are significantly higher levels of DA D1 (D1R) and D2 (D2R) receptors in the striatum during the prepubertal period, which undergo substantial pruning during puberty to reach adult levels (Teicher, Andersen, & Hostetter, 1995) Although the implications of these, and other, age-dependent differences in the mesocorticolimbic DA system are not fully understood, it is

clear that psychostimulant drugs act on functionally and structurally different systems in developing animals. The differing effects of stimulants on the DA system in juvenile and adult rodents result in differences in gene and protein expression in the mesocorticolimbic pathways, which could contribute to age-dependent behavioural effects of stimulants (Andersen et al., 2002; Cao, Lotfipour, Loughlin, & Leslie, 2007). Recent research suggests that there are stimulant-induced, age-dependent changes in protein expression in the Netrin-1 system (Yetnikoff, Almey, Arvanitogiannis, & Flores, 2008), which have been linked to locomotor sensitization (Grant et al., 2007).

Netrin-1

Netrin-1 is a protein, known as a guidance cue, which is critical in directing axon growth in early brain development. Most research on the Netrin-1 system has focused on prenatal development, as Netrin-1 dysfunction during this period results in fatal malformations of the corpus callosum, the hippocampal commisure, and the ventral spinal commisure (Serafini et al., 1996). Research has demonstrated that the Netrin-1 system plays a vital role in prenatal organization of brain circuitry through two primary receptor subtypes, DCC and UNC-5 (Moore, Tessier-Lavigne, & Kennedy, 2007). In the embryonic brain, Netrin-1 protein is released from specific regions of the neural plate. It diffuses, and binds to DCC and UNC-5 receptors found on the growth cones at the tip of extending axons (Moore et al., 2007). Binding at the DCC receptor mediates attraction, causing the axon to grow towards the source of Netrin-1 (Baker, Moore, Jarjour, & Kennedy, 2006). In contrast, binding at the UNC-5 receptor mediates repulsion over short distances, and binding at a DCC/UNC-5 homologue mediates repulsion over longer

distances, causing the axon to grow away from the source of Netrin-1 (Baker et al., 2006). Through receptor mediated attraction and repulsion, axons in the prenatal brain are directed to the appropriate targets.

Netrin-1 and its receptors continue to be expressed in the postnatal brain, after the majority of axon growth is completed (Baker et al., 2006). Preliminary research indicates that Netrin-1 in the postnatal brain is bound to the extracellular matrix, instead of being secreted and diffusing to its target (Manitt & Kennedy, 2002). Thus, it has been hypothesized that Netrin-1 binding at DCC and UNC-5 in the adult brain may be responsible for communication over short distances, localizing the cell to neighboring neurons, and participating in synaptic connectivity. Additionally it has been theorized that Netrin-1 can function as an autocrine cue, binding to receptors on the surface of the cell where it is produced to direct protein production and placement within that cell (Manitt & Kennedy, 2002). However, research on where and how Netrin-1 is produced in the postnatal central nervous system, and its specific role in neural organization after birth, has not yet been conducted.

Psychostimulant drugs have been shown to induce dendritic arborisation within the mesocorticolimbic pathway of both developing (Diaz Heijtz, Kolb, & Forssberg, 2003; Mueller, Chapman, & Stewart, 2006) and adult animals (Robinson & Kolb, 1997, 1999). Furthermore, research has demonstrated that Netrin-1 receptors in the postnatal brain are expressed on dopaminergic neurons in the mesocorticolimbic pathway (Grant et al., 2007; Yetnikoff, Labelle-Dumais, & Flores, 2007). Because Netrin-1 is localized on these DA neurons, and Netrin-1 activity can direct the placement of new proteins and connections between neighboring neurons, it was hypothesized that this protein might

play a role in stimulant induced structural changes in neurons (Yetnikoff et al., 2007). Repeated AMPH treatment, which typically induces locomotor sensitization and place preference in adult mice, does not elicit these behaviours in DCC deficient mice (Flores et al., 2005; Grant et al., 2007). Additionally, repeated AMPH treatment in adult rats results in elevated levels of DCC and UNC-5 in the VTA (Yetnikoff et al., 2007). There appears to be a relationship between psychostimulants and the Netrin-1 system, since the behavioural profile associated with stimulants is not seen in DCC deficient mice, and AMPH treatment induces changes levels of Netrin-1 receptor expression.

Netrin-1's role shifts from the long-range direction of axon growth in the prenatal brain, to short-range communication between cells in the postnatal brain. In addition, the UNC-5 receptor is not fully expressed until early adulthood (Manitt, Thompson, & Kennedy, 2004). Since the function of the Netrin-1 system and UNC-5 expression change throughout development, and stimulant drugs affect this system, it is possible that psychostimulants have developmentally-dependent effects on DCC and UNC-5 expression. To investigate this possibility, AMPH-induced changes in Netrin-1 receptors in prepubertal and pubertal rats were assessed and compared to previous findings in adult animals (Yetnikoff, et al., 2008). AMPH treatment resulted in decreased levels of UNC-5 and DCC expression in prepubertally treated animals, increased UNC-5 in pubertally treated animals, and increased DCC and UNC-5 in rats treated in adulthood (Yetnikoff et al., 2008). These age-dependent changes in DCC and UNC-5 receptors suggest a Netrin-1/DA system interaction across development, which could participate in the enduring effects of juvenile exposure to stimulants on mesocorticolimbic DA circuitry. Further

research is needed to extend these findings, and elucidate the behavioural implications of these changes in Netrin-1.

Experiment 1

Although it is clear that some behavioural responses to psychostimulants are developmentally-dependent, the findings in this field remain incomplete. In particular, additional research is needed clarify how prepubertal locomotor sensitization compares to that seen in adults. The current experiment assessed locomotor sensitization in response to MPH treatment in juvenile and adult rats in an effort to clarify whether this behavioural response to psychostimulants is age-dependent. Specifically, prepubertal animals and adult animals received two injections of MPH (2mg/kg) or saline daily for 10 days, after which half of the rats in each age group underwent a 1 week withdrawal period, and the other half underwent a 4 week withdrawal period. Following this drug free period, all animals were administered an acute dose of COC (10mg/kg), and placed in a locomotor testing box that recorded their activity to assess whether sensitization had developed.

The unique behavioural response to stimulants seen in adulthood, resulting from psychostimulant administration during development, was of particular interest in this experiment. This behavioural response has been referred to as a developmental drug effect, because the drug free period after treatment allows the brain of these young animals to fully develop before testing (Tirelli et al., 2003). Thus, any changes in behaviour observed at the time of testing result from an interaction between drug effects and the developing brain. With the assessment of developmental drug effects as a

priority, rats were treated during the prepubescent period and tested for locomotor activity during puberty (1 week following treatment) or adulthood (4 weeks following treatment). These prepubertally treated rats were compared to rats that received MPH in adulthood to determine whether locomotor sensitization is different in these two age groups. This procedure allowed for the longitudinal assessment of the development and persistence of locomotor sensitization.

MPH was selected as the stimulant drug in this experiment because it has greater ecological validity than other stimulants, as it is routinely prescribed and administered to children to treat ADHD (Mayes, Bagwell, & Erkulwater, 2008). The two daily 2mg/kg injections of MPH administered to rats in this experiment were selected because research has shown that this dose elicits plasma levels of MPH in rats similar to those seen in children in response to a therapeutic dose of MPH (Brandon et al., 2001). Furthermore, MPH was administered twice daily, because MPH is traditionally prescribed to children with ADHD in two daily doses to maintain levels of the drug in the bloodstream throughout the day, and ensure consistent behavioural results (Findling et al., 2006). Previous research in our laboratory has used this dose and treatment regime, thereby facilitating an accurate comparison between experiments. Although we did not intend to specifically model ADHD in the rodent, all efforts were made to ensure clinical-relevance in the treatment regime in an effort to increase the ecological validity of the experiments.

The goal of experiment 1 was to determine whether there were age-dependent differences in locomotor sensitization in response to a clinically-relevant dose and regime of MPH. Based on previous research with similar methodology, it was hypothesized that

rats treated prepubertally would show significantly elevated locomotor sensitization when compared to rats treated as adults.

Experiment 2

Previous research has demonstrated that stimulants induce alterations in the Netrin-1 system, which is hypothesized to play a role in long-term neuronal changes underlying the behavioural responses to these drugs (Flores et al., 2005; Grant et al., 2007; Yetnikoff et al., 2007). Such changes to the Netrin-1 system are age-dependent, as western blot analyses revealed that prepubertal AMPH treatment results in decreased DCC and UNC-5 receptor expression in the VTA, while adult AMPH treatment results in increased receptor expression in the VTA (Yetnikoff et al, 2008). The goal of experiment 2 was to determine whether a clinically-relevant MPH treatment would elicit similar changes in DCC and UNC-5 expression in the VTA, to those seen in response to an AMPH treatment.

Accordingly, this experiment used Western blot analyses to assess both DCC and UNC-5 levels in the VTA of prepubertal and adult animals from experiment 1. Although all animals received an acute dose of COC one hour prior to decapitation (see experiment 1), it has been shown that an acute dose of AMPH two hours prior to decapitation does not induce changes in Netrin-1 receptors (Yetnikoff et al., 2007). As a result, any changes seen in DCC and UNC-5 in experiment 2 should be attributed to the MPH treatment, and not to the acute dose of COC administered prior to locomotor testing. Western blot analyses focused on the VTA, as previous research assessed Netrin-1 receptor levels throughout the mesocorticolimbic DA system and AMPH induced changes were

observed only in the VTA (Yetnikoff et al., 2007). This experiment examined the effects of MPH both 1 and 4 weeks after the end of treatment, so the persistence of any changes in Netrin-1 receptors in prepubertal or adult rats could be assessed.

The premise of this experiment was to extend previous research demonstrating that an AMPH treatment induces age-dependent changes in Netrin-1 receptors, DCC and UNC-5. It was hypothesized that the MPH treatment employed in this experiment would have similar effects to AMPH treatment, decreasing Netrin-1 receptors in rats treated prepubertally, and increasing these receptors in rats treated during adulthood.

Methods

Subjects

The subjects in these experiments were 80 male Sprague-Dawley rats purchased from the Charles River breeding farm (St. Constant, Quebec). All animals were pair-housed in transparent polyethelene shoebox cages (50 × 26.8 × 36.4 cm) lined with wood chips in a humidity- and temperature controlled animal colony (~22 C, 40-45%). The rats were maintained on a 12h light/dark cycle; lights were turned on at 8:00hrs and off at 20:00hrs. They had food and water available *ad libitum* except during locomotor testing.

Subjects were obtained from the breeder at either PND 21(weaning, n=40) or PND 56 (n=40). The animals were given four days to acclimatize to the facilities before the experiments began at PND 25 (juvenile) or PND 60 (adult), respectively. During the four day acclimatization period, all animals were handled daily to familiarize them with the experimenter. All efforts were made to maximize the animals' health and wellbeing,

and experimental procedures and protocols were in accordance with guidelines from the Animal Care Committee of Concordia University.

Drug Administration

Both MPH hydrochloride (Medisca, Québec, Canada) and COC hydrochloride (Medisca, Québec, Canada) were used in these experiments. The drugs were dissolved in 0.9% isotonic saline. Animals in the experimental treatment group received 4mg/kg of MPH daily via two intraperitoneal (i.p.) injections (2mg/kg per injection) during the treatment phase. Animals in the control group received two daily i.p injections of 0.9% isotonic saline during this treatment phase. All animals received a 10 mg/kg challenge dose of COC by i.p. injection on the locomotor test day.

Apparatus

Locomotor testing in response to COC was carried out in boxes specifically designed to measure horizontal locomotion in rodents. These boxes were 41cm x 25 cm x 17cm, and were made of transparent Plexiglas front, back, and top. The sidewalls were aluminum, and the floor was made of stainless steel rods that ran width wise across the box at 1.5cm intervals. Locomotor activity was monitored by eight pairs of photocells located along the longitudinal axis of each box, 4.8 cm above the floor. The locomotor activity boxes were connected to a computer that recorded photocell beam interruptions. Data collection was performed using a MED-PC IV software program, which recorded the number of photo beam interruptions during the habituation session and the locomotor test.

Procedure

This experiment utilized cohorts of same-aged animals (n = 20): two cohorts of prepubertal rats and two cohorts of adult rats. In each cohort half of the animals were randomly assigned to the MPH treatment group, and half served as saline controls.

Experiment 1 – Treatment and Behavioural Testing. On the final day of acclimatization half of each cohort of rats was randomly assigned to the experimental treatment group and the other half was assigned the control group. At this time all animals were weighed to determine the appropriate dose of MPH/saline for the first day of treatment. The animals in the experimental treatment group received two daily injections of MPH (2mg/kg) or saline, the first at 9:00hrs and the second at 15:30hrs. During the afternoon injection session, the animals were weighed to determine the appropriate drug/saline dose for the injections the following day. This treatment phase lasted for 10 days, so rats received pre-treatment from either PND 25-35 in the prepubertal groups, or PND 60-70 in the adult groups.

After the final treatment day, animals were housed in the animal colony during a withdrawal period before their locomotor test. One cohort of juvenile and one cohort of adult animals were left untested 1 week between their treatment and locomotor test. The other two cohorts of rats, one juvenile and one adult, were left for 4 weeks between their treatment and locomotor testing. No experimental manipulations were performed during this period, although the animals were handled approximately every third day.

The day before the locomotor test, either 6 or 27 days after pre-treatment ended, all animals in a cohort were habituated to the locomotor testing boxes. In the habituation session, rats were transported from the animal colony to the laboratory and placed in the

locomotor boxes for 1hr, during which photocell beam interruptions were recorded, before they were returned to the colony. This was done to reduce the anxiety associated with transportation and introduction to a novel environment on locomotor testing, to diminish the effects of these variables on test results. At the end of the habituation session all rats were weighed to determine the appropriate dose of COC for the locomotor test the following day. The locomotor test assessed horizontal locomotion of animals in response to an acute injection of COC. Following COC administration the rats were immediately placed into a locomotor box, and the number of photocell beam interruptions were recorded for 1hr, and stored for later analysis. Immediately following the locomotor test the animals were decapitated, and their brains were removed and flash frozen to preserve them for later analysis.

Experiment 2 - Western Blot Analyses. Brains were sectioned on a cryostat at -15° C, and tissue punches were taken from the VTA. Specifically, two 500μm slices were taken, the first 5.2mm posterior to Bregma, and the second 5.7mm posterior to Bregma (Paxino and Watson, 1998). A stainless steel punch, with an inner diameter of 1.2mm, was used to remove tissue from the VTA, and these samples were stored at -80C. Figure 1 indicates the approximate region from which VTA samples were obtained. VTA samples from each cohort of rats were processed by Western blot analysis to compare levels of Netrin-1 receptors, DCC and UNC-5, in MPH and saline treated animals.

Ice cold lysis buffer containing 1M Tris, 1% NP40, 4M NaCl, 2mM $NaV0_4$, 10mM NaF, and a protease inhibitor cocktail (Sigma, P2714), was added to the VTA tissue samples, and each was sonicated to break up the tissue. The resulting homogenate was centrifuged (10 000 RPM) at 4°C for 5min, and the supernatant was collected.

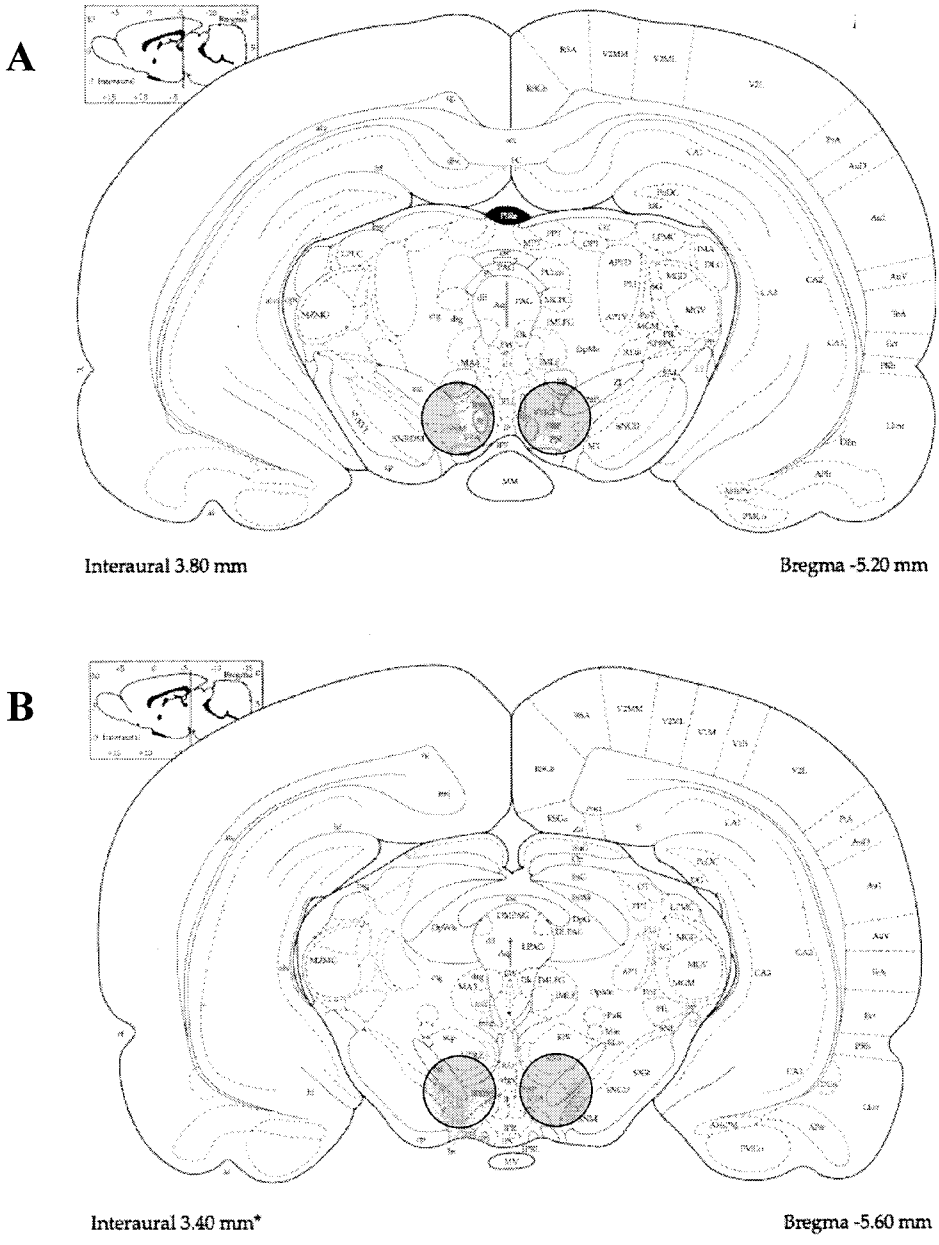

Figure 1 The 500μm brain slices containing VTA tissue taken for Western analyses. (**A**) Approximate location of the first slice, and the specific location of VTA punches. (**B**) Approximate location of the second slice, and the specific location of VTA tissue punches. ◯ represents VTA punches.

A BCA protein assay (Thermoscientific, 23225) was run on all samples in a cohort, and protein concentration in each sample was standardized. Aliquots of each tissue sample from one cohort, containing 25μg of protein, were separated by "Criterion XT" 4-12% Bis-Tris pre-cast gels (BioRad Laboratories, 345-0124), and transferred onto PVDF membrane (BioRad Laboratories, 162-0177).

The membrane was cut at approximately 100kDa, so DCC (180kDa) and UNC-5 (130kDa) proteins were on the top half of the membrane, and tubulin (50kDa) was on the bottom half. These two pieces of membrane were blocked for 45min at room temperature in PBS-T solution (1.5M NaCl, 37mM KCl, 12.5mM $Na_2HPO_47H_2O$, 57mM KH_2PO_4, and 0.2% Tween 20) containing 5% non-fat powdered milk. Then top and bottom halves of the membrane were probed in blocking solution containing the DCC primary antibody and tubulin primary antibody, respectively, for ~ 15hrs (DCC, 1:1000, BD Pharmagen [554222]; Tubulin, 1:2000, Millipore [AB3201]). After exposure to the primary antibody, the membrane was blocked again for 45min at room temperature, and then incubated in either anti-mouse or anti-rabbit (depending on how the primary antibody was raised) IgG HRP labeled secondary for 45min (anti-mouse IgG, 1:4000, Vector Lab [PI-2000]; anti-rabbit IgG, 1:4000, Vector Lab [PI-1000]). Finally the membranes were washed for 45min in PBS-T before chemiluminescent (Perkin Elmer, 203-081001) was applied to allow for visualization of proteins of interest. The immunoreactivity (IR) signal from the membrane was captured using a Kodak Machine, and Kodak software (2007) was used to analyze the IR of the DCC and tubulin signal for each sample.

After the DCC and tubulin IR signals were photographed with the Kodak Machine, both halves of the membrane were rinsed in boiling PBS for 45min to strip all

antibodies off the membrane. After the membranes were stripped, the top was probed with UNC-5 primary antibody (UNC-5, 1:7500, Flores laboratory private supplier), and the bottom was probed again with tubulin primary antibody, for ~15hrs. Following removal from the primary antibody, the procedures described above were used to analyze the UNC-5 and tubulin IR signals from the membrane.

Statistical Analyses

Experiment 1. Locomotor activity scores for the habituation and test days of each cohort of rats were analyzed in separate 2 x 6 mixed ANOVAs. The between-subjects variable was drug treatment (saline or MPH). The within-subjects variable was time, with six 5min bins used to assess behaviour over the first 30min of the habituation session and locomotor test. Any significant effects of this ANOVA were further analyzed with Boneferroni corrected pairwise comparisons.

Experiment 2. Prior to analysis of the Western blots, DCC and UNC-5 receptor densities were normalized relative to tubulin. Tubulin is a structural protein that is not affected by psychostimulant treatment; if all samples were loaded accurately, each sample should have the same tubulin IR. Samples were normalized by dividing the IR signal of DCC or UNC-5 by the IR signal of tubulin from the corresponding sample, which resulted in an IR signal of DCC/Tub and UNC-5/Tub. Any experimental error in sample loading is reflected as a difference in tubulin levels; through normalization, this source of experimenter error is eliminated.

Once DCC and UNC-5 intensities were normalized, the results from each Western blot were analyzed using an independent samples t-test. The MPH-treated animals' mean

intensity IR signal of DCC/Tub and UNC-5/Tub were compared to the mean intensity IR signal of saline-treated animals. These analyses determined whether MPH treatment resulted in any significant changes in the levels of Netrin-1 receptors in the VTA.

Results

Experiment 1

Habituation data. Figure 2A illustrates locomotor results from the habituation session of rats treated with MPH or saline in prepubescence, and habituated 6 days after treatment. A 2x6 ANOVA revealed no significant interaction between time and drug treatment, and no main effect of drug treatment. Locomotor activity from the habituation session of adult rats treated with MPH or saline, and habituated 6 days after treatment ended, also reveal no significant interaction between the variables and no main effect of drug treatment (see figure 2B). The data from prepubescent rats, treated with MPH or saline, and habituated to the locomotor testing environment 27 days after treatment is depicted in figure 3A. An ANOVA demonstrated that there was no interaction between the two independent variables, and no main effect of drug treatment in this cohort. Similarly, in rats treated in adulthood, and habituated to the locomotor boxes 27 following treatment, an ANOVA revealed no significant interaction or main effect of drug treatment in this cohort (see figure 3B). This indicates that the MPH treatment did not affect the locomotor response to the novel testing environment in rats treated prepubertally or in adulthood, either 6 or 27 days after treatment was terminated.

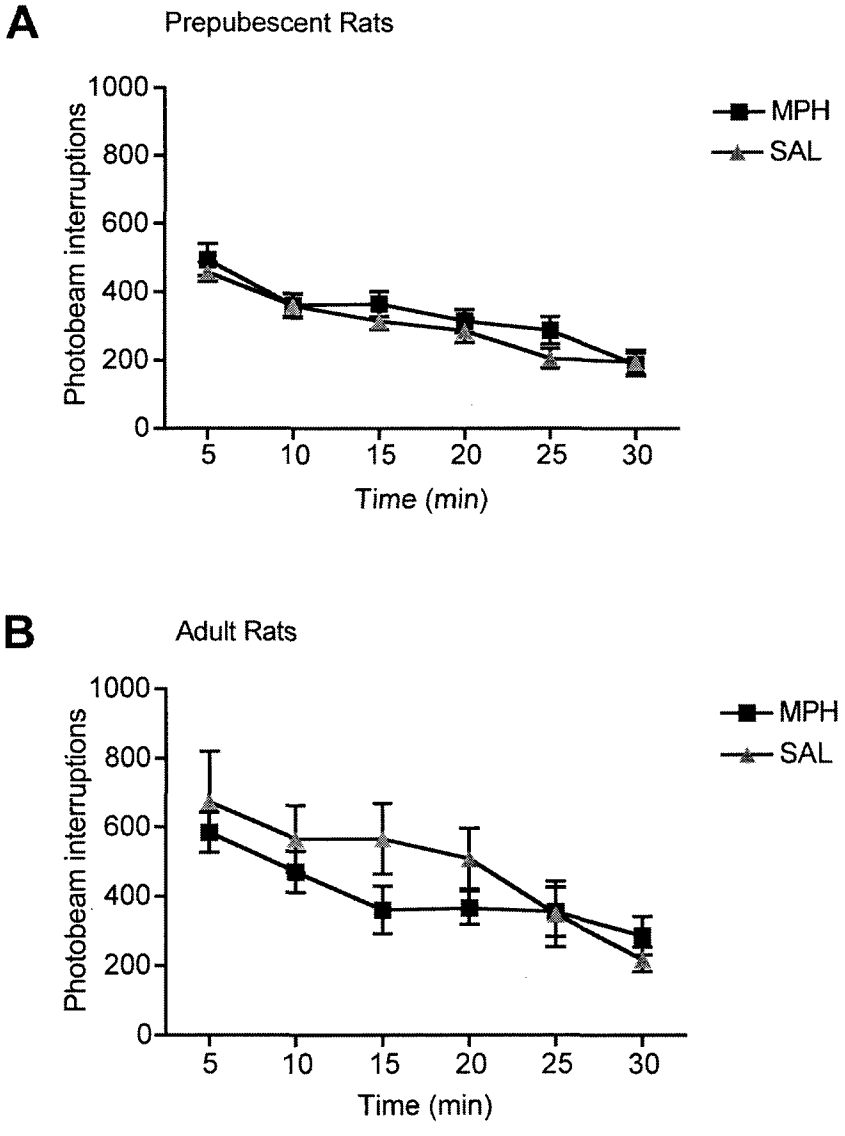

Figure 2 Locomotor activity counts (mean photocell beam interruptions +/- SEM) during the habituation session 6 days following MPH treatment. **(A)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in prepubescence when compared to saline controls. **(B)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in adulthood when compared to saline controls.

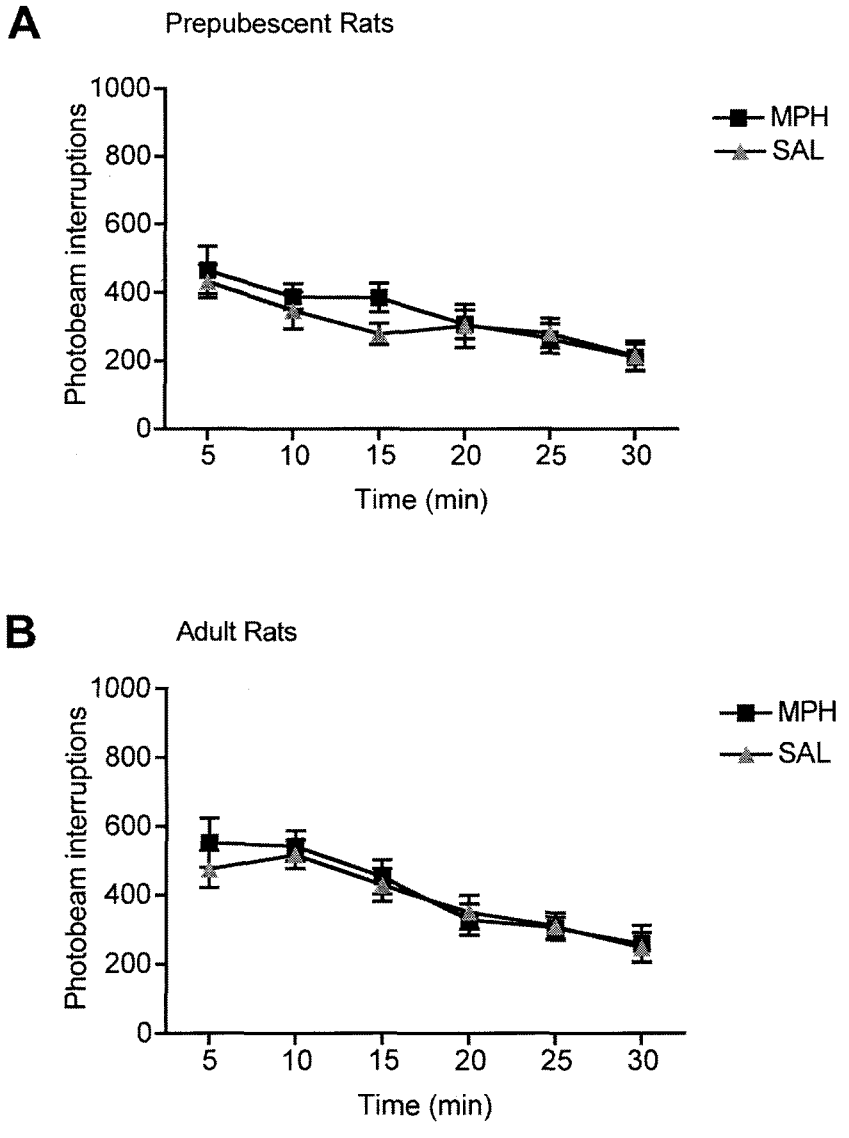

Figure 3 Locomotor activity counts (mean photocell beam interruptions +/- SEM) during the habituation session 27 days following MPH treatment. **(A)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in prepubescence when compared to saline controls. **(B)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in adulthood when compared to saline controls.

Tests 1 week after treatment. Figure 4A depicts the locomotor results from rats treated prepubertally with MPH or saline, and tested 1 week after treatment with an acute COC challenge. Mauchlys test of sphericity was violated ($W = 0.88$, $p = 0.000$), so Greehhouse-Geisser corrections were applied to the data. There was no significant interaction between the two independent variables, and no main effect of drug. Therefore, sensitization did not develop in these rats, as the MPH- and saline-treated animals showed similar locomotor activity in response to COC. A significant main effect of time was found ($F(2.604, 46.88) = 4.590$, $p = 0.001$), and Bonferroni corrected pairwise comparisons were used to investigate this effect. These comparisons show that locomotor activity counts at 5min ($p = 0.042$), 10min ($p = 0.017$) and 15min ($p = 0.007$) were significantly higher than those seen at 30min.

The locomotor activity data of adult rats treated with MPH or saline, and tested 1 week following treatment, also violated Mauchlys test of sphericity ($W = 0.226$, $p = 0.48$) so Greenhouse-Geisser corrections were again employed (see Figure 4B). An ANOVA revealed no significant interaction and no significant main effect of drug treatment, which indicates that MPH treatment did not affect COC-induced locomotion, and suggests that sensitization did not develop. There was a significant main effect of time ($F(3.28, 59.11) = 15.659$, $p = 0.000$), which was further analyzed using Bonferroni corrected pairwise comparisons. These tests showed that there was significantly higher locomotor activity counts at 5 min than at 20min ($p = 0.019$), 25min ($p = 0.000$), or 30min ($p = 0.001$). Locomotor activity at 10min was significantly greater than at 20min ($p = 0.050$), 25min ($p = 0.005$), or 30min ($p = 0.002$). Finally, at 15min significantly higher locomotor activity counts were observed than at 25min ($p = 0.007$), or 30min ($p = 0.001$).

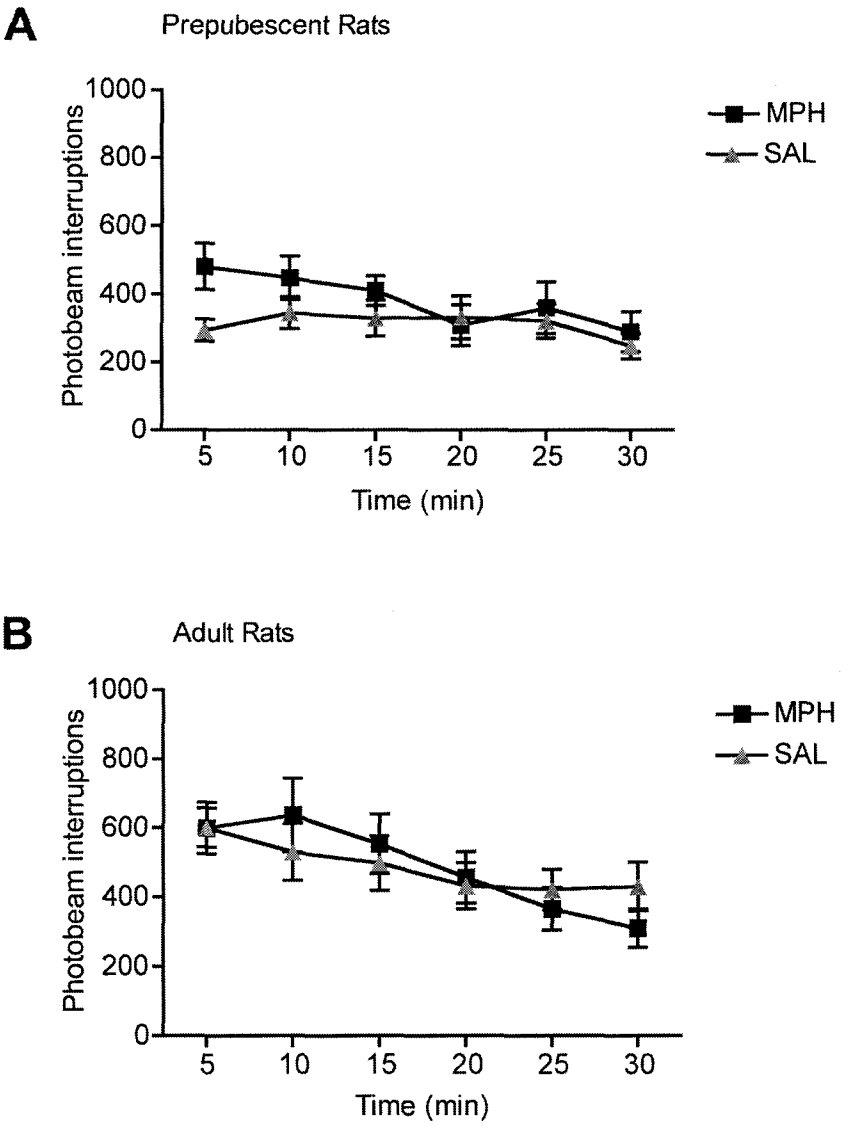

Figure 4 Locomotor activity counts (mean photocell beam interruptions +/- SEM) in response to an acute dose of COC (10mg/kg) 1 week following MPH treatment. **(A)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in prepubescence when compared to saline controls. **(B)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in adulthood when compared to saline controls.

Tests 4 weeks after treatment. Figure 5A illustrates data comparing locomotor activity in response to an acute dose of COC in prepubertal rats treated with MPH or saline. Mauchlys test of sphericity was violated ($W = 0.177$, $p = 0.026$), so Greenhouse-Geisser corrections were applied. There was a significant interaction between the two independent variables ($F(3.31, 56.29) = 3.034$, $p = 0.032$). To further investigate this interaction, Bonferroni corrected pairwise comparisons were run, revealing that MPH treated animals exhibit significantly higher locomotor activity counts than saline controls at 5min ($p = 0.17$) and 10min ($p = 0.47$). No significant difference was found between locomotor activity of MPH and saline treated animals at any other time. These results show that MPH treatment during the prepubertal period resulted in COC-induced sensitization of locomotor activity 4 weeks after the treatment was terminated. There was also a main effect of time in these prepubertally treated rats ($F(3.31, 56.29) = 19.594$, $p = 0.000$). Bonferroni corrected pairwise comparisons indicated that when locomotor activity was examined, regardless of experimental treatment, locomotor activity counts were significantly higher at 5min than at 15min ($p = 0.042$), 20min ($p = 0.015$), 25min ($p = 0.000$), or 30min ($p = 0.000$). These tests also showed that activity counts at 10min time were significantly greater than those seen at 20min ($p = 0.002$), 25min ($p = 0.003$), or 30min ($p = 0.000$). Additionally, locomotor counts at 15min ($p = 0.002$) and 25min ($p = 0.014$) were significantly higher than at 30min.

Figure 5B depicts the locomotor data from adult rats treated with MPH and saline, and challenged with an acute dose of COC 4 weeks later. These data also violated the

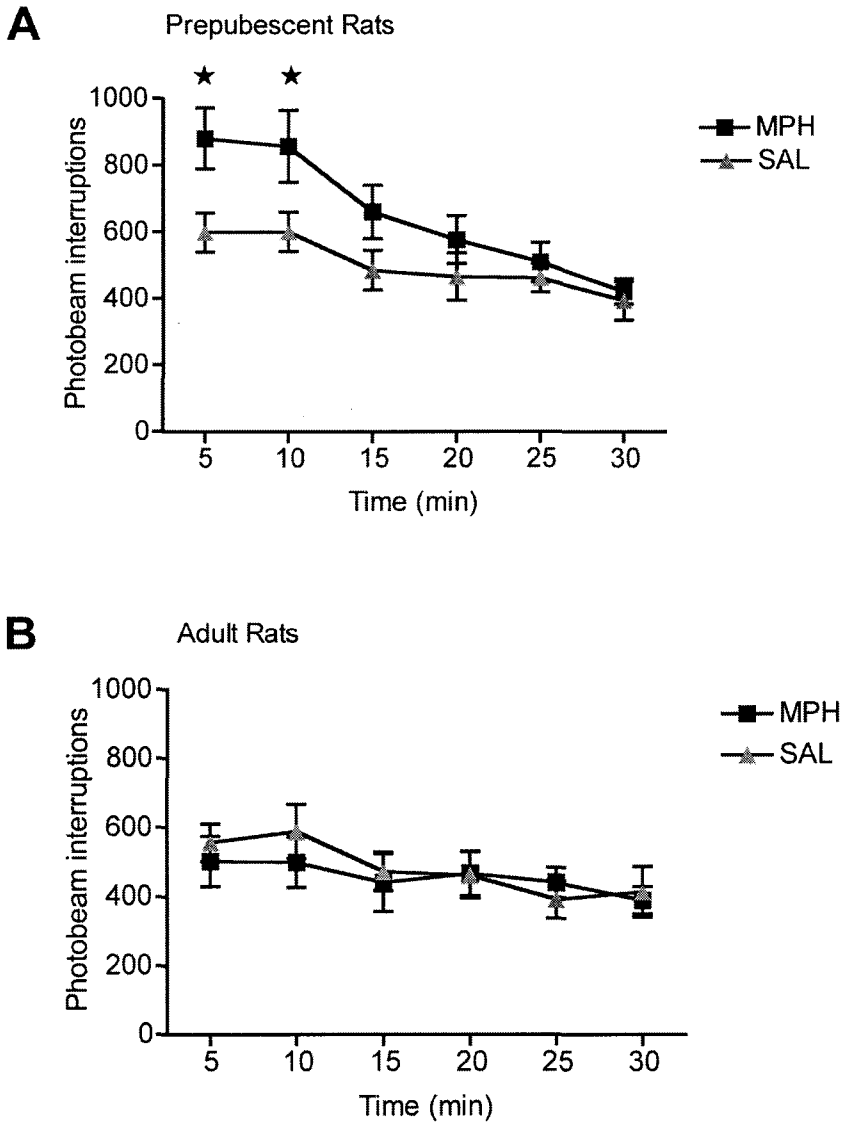

Figure 5 Locomotor activity counts (mean photocell beam interruptions +/- SEM) in response to an acute dose of COC (10mg/kg) 4 weeks following MPH treatment. **(A)** Significantly higher locomotor activity was observed in rats treated with MPH (2mg/kg, twice daily) during the prepubertal period at 5min (p=0.017) and 10min (p=0.047), when compared to saline controls. **(B)** No differences were observed in rats treated with MPH (2mg/kg, twice daily) in adulthood when compared to saline controls.

★ $p < 0.05$, MPH > saline

assumption of sphericity according to Mauchlys test ($W = 0.172$, $p = 0.013$), so Greenhouse-Geisser corrections were used. An ANOVA showed no significant interaction between the drug treatment and time, and no main effect of drug. This demonstrates that the MPH treatment did not result in locomotor sensitization in adult rats, as MPH and saline treated animals exhibited comparable levels of locomotor activity 4 weeks after treatment. However, there was a main effect of time ($F(3.06, 55.05) = 2.902$, $p = 0.042$), which was further investigated using Bonferroni corrected pairwise comparisons. These comparisons showed no significant differences in locomotor activity counts between the time bins. Although there was a significant main effect, it appears that the stringency of the Bonferroni correction resulted in none of these comparisons reaching significance.

Experiment 2

Receptor IR 1 week after treatment. The antibody used to detect DCC protein in the VTA samples resulted in two bands: one strong band migrating at ~185Kd, the molecular weight of DCC protein, and one faint band migrating at ~155Kd that is a breakdown product of DCC. The UNC-5 antibody also detected two bands: a band migrating at ~135kDa that corresponds to the molecular weight of UNC-5, and a band at ~95kDa that appears to be a breakdown product of UNC-5. Finally, the tubulin antibody detected a single band at 50kDa.

T-tests were used to compare the mean density of DCC/Tub IR and UNC-5/Tub IR in the VTA of MPH and saline treated animals. Figure 6A presents data comparing DCC/Tub in MPH versus saline animals treated during the prepubertal period. This test

revealed no difference between the experimental groups. The t-test comparing UNC-5/Tub levels in this cohort of animals also revealed no significant difference between MPH and saline treated animals (see Figure 6B). Figure 7A depicts the levels of DCC/Tub in adult rats 1 week after MPH or saline treatment. The t-test revealed no significant difference in mean intensity of DCC/Tub in the two treatment groups. Finally, the t-test assessing UNC-5 IR in the VTA of animals treated during adulthood showed no difference in UNC-5 IR between MPH and saline treated rats (see figure 7B). This demonstrates that 1 week after treatment, there was no effect of MPH treatment on levels of either DCC or UNC-5 receptors in rats treated during prepubescence or adulthood.

Receptor IR 4 weeks after treatment. Figure 8 illustrates the DCC/Tub IR (figure 8A) and UNC-5/Tub IR (figure 8B) in prepubescent animals 4 weeks following treatment with MPH or saline. T-tests showed no significant difference in DCC/Tub IR or UNC-5 IR between the groups in this cohort. The t-test run on data from adult rats four weeks following treatment revealed that MPH treatment did not affect DCC/Tub IR (see Figure 9A). This finding extended to UNC-5 IR, illustrated in Figure 9B, as no significant differences were observed as a result of MPH treatment. Thus, MPH treatment did not induce changes in levels of Netrin-1 receptors in prepubertal or adult rats 4 weeks after treatment ended.

Discussion

Experiment 1 – Locomotor sensitization

The behavioural results of this experiment demonstrate that 1 week after MPH

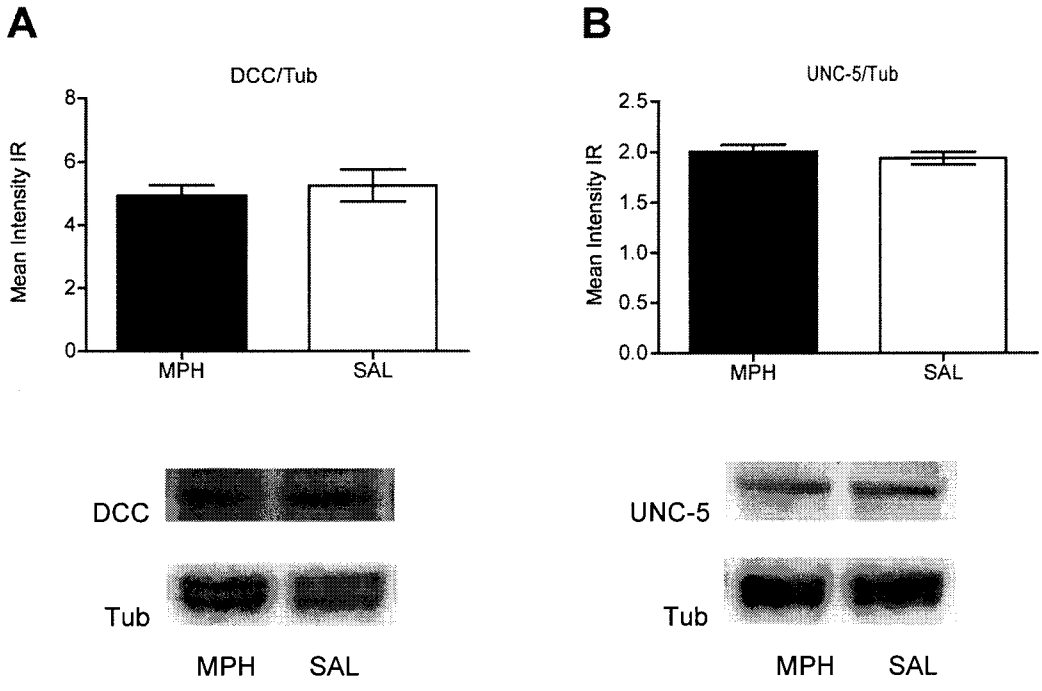

Figure 6 DCC and UNC-5 IR signal in VTA tissue 1 week following MPH or saline treatment during the prepubertal period. Examples of Western blots are shown below the corresponding graph. **(A)** MPH treatment did not alter levels of DCC/Tub in the VTA when compared to saline controls. **(B)** MPH treatment did not alter levels of UNC-5/Tub in the VTA when compared to saline controls. All data are presented as mean intensity IR signal +/- SEM.

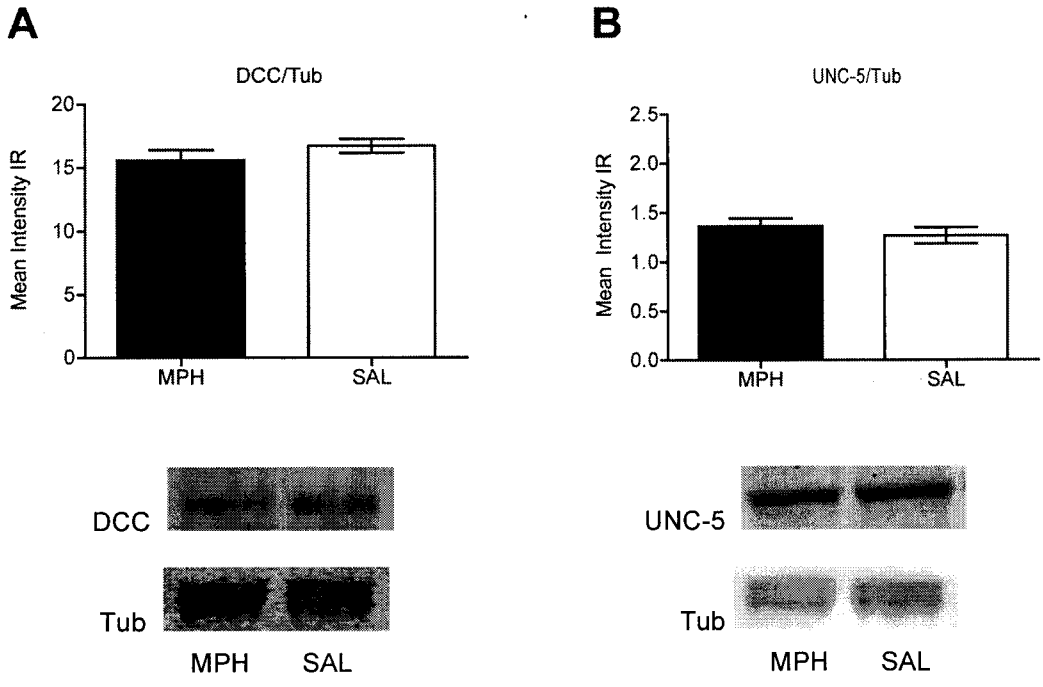

Figure 7 DCC and UNC-5 IR signal in VTA tissue 1 week following MPH or saline treatment in adulthood. Examples of Western blots are shown below the corresponding graph. **(A)** MPH treatment did not alter the mean intensity of DCC/Tub in the VTA when compared to saline controls. **(B)** MPH treatment did not alter the mean intensity of UNC-5/Tub in the VTA when compared to saline controls. All data are presented as mean intensity IR signal +/- SEM.

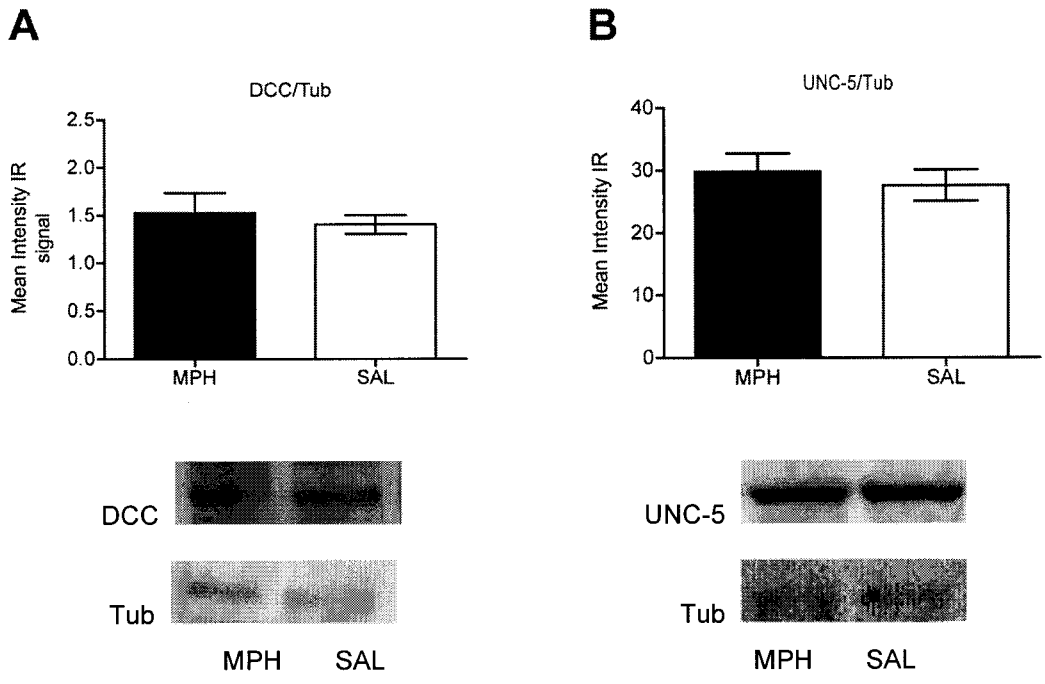

Figure 8 DCC and UNC-5 IR signal in VTA tissue 4 weeks following MPH or saline treatment during the prepubertal period. Examples of Western blots are shown below the corresponding graph. **(A)** MPH treatment did not alter levels of DCC/Tub when compared to saline controls. **(B)** MPH treatment did not alter levels of UNC-5/Tub when compared to saline controls. All data are presented as mean intensity IR signal +/- SEM.

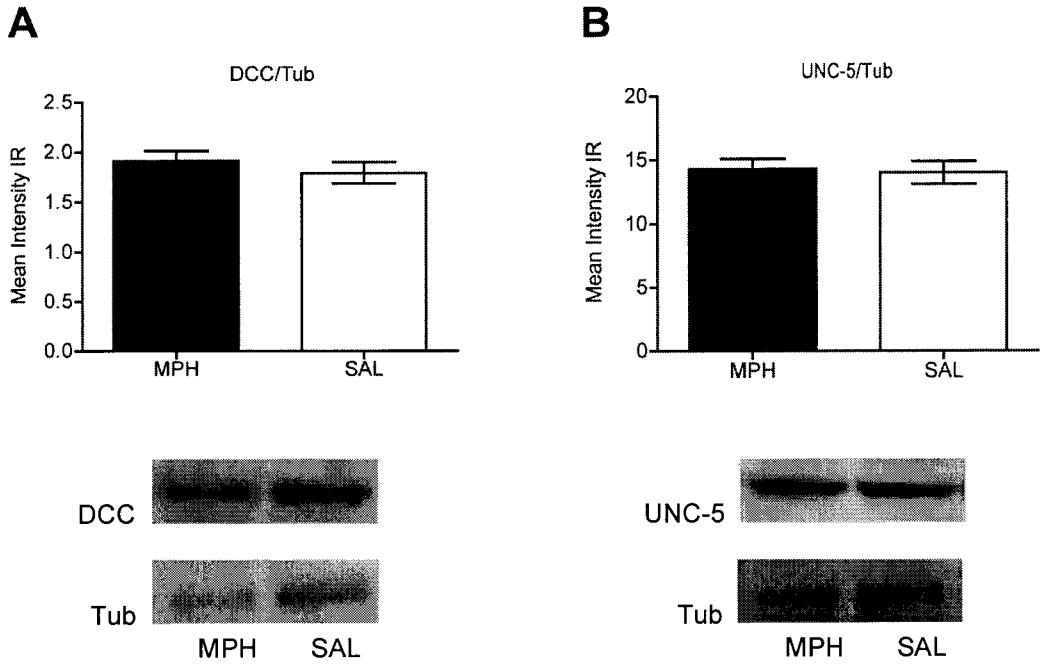

Figure 9 DCC and UNC-5 IR signal in VTA tissue 4 weeks following MPH or saline treatment in adulthood. Examples of western bands are shown below the corresponding graph. **(A)** MPH treatment did not alter levels of DCC/Tub when compared to saline controls. **(B)** MPH treatment did not alter levels of UNC-5/Tub when compared to saline controls. All data are presented as mean intensity IR signal +/- SEM.

treatment, when challenged with an acute dose of COC, significantly higher locomotor activity was not observed in either prepubertal or adult rats treated with MPH, when compared to saline controls. Although, data from these prepubertally treated rats were not significant, there was a trend towards sensitization, which likely resulted from elevated locomotor activity in the MPH treated animals early in the test (see Figure 4A). Four weeks after MPH treatment a sensitized locomotor response was observed in prepubertal rats in response to a challenge dose of COC, when compared to saline controls. In contrast, adult animal treated with MPH continued to show no locomotor sensitization to the challenge dose of COC 4 weeks after treatment. Taken together these results demonstrate developmental effects of repeated MPH exposure in prepubertally treated rats, as a trend towards sensitization was observed when tested during puberty, and significant sensitization was observed when tested in adulthood. However, the same MPH treatment administered to adult rats did not result in significant locomotor sensitization, which indicates that this sensitized response is age-dependent.

This experiment replicates unpublished findings from our laboratory, which show prepubertal animals administered a clinically-relevant MPH treatment exhibit locomotor sensitization to a challenge dose of COC 4 weeks following treatment, while adult animals do not (Augustyniak et al., 2004). In addition, these findings are similar to those of Laviola and colleagues (1995), and Adriani and colleagues (1998), which indicate that repeated COC or AMPH administration results in greater locomotor sensitization to a drug challenge in pubertally treated rats than in adult rats. It is interesting to note that these two experiments, demonstrating elevated locomotor sensitization in pubertal rats, also show significantly higher stimulant-induced sensitization of grooming and sniffing

in adult rats (Adriani et al., 1998; Laviola et al., 1995). These authors theorized that locomotor sensitization might be expressed equally in these two age groups, but manifested as different behaviours, likely resulting from different neurobiological effects of stimulants in the developing versus adult brain. Comparison between these experiments and this thesis should be made with caution, as the work of Adriani et al. (1998) and Laviola et al. (1995) examined locomotor activity within 3 days of treatment instead of evaluating developmental drug effects in adulthood. Nonetheless, it would be interesting to examine whether adult rats examined in the same protocol used in the present research would exhibit more stereotyped behaviours than prepubertal rats. Video recording of the animals' behaviour during the test would yield more detailed behavioural information, allowing for the assessment of stereotypy as well as locomotor activity.

The findings of this thesis contradict previous work, in which rats treated with stimulants during puberty exhibited attenuated locomotor sensitization compared to adults (Collins & Izenwasser, 2002; Frantz et al., 2007). Frantz and colleagues (2007) administered COC (10mg/kg) every 5 days to pubertal and adult animals, and compared locomotor activity between the first dose and the fourth dose of COC to assess locomotor sensitization. This study used pubertal rats, not prepubertal rats, and employed a different administration schedule than the one used here, which could explain why the findings do not correspond to this thesis and other work in the field (Adriani et al., 1998; Laviola et al., 1995). In the experiment of Collins and Izenwasser (2002) methodological differences also may have caused these contradictory results. This experiment employed extremely high doses of COC (50mg/kg daily), which have different behavioural effects than the clinically-relevant MPH doses used in this experiment (Prieto-Gomez et al.,

2004; Yang et al., 2006). Additionally, both of these studies administered COC to rats, which is a different compound than MPH, with different pharmacodynamics, that could differentially affect behaviour (Holman, 1994). In the future, a dose response curve experiment, using a range of MPH doses administered to prepubertal, pubertal, and adult rats, would clarify whether drug dose modifies MPH-induced age-dependent locomotor sensitization.

Developmental dissociation of incentive properties and locomotion effects. Research on the behavioural effects of repeated stimulant administration in adult rats shows that the incentive properties and locomotor effects of these drugs sensitize in parallel (Robinson & Berridge, 2001; Shimosato & Ohkuma, 2000). However, when the current results are considered along with previous data from this laboratory, it appears that MPH treatment in prepubertal rats results in augmented locomotor sensitization and attenuated place preference when compared to adult rats (Augustyniak et al., 2006). Recently it was demonstrated that MPH treatment during the prepubertal period resulted in no preference for the drug-paired compartment following CPP, but robust COC-induced locomotor sensitization (Adriani et al., 2006). This experiment lends support to the concept that prepubertally and pubertally treated rats exhibit elevated sensitization to the locomotor effects of stimulants but attenuated sensitization of incentive properties when compared to adults. Taken together, these results imply a developmentally-dependent dissociation between the neurobiological pathways responsible for these two types of sensitization.

Research examining the development of the dorsal (CPu) and ventral (NAc) striatum, and the effect of psychostimulants in these regions, has implicated them in the

age-dependent dissociation of incentive sensitization and locomotor sensitization. Both the NAc and the CPu are innervated by dopaminergic afferents from the VTA, and stimulant drugs increase DA transmission in these regions (Holman, 1994). There is a dorso-lateral/ventro-medial gradient in the stratum's role in affect and behaviour, where the CPu shows a strong relationship to behavioural activity, while the NAc shows a more direct relationship to reward (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). Experiments examining COC-induced expression of the immediate early gene, c-*fos*, which is an indication of neuronal activation, show adult rats have significantly more c-*fos* expressing cells in the NAc than pubescent rats. This indicates that there is higher stimulant induced activation in the NAc of adult rats than prepubertal rats (Cao et al., 2007; Chase, Carrey, Brown, & Wilkinson, 2005). Furthermore, Cao and colleagues (2007) showed that the NAc develops laterally to medially, as prepubescent rats have significantly fewer c-*fos* expressing cells than adults in both the lateral and medial NAc, while pubescent rats only have significantly fewer c-*fos* expressing cells in the medial NAc. Since juvenile rats exhibit lower activation in the medial NAc in response to COC, which is associated with reward, this may contribute to their attenuated sensitization to the incentive properties of stimulants.

As previously mentioned, the CPu is strongly implicated in the sensorimotor responses to stimulant drugs, and D1R activation in this region is required for the development of psychostimulant induced locomotor sensitization (Vezina, 1996). Repeated AMPH administration causes D1Rs within the NAc and CPu to become supersensitive to DA, which is thought to be important in the development of behavioural sensitization (Wolf, White, & Hu, 1994). Interestingly, pre- and pubertal rats have

significantly more D1Rs and D2Rs in mesocorticolimbic pathways, which undergo pruning during late puberty to achieve adult levels of and distribution within the mesocorticolimbic system (Teicher et al., 1995). Taken together these findings indicate significantly higher levels of D1R in the striatum of juvenile versus adult rats. The age-dependent overexpression of D1R in the CPu, which is strongly implicated in locomotor sensitization, could contribute to prepubertal and pubertal rats exhibiting more robust locomotor sensitization than adult rats.

In summary, the NAc and CPu are involved in sensitization of incentive properties and locomotor effects of psychostimulants, and have different cellular activity and D1R density during different developmental periods. Therefore, these regions of the striatum have the potential to mediate the age-dependent dissociation between sensitization of locomotion and incentive properties of stimulant drugs. There is evidence to support this hypothesis, but substantial research with prepubescent and adult rats is needed to directly connect NAc activity to attenuated place preference, and D1 receptor density in the CPu to augmented locomotor sensitization, during development.

Context-dependent sensitization. There is an alternate explanation for the behavioural findings observed here, which involves the environmental cues present during drug treatment and testing. Research in adult rats has demonstrated that the environment can moderate both the induction of sensitization, during the initial stimulant treatment, and the expression of this behaviour, seen in response to a challenge dose of drug (Robinson, Browman, Crombag, & Badiani, 1998). In adult rats, if a stimulant drug is repeatedly administered in a novel environment it induces significantly higher locomotor activity than a stimulant treatment administered in the home environment

(Badiani, Anagnostaras, & Robinson, 1995; Robinson et al., 1998). In contrast, once locomotor sensitization is induced, testing for this behaviour in a novel environment elicits significantly attenuated locomotor activity compared to testing in the environment paired with treatment (Anagnostaras & Robinson, 1996; Fraioli, Crombag, Badiani, & Robinson, 1999). When these results are considered, the drug administration procedure in the current experiment should attenuate the expression of sensitization in adult rats, as the initial drug treatment was administered in the home environment, and testing occurred in a novel environment.

In prepubertal rats treated with stimulants, the relationship between the contextual cues present during stimulant treatment and the expression of the sensitized locomotor response remains unclear. It was established that postnatal and pre-weanling rats, tested one day after repeated COC treatment, only express locomotor sensitization if tested in the same environment (Wood et al., 1998). However, context has not been shown to modify sensitization in developing animals for longer than one day, which is attributed to the developing memory system in young rats being unable to retain the association between drug effects and contextual cues over long periods of time (McDougall et al., 2009; Tirelli et al., 2003). There is research demonstrating that rats treated with stimulants during the pubertal period express locomotor sensitization to COC in a novel testing environment, which would typically reduce the expression of sensitization in adult rats (Marin, Cruz, & Planeta, 2008). Therefore, it seems that context does not have the same control over the expression of locomotor sensitization in prepubertally treated animals.

In light of these findings, the data from this experiment can be interpreted as the novel testing environment suppressing the expression of sensitization in adult rats, both 1 and 4 weeks after MPH treatment. In the prepubertally treated rats the drug-environment association is maintained for 1 week, and like the adult rats, no locomotor sensitization is expressed. However, prepubertally treated rats are unable to retain the drug-environment association for 4 weeks, so locomotor sensitization is expressed in this test. Clearly, research is needed to determine whether prepubertally treated rats actually develop greater locomotor sensitization than adults, or whether our results reflect the novel test environment preventing the expression of sensitization in rats treated in adulthood, but not prepubertally. A clinically relevant MPH treatment administered in the testing environment to both prepubescent and adult rats would induce maximal locomotor sensitization in both groups, and would determine whether young animals genuinely exhibit greater sensitization than adults.

Experiment 2 – DCC and UNC-5 IR

Preliminary research indicates that Netrin-1 receptors are involved in age-dependent stimulant-induced changes in the mesocorticolimbic system (Yetnikoff et al, 2008). However, data reported here indicate that a clinically relevant MPH treatment does not affect expression of the Netrin-1 receptors, DCC and UNC-5, in rats treated either during prepubescence or adulthood. These results contrast the data of Yetnikoff and colleagues (2008), which show that AMPH treatment in prepubertal rats decreases levels of Netrin-1 receptors, while AMPH treatment in adult rats increases levels of these receptors.

It is intriguing that the MPH treatment in this experiment did not induce the same changes in Netrin-1 receptors as was seen in response to an AMPH treatment. One potential reason for this disparity is the difference in drug doses employed by these two experiments. Here a low drug dose was used (4mg/kg MPH daily), with similar pharmacodynamics as stimulant treatments prescribed to children, while the previous research employed higher doses of AMPH (3mg/kg daily). It is well established that when MPH is administered in low enough doses, it does not elicit the typical behavioural and neurobiological profile associated with repeated stimulant drug use (Yang, Swann, & Dafny, 2006, 2007). Thus, the MPH treatment may not have sufficiently stimulated the mesocorticolimbic system to induce the age-dependent Netrin-1 receptor changes seen in response to AMPH treatment. To investigate whether this is true, a MPH dose response experiment should be carried out with prepubertal and adult animals, and VTA levels of DCC and UNC-5 should be assessed. This experiment would demonstrate whether MPH is capable of altering Netrin-1 receptors at high doses, and it would indicate the threshold dose for inducing these changes.

An alternate hypothesis is that DCC and UNC-5 levels are affected by AMPH, but not MPH, because of the difference in pharmacodynamics of these two drugs. As mentioned in the introduction, AMPH is uptaken by DA neurons and induces transport of DA from the cytosol into the synapse (Jones et al., 1999; Sulzer et al., 1995), while COC and MPH bind to the DA transporter to prevent DA released into the synaptic cleft from being reuptaken into the presynaptic cell (Holman, 1994; Schiffer et al., 2006). Thus, all three psychostimulants increase DA availability in the synapse, but this achieved through different mechanisms. Research shows fourfold higher levels of extracellular DA in the

NAc in response to a clinically-relevant AMPH treatment than to a clinically-relevant MPH treatment in rats (Schiffer et al., 2006). This indicates that the differing mechanism of these two stimulant drugs may have different effects in the mesocorticolimbic system, which could result in different protein expression in response to these drug treatments. Thus, based on its pharmacodynamics MPH may not affect levels of Netrin-1 receptors in the VTA. The dose response experiment described in the preceding paragraph would determine if a higher dose of MPH would induce changes in levels of DCC and UNC-5, or if MPH mechanism of action does not affect levels of Netrin-1 receptors. Future work in this field should take into consideration age at the time of treatment, as both the Netrin-1 and other mesocorticolimbic systems continue to develop into early adulthood.

Netrin-1 receptors and locomotor sensitization

Considering the results from these two experiments together allows for examination of Netrin-1 receptors role in locomotor sensitization. In adult rats MPH treatment did not result in locomotor sensitization and did not alter levels of Netrin-1 receptors of the VTA. Previous research has shown that an AMPH treatment that induces sensitization in adult animals also increases levels of DCC and UNC-5 in the VTA (Yetnikoff et al., 2007). Additionally, DCC heterozygous mice, which have significantly fewer DCC receptors compared to normal animals, do not develop sensitization in response to AMPH treatment (Flores et al., 2005; Grant et al., 2007). Therefore, the current findings in adult rats seem to parallel previous research, since the clinically relevant MPH treatment used in these experiments may have been insufficient to induce both locomotor sensitization and Netrin-1 changes.

When the sensitization and Netrin-1 findings in prepubertally treated rats are considered together, interpretation is more challenging. This research shows that 1 week after MPH treatment, these young rats do not exhibit locomotor sensitization or changes in Netrin-1 receptors, similar to adult rats. However, 4 weeks after MPH treatment, prepubertally treated rats demonstrate locomotor sensitization, but no difference in Netrin-1 receptor expression, when compared to saline controls. This finding contradicts the previous reports indicating a link between sensitized locomotor activity and changes in Netrin-1 receptors (Flores et al., 2005; Grant et al., 2007; Yetnikoff et al., 2007).

As discussed in the preceding section, based on pharmacodynamic differences between MPH and AMPH, the MPH treatment in this experiment may not be capable of altering Netrin-1 receptors. Although age-dependant alterations in DCC and UNC-5 expression in the VTA are seen in response to an AMPH treatment that induced locomotor sensitization, currently there is no research that indicates these changes in expression of Netrin-1 receptors are necessary for the development of sensitization. Therefore, changes in DCC and UNC-5 result from AMPH treatment, but they may be unrelated to the development of locomotor sensitization. In this case, locomotor sensitization could occur without changes in the Netrin-1 system, which is what was seen in rats treated prepubertally with MPH. However, there is also the possibility that a higher dose of MPH would be capable of inducing both locomotor sensitization and alterations in Netrin-1 receptors in adult rats, which would parallel previous research in adults (Yetnikoff et al., 2007). If this is the case, then these results indicate that prepubescent rats can develop sensitization to a lower MPH dose than adults, and that this sensitized response does not require changes in Netrin-1 receptors. Research has

established that both the function of the Netrin-1 system and UNC-5 receptor levels change throughout development, but little research has directly examined the role of Netrin-1 in the postnatal developing brain. As this discussion has already established, research is needed before age-dependent locomotor sensitization and changes Netrin-1 receptor levels are properly understood. These data provide a starting point for future research examining how stimulant drugs affect Netrin-1 receptors in juveniles, and the longterm behavioural effects of these changes.

Conclusions

In summary, the data in the present studies are the first to show that repeated low dose MPH treatment induces locomotor sensitization in response to an acute dose of COC in prepubertally treated rats, but not adult rats. The sensitized response in prepubertally treated animals appears to be a developmental drug effect, since these rats continue developing into aduthood before locomotor sensitization is observed. This is also the first experiment to examine the effects of MPH on Netrin-1 receptors in the VTA. In contrast to research with AMPH, the MPH treatment employed by this experiment does not have any effect on DCC or UNC-5 receptors in rats treated either during prepubescence or adulthood. Interpretation of these results is difficult, as the relationship between the developing mesocorticolimbic and Netrin-1 systems is poorly understood. Nonetheless, the data provide insight into age-dependent locomotor sensitization and the effects of MPH on Netrin-1 receptor levels, and are an impetus for further research in this field.

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