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Characterization of the behavioral and neurochemical effects of nicotine withdrawal in adolescent and adult rats.

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CHARACTERIZATION OF THE BEHAVIORAL AND NEUROCHEMICAL EFFECTS OF
NICOTINE WITHDRAWAL IN ADOLESCENT AND ADULT RATS

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Dedicated in loving memory to Pedro Bertoldo, Toribio and Ramona Natividad, and

Roberto and Maria Luisa Aguilar.

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NICOTINE WITHDRAWAL IN ADOLESCENT AND ADULT RATS

by

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Abstract

Previous studies have demonstrated that the behavioral effects of nicotine withdrawal are lower in adolescent versus adult rats. However, the neurochemical mechanisms that mediate these developmental differences are presently unclear. Much work has shown that nicotine reward is mediated via enhanced dopamine neurotransmission in the mesolimbic pathway which originates in the ventral tegmental area (VTA) and terminates in several forebrain structures including the nucleus accumbens (NAcc). More recently, studies have shown that nicotine withdrawal produces a decrease in NAcc dopamine transmission, an effect that is believed to serve as a neurochemical marker of withdrawal in adult rodents. The goal of this project was to understand whether developmental sensitivity to nicotine withdrawal is mediated via dopaminergic mechanisms in adolescent versus adult rats. Thus, extracellular levels of dopamine in the NAcc were compared in adolescent and adult rats experiencing nicotine withdrawal. Following 13 days of nicotine exposure, the rats were implanted unilaterally with microdialysis probes into the NAcc and the ipsilateral VTA. The next day, dialysate samples were collected following administration of the nicotinic-receptor antagonist mecamylamine to precipitate withdrawal. The physical signs of withdrawal were also examined in the same animals during baseline and then following systemic mecamylamine administration. The results revealed that mecamylamine precipitated the physical signs of withdrawal in both age groups; however, the total number of physical signs was larger in nicotine-dependent adult versus adolescent rats. The microdialysis results revealed that mecamylamine produced a decrease in extracellular levels of dopamine in the NAcc that was larger in adults (44% decrease) versus adolescents (20%). A similar pattern of developmental differences was observed with the dopaminergic metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid). However, an assessment of the serotonergic metabolite (5-hydroxyindoleacetic acid) revealed that there were no developmental

differences in this measure during nicotine withdrawal. A follow-up study compared extracellular levels of NAcc dopamine in adolescent and adult rats receiving intra-VTA administration of bicuculline, which reduces gamma-aminobutyric acid (GABA) inhibition of dopamine neurotransmission. The results revealed that blockade of GABA receptors in the VTA produced a 2-fold increase in NAcc dopamine of adult, but not adolescent rats. The results of these studies provide a potential mechanism involving dopamine that mediates developmental differences in nicotine withdrawal. Specifically, they suggest that GABAergic systems are underdeveloped during adolescence, and this reduced inhibition of dopamine neurons in the VTA may lead to reduced decreases in NAcc dopamine of adolescent versus adult rats during nicotine withdrawal.

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Introduction

Adolescent tobacco use is a major health and economic concern: It is currently estimated that 21% of high school students in the USA are cigarette smokers (Centers for Disease Control; CDC, 2008). This trend of adolescent tobacco use is alarming in light of growing evidence that adolescents are particularly vulnerable to tobacco addiction. For example, clinical studies have found that people who begin smoking as adolescents are more likely to become heavy smokers later in life (Rigotti et al., 1990; Riala et al., 2004; Glynn et al., 1993). This is a major health concern since the development of many diseases such as lung cancer, coronary heart disease, and stroke are highly correlated with long-term tobacco use (U.S. Department of Health and Human Services, 2004). Moreover, the increasing rate of nicotine dependence and smoking-related diseases produce economic consequences such as rising healthcare costs and losses in worker productivity (CDC, 2005). Despite severe health and economic concerns regarding tobacco use during adolescence, the mechanisms that motivate tobacco abuse in this age group are not well understood.

Adolescent smokers display reduced withdrawal symptoms during abstinence: It is well established that nicotine withdrawal plays a major role in motivating continued use of tobacco products and in driving relapse during abstinence in adult tobacco users (Benowitz, 2008; Buchhalter et al., 2008; Carmody et al., 2007). However, to our knowledge, no one has directly compared the intensity of withdrawal symptoms in adolescent versus adult tobacco users. Thus, the contribution of nicotine withdrawal to adolescent tobacco abuse is unclear. Recent clinical reports suggest anecdotally that nicotine withdrawal symptoms are milder in adolescents versus adults, and that the negative effects of withdrawal do not appear to be related to relapse behavior in adolescent smokers (Smith et al., 2008a; Smith et al., 2008b). Moreover, clinical studies have

reported that treatment strategies that focus on alleviating the negative effects of nicotine withdrawal, such as the nicotine patch, do not improve abstinence rates in adolescent smokers (Hanson et al., 2003; Moolchan et al., 2005). One possible explanation for these findings is that adolescents do not experience the negative effects of withdrawal during abstinence from nicotine, and as a result, treatments that target withdrawal may not be expected to reduce tobacco abuse in this young age group. These differences in withdrawal symptoms and treatment outcomes suggest that the mechanisms that mediate nicotine withdrawal are different in adolescents as compared to adults.

Animal models of adolescence in rats: Animal models involving rodent preparations have been widely used to study developmental differences in the physiological mechanisms that mediate the behavioral effects of nicotine. Researchers studying developmental differences in rats have been challenged with determining the exact boundaries of the adolescent period. However, most researchers agree that the period of adolescence conservatively ranges from PND 28-42 (Spear, 2000). Although it is difficult to define an exact time frame of adolescence, most researchers agree that this phase of development is a period of transition that encompasses a series of events with no single event that signals an onset or termination, such as puberty that is signaled by sexual maturation. Adolescence reflects a period during which age-specific behavioral discontinuities from younger and older animals are most evident. In rats, most behavioral and physiological systems reach maximal maturation by PND 60, and are considered adults beyond this age. The present study tested adolescent rats between 41-43 days of age and adult rats beyond 60 days of age.

Studying nicotine withdrawal in rats: Nicotine withdrawal has been widely studied in adult rats that receive chronic administration of nicotine via subcutaneous osmotic pumps

(Kenny and Markou, 2001; Malin, 2001; O'Dell et al., 2006). Following nicotine exposure for at least 5-7 days, nicotine withdrawal is induced either by surgically removing the nicotine pump (i.e., spontaneous withdrawal) or by administering a nicotinic acetylcholine receptor antagonist such as mecamylamine to pharmacologically induce withdrawal (i.e., precipitated withdrawal).

Much work has shown that nicotine withdrawal, under both spontaneous and precipitated withdrawal conditions, produces an increase in negative affective states such as anxiety-like behavior and aversion for environmental cues that are repeatedly paired with nicotine withdrawal (O'Dell and Khroyan, 2009). Also, spontaneous and precipitated nicotine withdrawal produce an increase in physical somatic signs, including eye blinks, writhes, body shakes, teeth chatters, gasps, and ptosis. Studies examining the physical signs of nicotine withdrawal commonly report the total number of all of these signs because it is difficult to detect group differences with an analysis of any individual sign that occurs in low frequency. However, we have reported that group differences with total signs are consistent with an individual analysis of eye blinking (O'Dell et al., 2004).

Nicotine withdrawal is lower in adolescent versus adult rats: Rodent studies examining developmental differences in nicotine withdrawal have shown that both the physical and negative affective properties of nicotine withdrawal are lower in adolescent relative to adult rats. For example, work in our laboratory has demonstrated that adolescent rats display fewer physical signs of withdrawal relative to adults across a range of nicotine doses to produce dependence and across a range of mecamylamine doses to precipitate withdrawal (O'Dell et al., 2004, 2006). Subsequent behavioral studies in our laboratory demonstrated that adolescents also display a reduced place aversion to an environment paired previously with nicotine withdrawal relative to adults (O'Dell et al., 2007). Moreover, work in other laboratories has established that the

behavioral effects of nicotine withdrawal are lower in adolescent rats and mice relative to their adult counterparts. For example, Kota et al. (2007) demonstrated that nicotine-dependent adolescent mice display fewer signs of nicotine withdrawal under both spontaneous and precipitated conditions relative to adults. Shram et al. (2008) also demonstrated that adolescent rats given a range of nicotine doses to produce dependence display a lack of place aversion and physical signs of withdrawal relative to adults under spontaneous and precipitated withdrawal conditions. Collectively, these behavioral studies suggest that adolescence is a period of development characterized by reduced sensitivity to nicotine withdrawal.

The rewarding effects of nicotine are mediated via enhanced dopaminergic mechanisms:

Research has shown that the rewarding effects of nicotine are mediated in large part via enhanced dopamine neurotransmission in the mesolimbic pathway (Balfour, 2002; Corrigall, 1991; Mansvelder and McGehee, 2002; Watkins et al., 2000). This pathway originates in the ventral tegmental area (VTA) and projects to various forebrain structures, including the nucleus accumbens (NAcc) which plays a critical role in mediating the rewarding effects of many drugs of abuse. For example, neurochemical studies have shown that nicotine administration produces an increase in extracellular levels of dopamine in the NAcc from 50-100% above baseline values (Ferrari et al., 2002; Fu et al., 2000; Di Chiara and Imperato, 1988; Nisell et al., 1994). The close relationship between NAcc dopamine release and nicotine reward has been demonstrated in studies reporting that extracellular dopamine levels are enhanced in this region during nicotine self-administration and following exposure to environmental cues that were repeatedly paired with nicotine administration (Di Chiara, 2000; Lecca et al., 2006). These findings suggest that enhanced dopamine neurotransmission in the NAcc plays an important role in mediating the rewarding effects of nicotine.

The negative effects of nicotine withdrawal involve reduced dopaminergic mechanisms in adult rodents: Recent studies have demonstrated that the neurochemical effects of nicotine withdrawal are opposite to the effects produced by administration of this drug. Specifically, adult rats showing physical signs of nicotine withdrawal display a 20-35% decrease in extracellular dopamine levels in the NAcc relative to baseline values (Carboni et al., 2000; Gaddnas et al., 2002; Hildebrand et al., 1998, 1999; Rada et al., 2001). The latter studies also found that nicotine withdrawal produces a decrease in extracellular levels of the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the NAcc. These metabolites provide an additional measure of dopamine neurotransmission because they are breakdown products of dopamine via enzymatic degradation in the pre-synaptic terminal button of the neuron. Some researchers have argued that there are limitations in the extracellular sampling of dopamine via microdialysis techniques because of the high-affinity uptake of this neurochemical by the dopamine transporter. Thus, metabolite measurements provide a useful (albeit indirect) index of dopamine-related changes since they generally parallel the pattern of changes observed with dopamine and are present at much higher concentrations than dopamine.

Primary thesis question: Do dopaminergic mechanisms mediate developmental differences in nicotine withdrawal? The hypothesis that the neural mechanisms of nicotine withdrawal involve decreased NAcc dopamine is consistent with studies showing reduced extracellular levels of NAcc dopamine in rodents experiencing withdrawal from several drugs of abuse including alcohol, opiates, and cocaine (Pothos et al., 1991; Rada et al., 2004; Weiss et al., 1992). Thus, decreased NAcc dopamine is believed to be a common neurochemical marker of withdrawal from drugs of abuse including nicotine. To our knowledge, however, changes in NAcc dopamine have not been compared in adolescent and adult rats experiencing nicotine

withdrawal. Thus, the goal of this study was to compare changes in extracellular levels of dopamine in the NAcc of adolescent and adult rats experiencing nicotine withdrawal. We also compared changes in extracellular levels of the dopamine metabolites DOPAC and HVA to further assess the role of dopamine in mediating developmental differences in nicotine withdrawal. In addition, this study also included a comparison of developmental differences in NAcc levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). Previous neurochemical studies have demonstrated that adult rats experiencing nicotine withdrawal do not display changes in 5-HIAA levels in the NAcc (Gaddnas et al., 2002; Hildebrand et al., 1998). Thus, our measures of NAcc 5-HIAA served as a negative control condition whereby neurochemical differences were not expected across our experimental conditions. Lastly, we included measures of the physical signs of withdrawal during microdialysis testing to provide a behavioral index of developmental sensitivity to nicotine withdrawal.

Secondary thesis question: Do inhibitory mechanisms contribute to developmental differences in nicotine withdrawal? Research has shown that dopamine release in the NAcc is inhibited by gamma-aminobutyric acid (GABA) neurotransmission in the dopamine cell body region of the VTA. The inhibition of dopamine in the VTA occurs via a population of GABA interneurons that form synapses onto VTA dopamine neurons that project to the NAcc (Johnson and North, 1992; Kalivas, 1993; Mansvelder and McGehee, 2002; Mansvelder et al., 2002). Microdialysis studies have shown that intra-VTA infusions of a GABA_A receptor antagonist produced an increase in extracellular dopamine levels in the NAcc (Ikemoto et al., 1997; Westerink et al., 1996), whereas intra-VTA infusions of a GABA_A agonist produced a decrease in NAcc dopamine (Westerink et al., 1996). Although the inhibition of dopamine release via

GABA systems has been well established, no one has compared the ability of GABAergic systems in the VTA to inhibit dopamine release in the NAcc of adolescent and adult rats.

A developmental difference in the ability of VTA GABA to inhibit NAcc dopamine might be expected based on previous studies showing that GABA systems are underdeveloped during adolescence. For example, adolescent rats display lower levels of GABA, GABA-converting enzymes, and GABA receptors in the brain compared to adults (Coyle and Enna, 1976; Hedner et al., 1984). More recently, immunohistochemistry studies have demonstrated that adolescent rats express lower levels of various GABA_A receptor subtypes relative to adults (Fritschy et al., 1994; Paysan et al., 1994; Yu et al., 2006). In addition, Fleming et al. (2007) demonstrated that inhibitory currents in granule cells of the dentate gyrus are lower in adolescent versus adult rats. Based on these findings, we suggest that GABA inhibition of the dopamine cell bodies in the VTA is reduced during adolescence, and this provides a potential mechanism to explain why adolescents might display reduced changes in NAcc dopamine during nicotine withdrawal. To address this hypothesis, the present study compared developmental differences in neurochemical changes of NAcc dopamine produced by intra-VTA administration of a GABA_A antagonist.

Background information regarding the use of in-vivo microdialysis to assess extracellular levels of neurotransmitters: In-vivo microdialysis is a technique used in live animals to extract samples of the extracellular environment in discrete brain regions to estimate changes in neurotransmission. A probe consisting of a semi-permeable membrane tip is inserted into the brain region of interest. A Ringer's buffer medium known as artificial cerebral spinal fluid (ACSF) is then delivered through the inlet of the probe at a constant rate by a syringe pump, creating a concentration gradient across the microdialysis membrane. Molecules of a

certain size enter the microdialysis probe via diffusion properties and the flow of ACSF containing neurotransmitters (i.e., dialysate) is deposited into a collection vial through the outlet of the probe. Reverse dialysis can also be used to deliver drugs into specific brain nuclei by continuously perfusing the probe with ACSF that contains a given drug. The passage of molecules across the membrane tip is determined by the concentration of neurotransmitter in the area where the probe is implanted, the flow rate of the perfusate applied through the probe, and the type of membrane used. The external diameter of the membrane is about 250-300 μm with molecular weight cut-offs of 6,000-20,000 Daltons.

High performance liquid chromatography (HPLC) is used to separate components of a sample (i.e., analytes) via reverse-phase ion-pairing retention on a chromatographic column. This occurs via the use of a pump apparatus that circulates a mobile phase solution at high pressure to deliver the sample through the separation column. The column consists of tightly packed silicon beads containing alkyl-carbon chains (i.e., stationary phase) that interact with the analyte based on its degree of hydrophobicity. Thus, the elution time of the analyte from the column depends on its ability to interact with the stationary phase in the column. For example, the more hydrophobic the analyte, the more it will interact with the column packing, and thus the more time it will take to elute from the column. The elution time is also a function of the alkyl chain length in the column packing, the mobile phase composition, and the flow rate applied to the system. The time at which a specific analyte elutes is called retention time and is considered to be a unique characteristic of the analyte.

HPLC is then coupled with electrochemical detection for quantification of the individual analytes. The electrochemical detector consists of 2 electrodes that carry an electrical potential which is set to oxidize in one electrode and reduce in the other as the separated analytes pass

through the pre-set field potentials. Oxidation and reduction produced by the field potentials then generate a measurable current that reflects the quantity of the analytes. The electrical current is translated into a computerized image of the reaction in peak format, which can be quantified and compared against a current that is generated from a standard containing a known concentration of the neurotransmitter of interest. The area-under-the-curve or peak heights from a series of standard injections are linearly related to the amount of analyte. Thus, a linear regression of these standard injections is used to predict the amount of analyte in a sample containing an unknown quantity.

Methods

Animals: Male Wistar adolescent and adult rats (n=7-8 per group) were used. Adolescents were between post-natal day (PND) 28-30 and adults were between PND 60-75 at the time of the pump implantation surgery. All rats were handled for 5 days prior to the start of experimentation and were given free access to food and water throughout the study. Rats were housed in groups of 2-3 per cage in a humidity- and temperature-controlled (20-22°C) vivarium using a 12-/12-hour light/dark cycle with lights on at 8:00 AM. The home cages consisted of a rectangular Plexiglas® hanging cage (41.5 cm long x 17 cm wide x 21 cm high) with pine bedding. The food and water were located above the animals' living space on a wire platform encased within a filtered top cover. Testing procedures were conducted during the light phase of the rats' light/dark cycle. The rats were bred in the Psychology Department from a stock of out bred Wistar rats from Harlan, Inc (Indianapolis, IN). Rats were bred onsite to minimize stress in adolescents that might have occurred if they had been shipped and tested close in time. All procedures were approved by the University of Texas at El Paso Animal Care and Use Committee and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs: The drugs used in this experiment were (-) nicotine-hydrogen tartrate and mecamylamine-hydrochloride purchased from Sigma Aldrich Inc. (St. Louis, MO), and bicuculline-methochloride purchased from Tocris Biosciences, Inc (Ellisville, MO). Mecamylamine was dissolved in 0.9% sterile saline and injected via the intraperitoneal (IP) route of administration in a volume of 1 ml/kg. Bicuculline was dissolved in artificial cerebrospinal fluid (ACSF) and administered via reverse dialysis through the microdialysis probe that was in the VTA.

Surgical preparation of osmotic pumps: Rats were anesthetized with an isoflurane/oxygen mixture (1-3% isoflurane) prior to surgical preparation of 14-day Alzet osmotic pumps purchased from Durect Corporation (model 2ML2; 1.0 μ l/hour; Cupertino, California) that were implanted subcutaneously on the back of the animal parallel to the spine. Pumps were filled with nicotine (4.7 mg/kg/day for adolescents or 3.2 mg/kg/day for adults; expressed as base). The concentration of nicotine in the pump was adjusted according to the weight of the rat at the time of the surgery. The nicotine concentrations were based on previous studies demonstrating that the infusion rate of nicotine was 1.5 times lower in adolescent versus adult rats after 17 days of exposure to the same nicotine dose as used in the present study (see Trauth et al., 2000). After surgery, the surgical incision was closed with 9-mm stainless steel wound clips and treated with a topical antibiotic ointment.

Stereotaxic implantation of microdialysis probes: Thirteen days after the pump surgery, rats were implanted unilaterally with 2 probes into the NAcc and the ipsilateral VTA. Rats were implanted between PND 40-42 for adolescents and PND 72-87 for adults. The probes were purchased from CMA-Microdialysis (model CMA 11; Solna, Sweden) with an active membrane length of 2 mm in the NAcc and 1 mm in the VTA. The probes were perfused for at least 1 hour prior to implantation at a rate of 0.5 μ l/minute with ACSF composed of 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 5.4 mM d-glucose, and 0.25 mM ascorbic acid and adjusted to a pH of 7.2-7.4. The probes were stereotaxically implanted into the brain regions using the following coordinates for the NAcc from bregma [adolescent placements- anterior-posterior (AP) = +2.2, medial-lateral (ML) = \pm 0.8, dorsal-ventral (DV) = -7.1; and adult placements- AP = +1.7, ML = \pm 1.4, DV = -8.1] and the ipsilateral VTA [adolescent placement-AP = -4.0, ML = \pm 0.6, DV = -7.4; and adult placements- AP = -4.8, ML = \pm 0.8, DV = -8.5]. Adolescent placements

were derived from Philpot et al. (2001) and Pistis et al. (2004) and adult placements were derived from O'Dell and Parsons (2004). The hemisphere that was implanted with the probe was randomized across treatment groups to control for possible hemispheric differences across age groups.

Following surgery, adolescent and adult animals were transferred to similar sized test cages that consisted of a square Plexiglas[®] cage (24 cm long x 24 cm wide x 31 cm high) with pine bedding. Food and water were available throughout dialysis testing. When comparing adolescent and adult rats, some researchers are careful to adjust for the size of the cage since this factor has been shown to influence exploratory behaviors such as sniffing, rearing, and locomotor activity. This may be particularly important for studies assessing developmental differences in affective measures such as anxiety-like behavior that are influenced by exploratory behavior. However, previous work in our laboratory using different sized chambers has consistently revealed that adolescent and adult rats tested in chambers of different sizes and shapes (round versus square) display similar basal and somatic signs of withdrawal (O'Dell et al., 2004 and 2006).

Microdialysis testing: The next day after probe implantation, the perfusate flow rate was increased to 1.0 μ l/minute for 1 hour to allow equilibration of the probes. Samples were then collected in 10-minute intervals for 1 hour to establish a baseline period, and then for 3 additional 1-hour sampling periods following systemic administration of saline and 2 doses of mecamylamine in increasing order (1.5 and 3.0 mg/kg, expressed as salt; IP). The doses of mecamylamine were chosen based on previous studies demonstrating that they produce a place aversion to environmental cues previously associated with withdrawal in nicotine-dependent rats (O'Dell et al., 2007). In addition, reports from other laboratories have demonstrated that similar

doses of mecamylamine produce decreases in extracellular levels of NAcc dopamine (approximately 20-35% from baseline) in nicotine-dependent adult rats experiencing withdrawal (Carboni et al., 2000; Gaadnas et al., 2002; Rada et al., 2001).

The last series of dialysate samples were collected during a 1-hour perfusion of bicuculline-methochloride into the VTA probe (100 μ M). This manipulation was done in order to compare developmental differences in the ability of GABA_A receptor blockade to produce increases in extracellular levels of NAcc dopamine. All dialysate samples collected from the NAcc were diluted with 10 μ l of perchloric-acid (0.05 N) in order to preserve our samples and prevent degradation of dopamine and the metabolites. After collection, the samples were immediately frozen on dry ice and stored in a -70°C freezer until they were analyzed.

Assessment of the physical signs of nicotine withdrawal: Rats were monitored for somatic signs of nicotine withdrawal for 10-minute observation periods following the administration of saline and 2 doses of mecamylamine. The observed signs included blinks, writhes, body shakes, teeth chatters, gasps, and ptosis. These measures of withdrawal have been widely used as a reliable index of the physical signs of withdrawal in nicotine-dependent rats after systemic administration of mecamylamine (Malin et al., 1994; O'Dell et al., 2004, 2006; Shram et al., 2008). Animals were continuously observed for 10 minutes during which time the frequency of any of the above signs displayed were recorded. Multiple successive counts of any sign required a distinct pause between episodes. If present continuously, ptosis was counted only once. The total number of somatic signs was defined as the sum of individual occurrences of the aforementioned withdrawal signs during the entire 10-minute observation period.

Neurochemical analysis of dopamine, DOPAC, HVA and 5-HIAA: Dopamine and the metabolites were quantified from a 10- μ l sample injected into a HPLC system equipped with an

ESA HR-80 80x4.6 mm column (3 μ m BetaBasic packing material, C-18 stationary phase, Chelmsford, MA) and eluted using a mobile phase composed of a 75 mM NaH_2PO_4 (monohydrate, monobasic) buffer (pH 3.75) with 10% acetonitrile, 0.025 mM sodium-EDTA, 0.4% (v/v) triethylamine and 1.7 mM 1-octanesulfonic acid sodium salt delivered at 1 ml/minute by an ESA model 580 syringe pump (Chelmsford, MA). Quantification was achieved via an ESA Coulochem II detector equipped with a coulometric sensor containing dual glassy carbon working electrodes (Chelmsford, MA) set at +350 mV for the metabolites and -150 mV for dopamine. The extracellular levels of dopamine and the metabolites were estimated using external calibration curves with standards containing known concentrations of these neurochemicals.

Histology: At the end of the experiment, all rats were deeply sedated with pentobarbital (100 mg/kg, salt; IP) and perfused using 0.85% saline and then a 4% paraformaldehyde solution. Following the perfusion, the brains were extracted and stored in formalin solution until they were sectioned. Verification of the probe placements was achieved during tissue sectioning using the Paxinos and Watson (1998) atlas. The probe placements were focused in the NAcc core region for both adolescents and adults, as determined during sectioning of the brain tissue. The VTA placements were all confined in this small brain region. As a final elimination criterion, each animal's baseline values had to fall within a range that was less than 2 standard deviations from the group mean. Based on these criteria, n=3 adolescents and n=2 adults were excluded from the study.

Statistical analyses: The physical signs of withdrawal were analyzed using repeated-measures ANOVA with age (adolescent and adult) as a between-subjects factor and drug treatment (saline and mecamylamine) as a within-subject factor. Repeated-measures ANOVA

was first conducted to examine whether there were age differences across the 6 baseline samples of dopamine, DOPAC, HVA, and 5-HIAA. The data revealed that there were no age differences in basal levels of dopamine or any of the metabolites. Our subsequent analyses were conducted on values that were converted to % change from baseline [i.e., (dialysate value/average baseline value) x 100%] in order to more clearly illustrate group differences across our experimental conditions. The dialysate data as % change from baseline were then analyzed using repeated-measures ANOVA with age (adolescent and adult) as a between-subjects factor and time (10-minute intervals) as a within-subject factor. Changes in neurotransmitter levels during the intra-VTA bicuculline infusion were analyzed separately using repeated-measures ANOVA with age (adolescent and adult) as a between-subjects factor and time (10-minute intervals during the 3 samples prior to and the 6 samples following intra-VTA bicuculline infusion) as a within-subject factor. Wherever appropriate, significant interaction effects were further analyzed using Fisher's least significant difference tests with a modified Bonferroni correction factor for alpha inflation.

Results

Total physical signs of withdrawal: Figure 1 illustrates the total physical signs of nicotine withdrawal in adolescent and adult rats. In summary, the results revealed that mecamylamine precipitated the physical signs of withdrawal to a greater extent in nicotine-dependent adult versus adolescent rats. Our analyses revealed that baseline withdrawal signs were not different between adolescent (5.8 ± 1.7) and adult (6.4 ± 2.4) rats [$F(1, 12) = 0.2$; ns]. A significant interaction effect was observed between age and drug treatment [$F(2, 22) = 4.3$; $P < 0.05$]. Subsequent post-hoc analyses revealed that both age groups displayed an increase in the physical signs of withdrawal relative to baseline, and this effect was larger in adult versus adolescent rats ($P_s < 0.05$).

Individual signs of withdrawal: The table below illustrates the individual signs of withdrawal. These behaviors are presented separately in order to examine whether the pattern of developmental differences observed with the total signs is similar across individual behaviors.

Mean frequencies of individual withdrawal signs (\pm SEM) in adolescents and adults

| Somatic Signs (total \pm SEM) | | Baseline (Saline) | Mecamylamine (1.5 mg/kg) | Mecamylamine (3.0 mg/kg) |
|---|-------------------|---------------------------------|-------------------------------------|-------------------------------------|
| Gasps | Adolescent | 2.3 ± 0.3 | 4.4 ± 0.6 | 5.1 ± 1.4 |
| | Adult | 2.7 ± 1.1 | 6.0 ± 0.9 | 8.8 ± 1.0 |
| Body Shakes | Adolescent | 1.1 ± 1.0 | 4.1 ± 1.4 | 6.0 ± 2.2 |
| | Adult | 1.3 ± 0.6 | 5.0 ± 1.6 | 7.1 ± 1.8 |
| Blinks | Adolescent | 1.9 ± 0.5 | 2.0 ± 0.6 | 1.3 ± 0.6 |
| | Adult | 2.0 ± 0.6 | 4.0 ± 1.0 | 4.7 ± 1.9 |
| Teeth Chatters | Adolescent | 0 | 2.8 ± 1.1 | 1.6 ± 0.6 |
| | Adult | 0 | 4.0 ± 1.5 | 3.0 ± 1.4 |
| Writhes | Adolescent | 0 | 1.1 ± 0.6 | 2.1 ± 0.5 |
| | Adult | 0 | 1.4 ± 0.6 | 3.4 ± 0.9 |
| Ptoxis | Adolescent | 0 | 1.0 ± 0.0 | 1.0 ± 0.0 |
| | Adult | 0 | 1.0 ± 0.0 | 1.0 ± 0.0 |

In general, the pattern of enhanced signs of withdrawal in adults versus adolescents was consistent between the total and individual signs of withdrawal. To further illustrate this point, Figure 2 reflects gasping behavior which was the most frequent and objective individual sign of withdrawal. Our analysis of gasping revealed a significant drug treatment effect [$F(1.3, 24) = 10.4$; $P < 0.05$], with mecamylamine producing an increase in gasping relative to baseline in both groups. We also observed an age effect [$F(1, 12) = 7.8$; $P < 0.05$], and a subsequent post-hoc analysis revealed that adult rats displayed higher levels of gasping relative to adolescents following the highest dose of mecamylamine ($P < 0.05$).

Baseline concentrations of NAcc dopamine and the metabolites: The table below reports the raw (nM \pm SEM) values of dopamine and the metabolites DOPAC, HVA and 5-HIAA averaged across the 1-hour baseline period. This was done to illustrate that there were no group differences in baseline values in any of our neurochemical measures. Specifically, our analyses of these data revealed that there were no age differences in our baseline measures of dopamine [$F(1, 14) = 0.3$; ns], DOPAC [$F(1, 12) = 0.2$; ns], HVA [$F(1, 12) = 1.4$; ns], or 5-HIAA [$F(1, 12) = 0.1$; ns].

Mean baseline concentrations of dopamine and the metabolites (\pm SEM)

| Baseline Levels (nM \pmSEM) | Dopamine | DOPAC | HVA | 5-HIAA |
|---|---------------------------------|------------------------------------|------------------------------------|------------------------------------|
| Adolescent | 2.8 \pm 0.2 | 487.8 \pm 52.1 | 395.7 \pm 41.3 | 169.2 \pm 29.0 |
| Adult | 2.9 \pm 0.1 | 517.9 \pm 47.9 | 328.7 \pm 38.6 | 225.3 \pm 49.0 |

NAcc dopamine during nicotine withdrawal: Figure 3 illustrates % change in extracellular levels of NAcc dopamine (\pm SEM) in adolescent and adult rats experiencing nicotine withdrawal. Overall, the results revealed that mecamylamine produced a decrease in

NAcc dopamine that was larger in nicotine-dependent adult versus adolescent rats. Our analyses revealed a significant interaction between age and time [$F(23, 322) = 2.0$; $P < 0.05$], with both age groups displaying a decrease in dopamine following mecamylamine administration that was larger in adult versus adolescent rats. Specifically, adult rats displayed a larger decrease in NAcc dopamine (average decrease of $44.1 \pm 5.5\%$ from baseline levels) versus adolescent rats (average decrease of $20.1 \pm 5.3\%$ from baseline levels). Subsequent post-hoc analyses revealed that the adult rats displayed a significant decrease relative to baseline at all time points following mecamylamine except for the sample that was collected before administration of the highest dose of mecamylamine ($P_s < 0.05$). In contrast, adolescents only displayed a significant decrease from baseline during the 2nd-5th, 9th and 11th-12th time points after mecamylamine administration ($P_s < 0.05$). Post-hoc analyses examining age differences revealed that adults displayed larger decreases in NAcc dopamine relative to adolescents at the 1st, 2nd, 7th and 12th time points after mecamylamine administration ($P_s < 0.05$).

NAcc DOPAC during nicotine withdrawal: Figure 4 illustrates % change in extracellular levels of NAcc DOPAC (\pm SEM) in adolescent and adult rats experiencing nicotine withdrawal. Overall, the results revealed that mecamylamine produced a decrease in NAcc DOPAC that was larger in nicotine-dependent adult versus adolescent rats. Our analyses revealed a significant interaction between age and time [$F(23, 276) = 1.6$; $P < 0.05$], with adults displaying a decrease in NAcc DOPAC following mecamylamine administration that was larger than the adolescent rats. Specifically, adult rats displayed a decrease in NAcc DOPAC (average decrease of $20 \pm 14.3\%$ from baseline levels) that was not altered in adolescent rats. Post-hoc analyses revealed that adult rats displayed a significant decrease relative to baseline during the final time point after mecamylamine administration ($P < 0.05$). Also, the post-hoc analyses examining age

differences revealed that adults displayed larger decreases in NAcc DOPAC relative to adolescents during the 1st, 5th, and 12th time points after mecamylamine administration (P s < 0.05). It should be noted that adults displayed an increase in NAcc DOPAC during the 3rd, 5th, and 6th time points after saline administration (P s < 0.05). However, adolescents did not display this effect following saline administration.

NAcc HVA during nicotine withdrawal: Figure 5 illustrates % change in extracellular levels of NAcc HVA (\pm SEM) in adolescent and adult rats experiencing nicotine withdrawal. Overall, the results revealed that mecamylamine produced a decrease in NAcc HVA that was larger in nicotine-dependent adult versus adolescent rats. Our analyses revealed a significant interaction between age and time [F (23, 276) = 1.9; P < 0.05] with adults, but not adolescents displaying a time-dependent decrease in NAcc HVA. Specifically, adult rats displayed a decrease in NAcc HVA (average decrease of $21 \pm 8.4\%$ from baseline levels) that was not altered in adolescent rats. Post-hoc analyses revealed that adults displayed a significant decrease relative to baseline during the final 3 time points after administration of the highest mecamylamine dose (P s < 0.05). Also, post-hoc analyses examining age differences produced by mecamylamine administration revealed that adults displayed a larger decrease in NAcc HVA relative to adolescents during the last time point after administration of the highest mecamylamine dose (P < 0.05). It should be noted that adults displayed an increase in NAcc HVA during the 5th and 6th time points after saline administration (P s < 0.05). However, adolescent rats did not display this effect following saline administration.

NAcc 5-HIAA during nicotine withdrawal: Figure 6 illustrates % change in extracellular levels of NAcc 5-HIAA (\pm SEM) in adolescent and adult rats experiencing nicotine withdrawal.

Mecamylamine did not produce any changes in NAcc 5-HIAA in nicotine-dependent adolescent or adult rats.

NAcc dopamine during intra-VTA administration of bicuculline: Figure 7 illustrates % change in extracellular levels of NAcc dopamine during the 3 samples prior to and the 6 samples following intra-VTA administration of bicuculline in adolescent and adult rats. Overall, the results revealed that blockade of GABA_A receptors in the VTA produced an increase in NAcc dopamine of adult but not adolescent rats. There were no age differences in dopamine during the 3 samples prior to bicuculline administration across adolescent (2.2 ± 0.2 nM) versus adult (1.6 ± 0.2 nM) rats [$F(1, 14) = 3.8$; ns]. Our analyses revealed a significant interaction between age and time [$F(8, 88) = 17.9$; $P < 0.05$], with adult rats displaying increases in NAcc dopamine that were higher versus adolescents. Specifically, adults displayed a 2-fold increase in NAcc dopamine following bicuculline infusion (i.e., from $56.0 \pm 7.5\%$ to $125.9 \pm 10.7\%$), whereas adolescents only showed a slight increase (i.e., from $77.4 \pm 5.5\%$ to $87.9 \pm 7.3\%$) in these measures. Post-hoc analyses revealed that during the final 3 time points, adults displayed significant increases in NAcc dopamine relative to the 3 samples prior to bicuculline ($P_s < 0.05$). In contrast, adolescents only displayed a significant increase in NAcc dopamine in the final time point relative to the 3 samples prior to bicuculline ($P < 0.05$). Also, post-hoc analyses examining age differences revealed that adults displayed significantly higher NAcc dopamine relative to adolescents during the final 3 time points after bicuculline ($P_s < 0.05$).

Discussion

Summary: The major finding of this report is that the physical signs of nicotine withdrawal and decreases in extracellular levels of dopamine in the NAcc were lower in adolescent versus adult rats. The pattern of developmental differences in dopamine was also consistent with the metabolites of this neurotransmitter, as decreases in extracellular levels of DOPAC and HVA were also lower in the NAcc of adolescent versus adult rats. The present report also demonstrated that intra-VTA administration of a GABA_A antagonist produced an increase in NAcc dopamine that was lower in adolescent versus adult rats. Thus, one possible mechanism to explain reduced changes in NAcc dopamine during withdrawal in adolescent rats is that inhibition of dopamine in the VTA is underdeveloped such that adolescents display less of a decrease in NAcc dopamine during withdrawal.

Developmental differences in the behavioral effects of nicotine withdrawal: The present study revealed that adolescents displayed fewer physical signs of nicotine withdrawal versus adult rats. These findings are consistent with previous behavioral studies. For example, work in our laboratory has demonstrated that adolescent rats display fewer physical signs of withdrawal relative to adult rats across a range of nicotine doses to produce dependence and across a range of mecamylamine doses to precipitate withdrawal (O'Dell et al., 2006). Moreover, recent work has demonstrated that adolescent mice display fewer signs of nicotine withdrawal versus adults under spontaneous withdrawal conditions (Kota et al., 2007). An examination of the individual signs of withdrawal revealed a similar pattern of developmental differences relative to the total physical signs of withdrawal. As an example, we observed that gasping behavior was significantly higher during withdrawal in adults versus adolescents. This finding supports our hypothesis that adolescent rats display less physical signs of withdrawal relative to adults.

The pattern of results obtained with the physical signs of withdrawal is consistent with studies comparing developmental differences in the negative affective properties of nicotine withdrawal. Specifically, previous work in our laboratory and others has demonstrated that the negative affective properties of nicotine withdrawal are also lower in adolescent versus adult rats (O'Dell et al., 2007; Shram et al., 2008; Wilmouth and Spear, 2006) and mice (Kota et al., 2007). For example, O'Dell et al. (2007) demonstrated that adolescent rats did not display aversion to environmental cues repeatedly paired with nicotine withdrawal. Shram et al. (2008) also demonstrated that adolescent rats display a lack of place aversion under both spontaneous and precipitated withdrawal conditions. Kota et al. (2007) and Wilmouth and Spear (2006) utilized the elevated plus maze to examine anxiety-like behavior in adolescents and adults experiencing withdrawal. This procedure assesses how animals respond to an approach-avoidance situation involving open elevated spaces that are avoided versus enclosed safe areas that are preferred. The Kota and Wilmouth studies both reported that adult rodents experiencing nicotine withdrawal displayed an increase in anxiety-like behavior, as measured by a decrease in open-arm time relative to controls. However, this effect was not observed in adolescents. Taken together, these studies suggest that adolescence is a period of development characterized by reduced sensitivity to the negative affective properties of nicotine withdrawal.

Dopaminergic mechanisms appear to mediate developmental differences in nicotine withdrawal: Our neurochemical results extend previous behavioral studies by providing a potential mechanism for reduced sensitivity to nicotine withdrawal during adolescence. This mechanism involves reduced dopamine neurotransmission in the NAcc, an effect that has been well established in adult rats experiencing withdrawal from nicotine (Carboni et al., 2000; Gaddnas et al., 2002; Hildebrand et al., 1998, 1999; Rada et al., 2001) and other drugs of abuse

(Poethos et al., 1991; Rada et al., 2004; Weiss et al., 1992). The latter studies regarding nicotine withdrawal have reported a 20-35% decrease in extracellular levels of NAcc dopamine during nicotine withdrawal, and the magnitude of this effect is consistent with the 44% decrease in NAcc dopamine observed in the present study. The major contribution of this report to the literature; however, is that adolescent rats only displayed a 20% decrease in extracellular levels of NAcc dopamine during nicotine withdrawal.

Consistent with the dopamine data, adolescent rats also displayed fewer changes in the dopaminergic metabolites DOPAC and HVA relative to adults. The metabolite data are useful for several reasons. First, they provide a supplementary (albeit, indirect) verification of the changes we observed with dopamine neurotransmission since the metabolites generally produced similar patterns of developmental differences during withdrawal. Second, they address an inherent limitation in the sampling of dopamine via microdialysis procedures that underestimates true concentrations of this neurotransmitter. This is because the high-affinity dopamine transporter quickly transports dopamine out of the synaptic cleft and into the pre-synaptic terminal button where it is metabolized into DOPAC and HVA. These inactive metabolites then diffuse into the extracellular environment, allowing them to be sampled through the microdialysis probe at higher concentrations relative to the retrieved levels of dopamine. Thus, our metabolite data provided a verification of the developmental differences observed with dopamine using a measure that produces a large signal. Third, the metabolite data allowed us to detect differences that were not observed with dopamine, such as the increases in DOPAC and HVA observed in adult rats following saline administration. The saline-induced increases in the metabolites might reflect acute stress in the adult animal produced by a systemic injection. This effect has also been observed in nicotine-dependent adult animals following intra-VTA

administration of saline (Hildebrand et al., 1999). Fourth, the metabolite data suggest that our developmental differences are specific to dopaminergic systems, since neither age group displayed changes in extracellular levels of 5-HIAA during withdrawal. This is consistent with previous studies showing that 5-HIAA is not altered in the NAcc of adult rats experiencing nicotine withdrawal (Gaddnas et al., 2001; Hildebrand et al., 1998).

In this study mecamylamine was used as a pharmacological tool to compare developmental differences in the neurochemical effects of withdrawal. Thus, our comparisons focused on adolescent and adult rats that were exposed to nicotine and then given mecamylamine to precipitate withdrawal. It may be argued that our observed changes in dopamine and its metabolites reflect age-dependent differences in response to mecamylamine given in combination with chronic nicotine treatment versus mecamylamine given alone. However, our previous place conditioning studies revealed that adolescents chronically exposed to nicotine still demonstrate less sensitivity to mecamylamine-precipitated withdrawal versus adults, even in separate groups of adolescents that received a 2-fold higher dose of mecamylamine or 7 additional days of nicotine exposure (O'Dell et al., 2007). Furthermore, in the absence of mecamylamine, the removal of a nicotine pump still produces less spontaneous signs of withdrawal in adolescent versus adult rats (Shram et al., 2008) and mice (Kota et al., 2007).

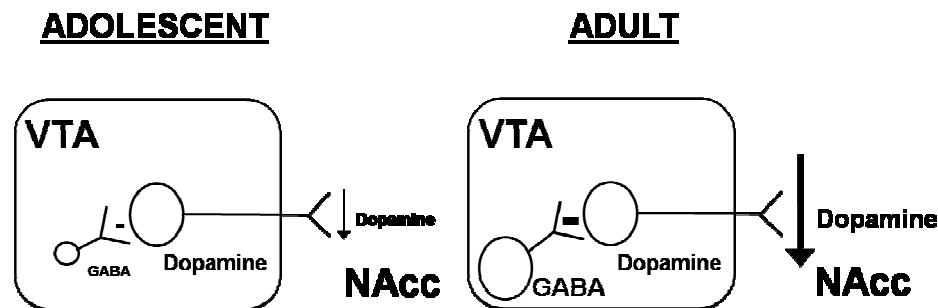
It is also unlikely that developmental differences observed in this study can be attributed to the effects of mecamylamine alone, since several reports have shown that this drug has little behavioral or neurochemical effects in the absence of nicotine. For example, administration of mecamylamine doses used in the present study do not alter the somatic signs of withdrawal or produce place aversion in adolescent versus adult rats (O'Dell et al., 2007; Shram et al., 2008) or mice (Kota et al., 2007). Also, separate laboratories have shown that mecamylamine alone does

not alter extracellular levels of dopamine in the NAcc of adult rats (Carboni et al., 2000; Gaddnas et al., 2002; Hildebrand and Svensson, 2000; Rada et al., 2001). Taken together, these studies suggest that our results are not influenced by developmental differences in response to mecamylamine. However, this potential limitation in the interpretation of the present findings might be addressed in future empirical studies comparing developmental differences in nicotinic receptor function.

It should be noted that adolescents display faster weight gain and metabolic rates of nicotine than adults. Thus, it may also be suggested that the lack of withdrawal in adolescents is due to lower levels of nicotine on the day of microdialysis testing relative to adults. However, this potential confound was likely avoided because we implanted the adolescent rats with a pump containing a 1.5 fold higher dose of nicotine as compared to adults. This adjustment factor was based on a study showing that after 17 days of nicotine pump exposure, the infusion rates of nicotine were 1.5 times lower in adolescent (3-4 mg/kg/day) versus adult rats (5 mg/kg/day; Trauth et al., 2000). Thus, one might expect that adolescents receiving 1.5 times more nicotine than adults would display equivalent nicotine levels as adults on the test day following 14 days of nicotine exposure. Moreover, we have demonstrated that place aversion produced by nicotine withdrawal is still lower in a group of adolescents that were tested after 21 days of nicotine exposure during which time they received a new pump containing an adjusted nicotine dose 14 days after the initial pump implantation (O'Dell et al., 2007). Also, Kota et al., (2007) demonstrated that adolescent mice given repeated systemic injections of nicotine that were adjusted for weight still display less physical and affective signs of withdrawal as compared to adults. Taken together, these studies suggest that the developmental differences observed in the present study are not likely due to developmental differences in nicotine dosing or tolerance;

however, future studies might directly assess this possibility at the time point that was used in the present study.

GABAergic inhibition of the dopaminergic mechanisms that mediate developmental differences in nicotine withdrawal: The diagram below depicts our hypothesis regarding developmental differences in nicotine withdrawal.



In adults, we hypothesized that administration of a GABA antagonist would increase extracellular levels of NAcc dopamine. Our hypothesis was based on the finding that blockade of GABA_A receptors in the VTA of adult rats produces a 40-80% increase in extracellular levels of NAcc dopamine, whereas stimulation of these receptors induces a 60% decrease in this measure (see Ikemoto et al., 1997; Westerink et al., 1996). Indeed, the present findings are consistent with previous reports showing that intra-VTA infusions of bicuculline produced a 69% increase in NAcc dopamine of adult rats.

The present study also explored the hypothesis that developmental differences in NAcc dopamine during withdrawal are mediated via GABAergic inhibition of dopamine cell bodies in the VTA. Our results demonstrated that blockade of GABA_A receptors in the VTA did not alter NAcc dopamine of adolescent rats as compared to adult rats that displayed a robust increase in this measure. The finding that adolescents displayed reduced changes in NAcc dopamine following intra-VTA bicuculline relative to adults is consistent with literature showing that

GABA systems are underdeveloped during adolescence. For example, GABA-mediated inhibition by postsynaptic GABA_B receptors is not functional early in life, and GABA currents in neonatal rat neurons are insensitive to benzodiazepine activation of GABA_A receptors, suggesting an immaturity in synaptic function during early development (Cherubini et al., 1991). Moreover, adolescents display lower levels of GABA, GABA-converting enzymes, and GABA receptors (Coyle and Enna, 1976; Hedner et al., 1984). Adolescents also express lower levels of the α subunits of GABA_A receptors compared to adult animals (Fritschy et al., 1994; Paysan et al., 1994; Yu et al., 2006). Thus, the finding that blockade of GABA receptors in the VTA does not alter NAcc dopamine in adolescent rats is likely due to underdeveloped inhibitory systems that mediate VTA dopamine neurons and release dopamine into the NAcc. As a result, it is possible that adolescent rats experiencing nicotine withdrawal display a lower magnitude of decreases in NAcc dopamine as compared to adults because of a reduced ability of VTA GABA to inhibit dopamine release in the cell body region. We recognize that our interpretation of these data may be limited on the basis of pharmacological studies, and future studies will need to more directly assess our hypothesis by comparing extracellular levels of VTA GABA in adolescent and adult rats experiencing nicotine withdrawal.

Clinical implications of the present study: Our findings may have clinical relevance for treating adolescent tobacco abuse. Specifically, the finding that adolescents display reduced withdrawal suggests that treatments focusing on alleviating nicotine withdrawal may be less effective in treating adolescent tobacco users. As an example, treatments that enhance dopamine neurotransmission such as bupropion may be less effective in adolescent smokers that experience fewer decreases in dopamine levels during withdrawal. There is clinical evidence to support this suggestion since long-term abstinence rates do not appear to be closely associated with nicotine

replacement therapies in adolescent smokers (Hanson et al., 2003; Hurt et al., 2000; Moolchan et al., 2005). Also, nicotine replacement does not prevent the expression of nicotine withdrawal symptoms in adolescent smokers (Killen et al., 2001). Moreover, a recent study that directly compared adolescent smokers to non-smokers found that young smokers only exhibited mild symptoms during withdrawal (anger and craving) that did not appear to be associated with self-reports of dependence or biological markers of cigarette use (Smith et al., 2008a). Another report from this laboratory found that withdrawal symptoms on the quit day were not related to relapse behavior in adolescent smokers (Smith et al., 2008b). These studies suggest that abstinence from chronic tobacco use only produces mild withdrawal symptoms that are not related to continued use or relapse behavior during adolescence. Thus, treatments focusing on alleviating withdrawal may be less effective in adolescent tobacco abusers. Future studies are needed to determine whether treatments that target nicotine withdrawal via enhanced dopamine neurotransmission are equally effective in adolescent and adult smokers.

References

- Balfour DJ. 2002. Neuroplasticity within the mesoaccumbens dopamine system and its role in tobacco dependence. *Current Drug Targets- CNS and Neurological Disorders* 1(4):413-421.
- Buchhalter AR, Fant RV, Henningfield JE. 2008. Novel pharmacological approaches for treating tobacco dependence and withdrawal: current status. *Drugs* 68:1067-1088.
- Benowitz NL. 2008. Neurobiology of nicotine addiction: implications for smoking cessation treatment. *American Journal of Medicine* 121:3-10.
- Carboni E, Bortone L, Giua C, Di Chiara G. 2000. Dissociation of physical abstinence signs from changes in extracellular dopamine in the nucleus accumbens and in the prefrontal cortex of nicotine dependent rats. *Drug and Alcohol Dependence* 58:93-102.
- Carmody TP, Vieten C, Astin JA. 2007. Negative affect, emotional acceptance, and smoking cessation. *Journal of Psychoactive Drugs* 39: 499-508.
- Center for Disease Control and Prevention. 2005. Annual Smoking-Attributable Mortality, Years of Potential Life Lost, and Productivity Losses- United States, 1997-2001. *Morbidity and Mortality Weekly Report* 54(25):625-628.
- Center for Disease Control and Prevention. 2008. Cigarette Use Among High School Students- United States, 1991-2007. *Morbidity and Mortality Weekly Report* 57(25):689-691.
- Cherubini E, Gaiarsa JL, Ben-Ari Y. 1991. GABA: an excitatory transmitter in early postnatal life. *Trends in Neuroscience* 14:515-519.
- Corrigall WA. 1991. Understanding brain mechanisms in nicotine reinforcement. *British Journal of Addiction* 86:507-510.

- Coyle JT, Enna SJ. 1976. Neurochemical aspects of the ontogenesis of GABAergic neurons in the rat brain. *Brain Research* 111(1):119-133.
- Di Chiara G, Imperato A. 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences in the U.S.A.* 85(14):5274-5278.
- Di Chiara G. 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *European Journal of Pharmacology* 393:295-314.
- Ferrari R, Le Novere N, Picciotto R, Changeux JP, Zoli M. 2002. Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. *European Journal of Neuroscience* 15(11):1810-1818.
- Fleming RL, Wilson WA, Swartzwelder HS. 2007. Magnitude and ethanol sensitivity of tonic GABA_A receptor-mediated inhibition in dentate gyrus changes from adolescence to adulthood. *Journal of Neurophysiology* 97(5):3806-11.
- Fritschy JM, Paysan J, Enna A, Mohler H. 1994. Switch in the expression of rat GABA_A-receptor subtypes during postnatal development: an immunohistochemical study. *Journal of Neuroscience* 14(9):5302-5324.
- Fu Y, Matta SG, Gao W, Brower VG, Sharp BM. 2000. Systemic nicotine stimulates dopamine release in nucleus accumbens: re-evaluation of the role of N-methyl-D-aspartate receptors in the ventral tegmental area. *Journal of Pharmacological Experimental Therapy* 294(2):458-465.
- Gaddnas H, Piepponen TP, Ahtee L. 2002. Mecamylamine decreases accumbal dopamine output in mice treated chronically with nicotine. *Neuroscience Letters* 330(3):219-222.

- Glynn T, Greenwald P, Mills S, Manley M. 1993. Youth tobacco use in the United States: progress, goals and potential solutions. *Preventative Medicine* 22:568-75.
- Hanson K, Allen S, Jensen S, Hatsukami D. 2003. Treatment of adolescent smokers with the nicotine patch. *Nicotine Tobacco Research* 5(4):515-526.
- Hedner T, Iversen K, Lundborg P. 1984. Central GABA mechanisms during postnatal development in the rat: neurochemical characteristics. *Journal of Neural Transmission* 59(2):105-18.
- Hildebrand BE, Nomikos GG, Hertel P, Schilstrom B, Svensson TH. 1998. Reduced dopamine output in the nucleus accumbens but not in the medial prefrontal cortex in rats displaying a mecamylamine-precipitated nicotine withdrawal syndrome. *Brain Research* 779:214–225.
- Hildebrand BE, Panagis G, Svensson TH, Nomikos GG. 1999. Behavioral and biochemical manifestations of mecamylamine-precipitated nicotine withdrawal in the rat: role of nicotinic receptors in the ventral tegmental area. *Neuropsychopharmacology* 21:560–574.
- Hildebrand BE, Svensson TH. 2000. Intraaccumbal mecamylamine infusion does not affect dopamine output in the nucleus accumbens of chronically nicotine-treated rats. *Journal of Neural Transmission* 107(8-9):861-872.
- Hurt RD, Croghan GA, Beede SD, Wolter TD, Croghan IT, Patten CA. 2000. Nicotine patch therapy in 101 adolescent smokers: efficacy, withdrawal symptom relief, and carbon monoxide and plasma cotinine levels. *Archives of Pediatrics and Adolescent Medicine* 154(1):31-37.

- Ikemoto S, Kohl RR, McBride WJ. 1997. GABA-A receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. *Journal of Neurochemistry* 69:137–143.
- Johnson SW, North RA. 1992. Two types of neurons in the rat ventral tegmental area and their synaptic inputs. *Journal of Physiology (London)* 450:455–468.
- Kalivas PW. 1993. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. *Brain Research Reviews* 18:75–113.
- Kenny PJ, Markou A. 2001. Neurobiology of the nicotine withdrawal syndrome. *Pharmacology Biochemistry Behavior* 70:531-549.
- Killen JD, Ammerman S, Rojas N, Varady J, Haydel F, Robinson TN. 2001. Do adolescent smokers experience withdrawal effects when deprived of nicotine? *Experimental Clinical Psychopharmacology* 9:176-182.
- Kota D, Martin BR, Robinson SE, Damaj MI. 2007. Nicotine dependence and reward differ between adolescent and adult male mice. *Journal of Pharmacology and Experimental Therapeutics* 322:399–407.
- Lecca D, Cacciapagali F, Valentini V, Gronli J, Spiga S, Di Chiara, G. 2006. Preferential increase of extracellular dopamine in the rat nucleus accumbens shell as compared to that in the core during acquisition and maintenance of intravenous nicotine self-administration. *Psychopharmacology* 184:435-446.
- Malin DH, Lake JR, Carter VA, Cunningham JS, Hebert KM, Conrad DL, Wilson OB. 1994. The nicotinic antagonist mecamylamine precipitates nicotine abstinence syndrome in the rat. *Psychopharmacology (Berlin)* 115(1-2):180-184.

- Malin DH. 2001. Nicotine dependence: studies with a laboratory model. *Pharmacology Biochemistry Behavior* 70:551-559.
- Mansvelder HD, McGehee DS. 2002. Cellular and synaptic mechanisms of nicotine addiction. *Journal of Neurobiology* 53:606–617.
- Mansvelder HD, Keath JR, McGehee DS. 2002. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* 33:905–919.
- Moolchan ET, Robinson ML, Ernst M, Cadet JL, Pickworth WB, Heishman SJ, Schroeder JR. 2005. Safety and efficacy of the nicotine patch and gum for the treatment of adolescent tobacco addiction. *Pediatrics* 115(4):e407-e414.
- Nisell M, Nomikos GG, Svensson TH. 1994. Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. *Synapse* 16(1):36-44.
- O'Dell LE, Parsons LH. 2004. Serotonin-1B receptors in the ventral tegmental area modulate cocaine-induced increases in nucleus accumbens dopamine levels. *Journal of Pharmacological and Experimental Therapeutics* 311(2):711-719.
- O'Dell LE, Bruijnzeel AW, Ghosland S, Markou A, Koob GF. 2004. Nicotine withdrawal in adolescent and adult rats. *Annals of the New York Academy of Sciences* 1021:167–174.
- O'Dell LE, Bruijnzeel AW, Smith RT, Parsons LH, Merves ML, Goldberger BA, Koob GF, Markou A. 2006. Diminished nicotine withdrawal in adolescent rats: implications for vulnerability to addiction. *Psychopharmacology* 186:612–619.
- O'Dell LE, Torres OV, Natividad LA, Tejeda HA. 2007. Adolescent nicotine exposure produces less affective measures of withdrawal relative to adult nicotine exposure in male rats. *Neurotoxicology and Teratology* 29:17–22.

- O'Dell LE, Khroyan TV. 2009. Rodent models of nicotine reward: what do they tell us about tobacco abuse in humans? *Pharmacology, Biochemistry, and Behavior* 91(4):481-8.
- Paysan J, Bolz J, Mohler H, Fritschy JM. 1994. GABAA receptor alpha 1 subunit, an early marker for area specification in developing rat cerebral cortex. *Journal of Comparative Neurology* 350(1):133-49.
- Paxinos G, Watson C. 1998. *The Rat Brain in Stereotaxic Coordinates*. 4th Edition. Academic Press, New York.
- Philpot RM, McQuown S, Kirstein CL. 2001. Stereotaxic localization of the developing nucleus accumbens septi. *Developmental Brain Research* 130:149-153.
- Pistis M, Perra S, Pillolla G, Melis M, Muntoni AL, Gessa GL. 2004. Adolescent exposure to cannabinoids induces long-lasting changes in the response to drugs of abuse of rat midbrain dopamine neurons. *Biological Psychiatry* 56(2):86-94.
- Pothos E, Rada P, Mark GP, Hoebel BG. 1991. Dopamine microdialysis in the nucleus accumbens during acute and chronic morphine, naloxone-precipitated withdrawal and clonidine treatment. *Brain Research* 566(1-2):348-350.
- Rada P, Jensen K, Hoebel BG. 2001. Effects of nicotine and mecamylamine-induced withdrawal on extracellular dopamine and acetylcholine in the rat nucleus accumbens. *Psychopharmacology* 157:105–110.
- Rada P, Johnson DF, Lewis MJ, Hoebel BG. 2004. In alcohol-treated rats, naloxone decreases extracellular dopamine and increases acetylcholine in the nucleus accumbens: evidence of opioid withdrawal. *Pharmacology, Biochemistry and Behavior* 79(4):599-605.

- Riala K, Hakko H, Isohanni M, Jarvelin MR, Rasanen P. 2004. Teenage smoking and substance use as predictors of severe alcohol problems in late adolescence and in young adulthood. *Journal of Adolescent Health* 35(3):245-254.
- Rigotti N. 1990. How can we help the remaining smokers to quit? *American Journal of Preventative Medicine* 6:258-267.
- Shram MJ, Siu EC, Li Z, Tyndale RF, Le AD. 2008. Interactions between age and the aversive effects of nicotine withdrawal under mecamylamine-precipitated and spontaneous conditions in male Wistar rats. *Psychopharmacology* 198:181–190.
- Smith AE, Cavallo DA, Dahl T, Wu R., George TP, Krishnan-Sarin S. 2008a. Effects of acute tobacco abstinence in adolescent smokers as compared with nonsmokers. *Journal of Adolescent Health* 43:46-54.
- Smith AE, Cavallo DA, McFetridge A, Liss T, Krishnan-Sarin S. 2008b. Preliminary examination of tobacco withdrawal in adolescent smokers during smoking cessation treatment. *Nicotine and Tobacco Research* 10:1253-1259.
- Spear LP. 2000. The adolescent brain and age-related behavioral manifestations. *Neuroscience Biobehavioral Review* 24:417-463.
- Trauth JA, Seidler FJ, Slotkin TA. 2000. An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions. *Brain Research* 867:29-39.
- U.S. Department of Health and Human Services. 2004. The health consequences of smoking: A report of the Surgeon General. Atlanta, Georgia: U.S. Department of Health and Human Services, Public Health Services, Centers for Disease Control and Prevention, National

- Centers for Chronic Disease Prevention and Health Promotion, office on Smoking and Health. Washington, D.C.: U. S. Government Printing Office.
- Watkins SS, Koob GF, Markou A. 2000. Neural mechanisms underlying nicotine addiction: acute positive reinforcement and withdrawal. *Nicotine and Tobacco Research* 2:19–37.
- Weiss F, Markou A, Lorang MT, Koob GF. 1992. Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. *Brain Research* 593(2):314-318.
- Westerink BH, Kwint HF, deVries JB. 1996. The pharmacology of mesolimbic dopamine neurons: a dual-probe microdialysis study in the ventral tegmental area and nucleus accumbens of the rat brain. *Journal of Neuroscience* 16(8):2605-2611.
- Wilmouth CE, Spear LP. 2006. Withdrawal from chronic nicotine in adolescent and adult rats. *Pharmacology Biochemistry Behavior* 85:648-657.
- Yu ZY, Wang W, Fritschy JM, Witte OW, Redecker C. 2006. Changes in neocortical and hippocampal GABA_A receptor subunit distribution during brain maturation and aging. *Brain Research* 1099(1):73-81.

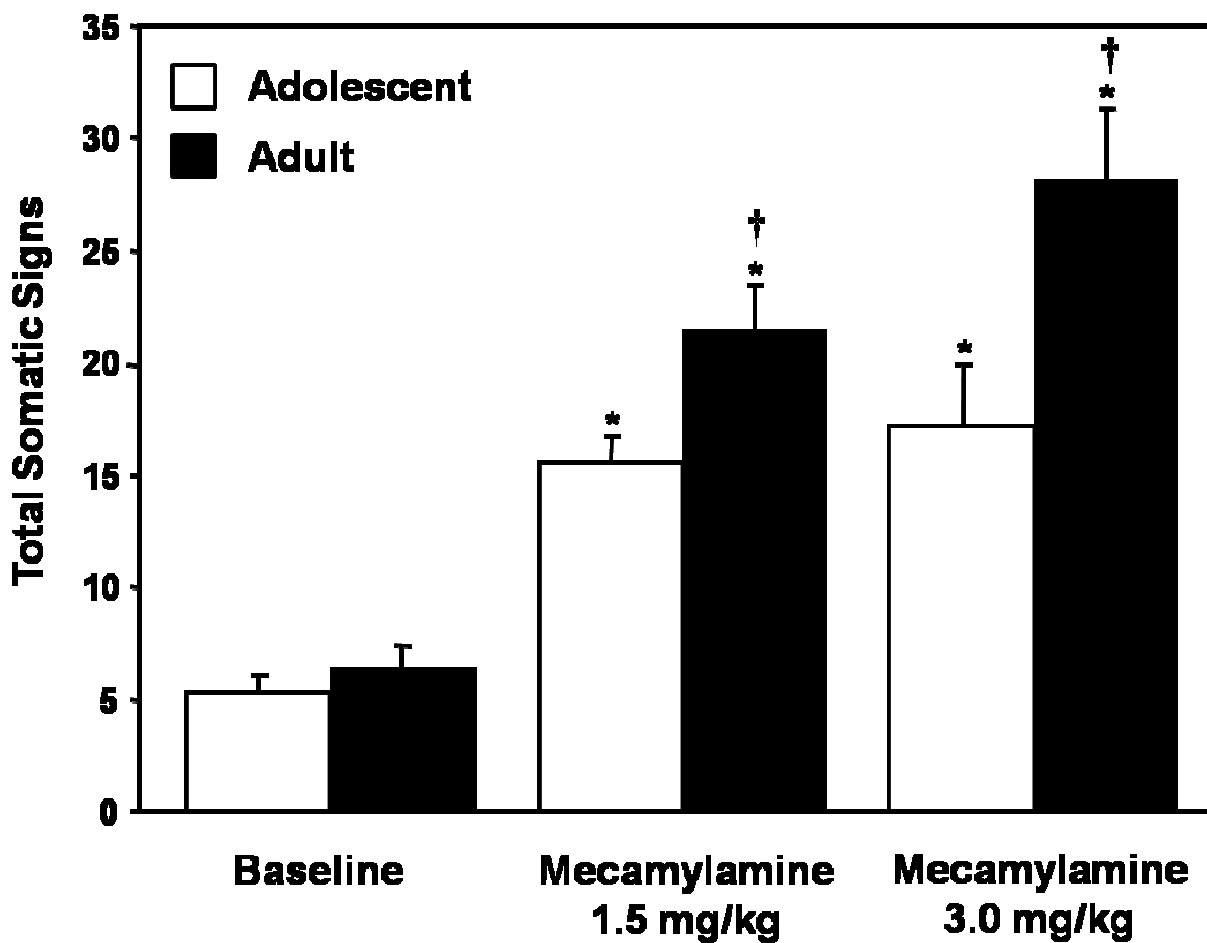


Figure 1: Data reflect total somatic signs of withdrawal (\pm SEM) exhibited in a 10-minute observation period during baseline and following mecamylamine administration in adolescent and adult rats ($n=7-8$ per group). Asterisks (*) denote a significant difference from baseline values ($P_s < 0.05$), and daggers (†) denote a significant difference between age groups ($P_s < 0.05$).

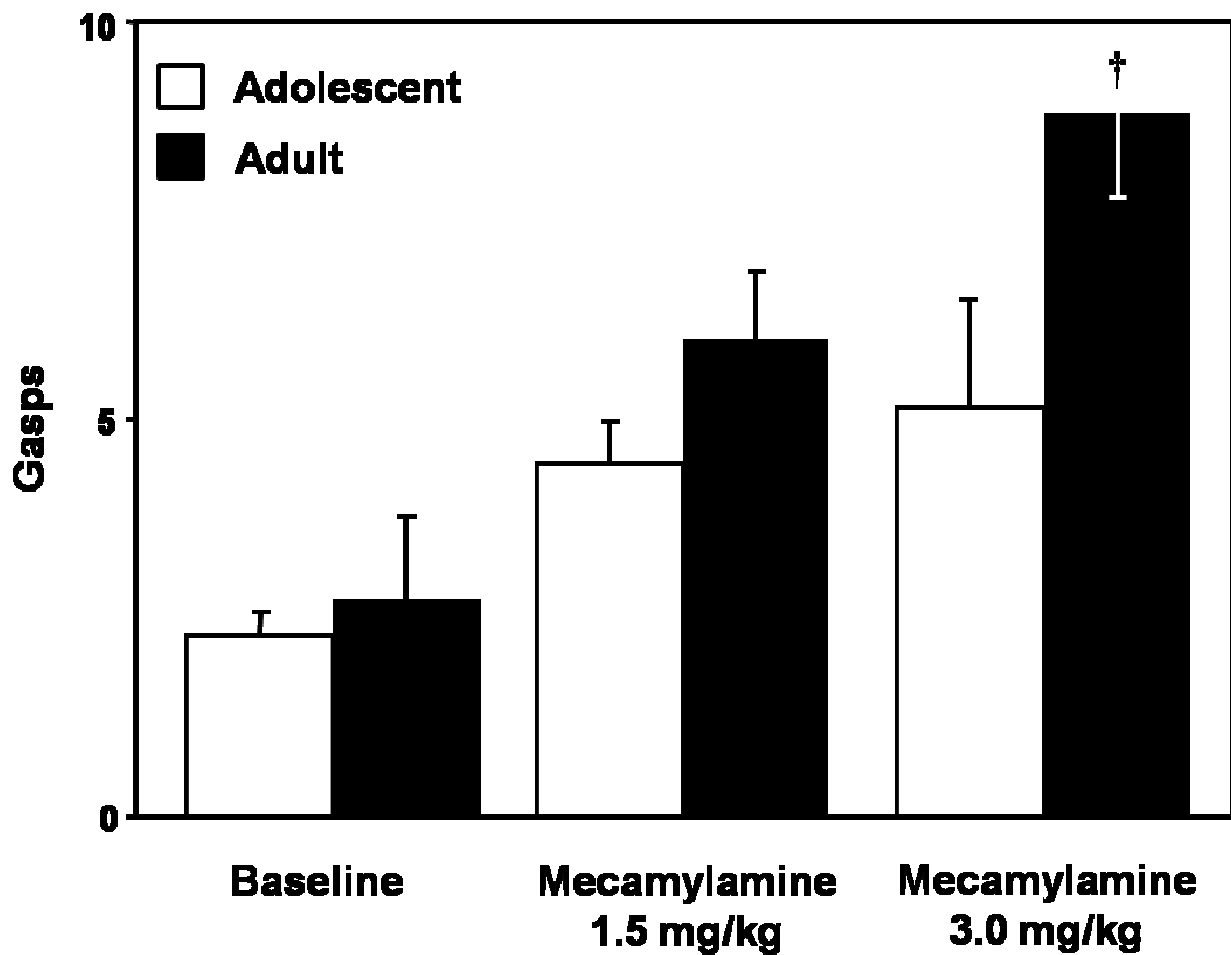


Figure 2: Data reflect the gasping behavior (\pm SEM) exhibited in a 10-minute observation period during baseline and following mecamylamine administration in adolescent and adult rats ($n=7-8$ per group). Dagger (\dagger) denotes a significant difference between age groups ($P < 0.05$).

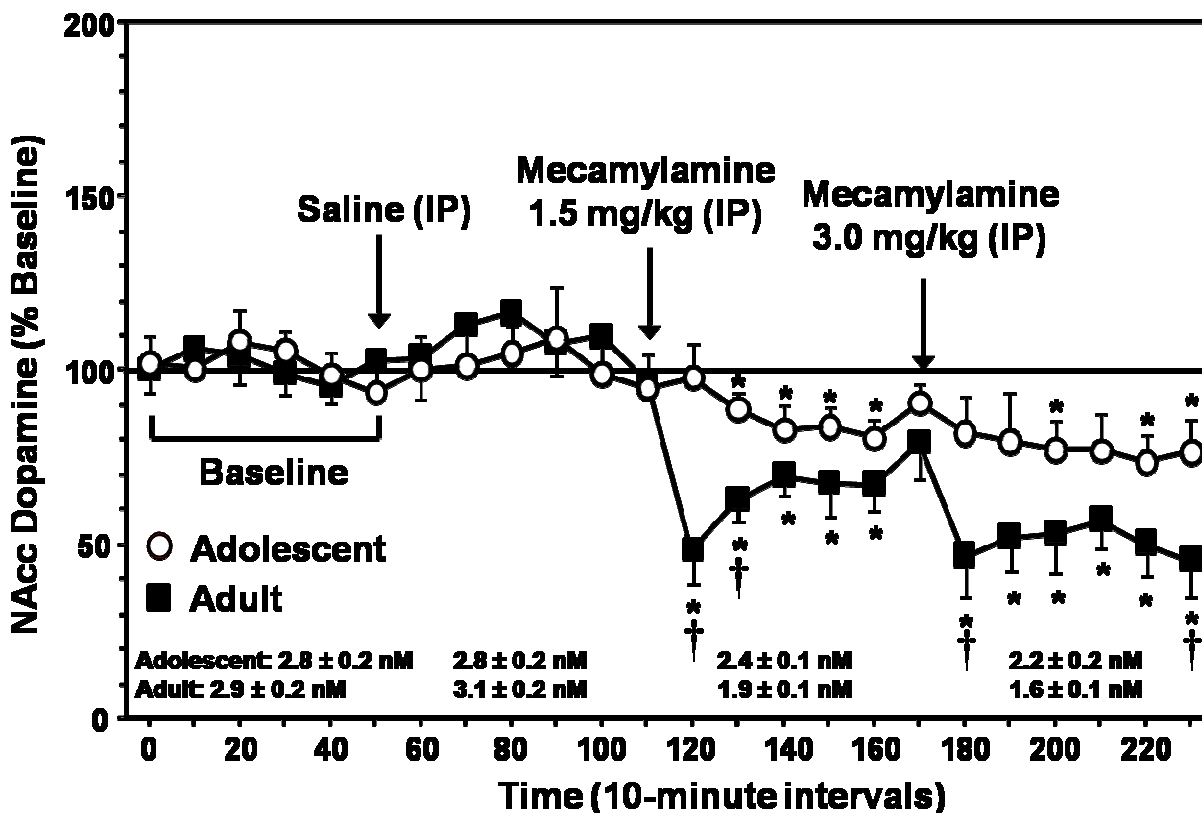


Figure 3: Data reflect % change in extracellular levels of NAcc dopamine (\pm SEM) plotted across 10-minute sample collections during baseline and following administration of saline and then 2 doses of mecamlamine to precipitate withdrawal in adolescent and adult rats ($n=7-8$ per group). The average dopamine nM concentration (\pm SEM) is noted below each treatment condition collapsed across the 1 hour of sample collection. The arrows indicate the onset of drug administration. Asterisks (*) denote significant differences from baseline levels ($P_s < 0.05$), and daggers (†) denote significant differences between age groups ($P_s < 0.05$).

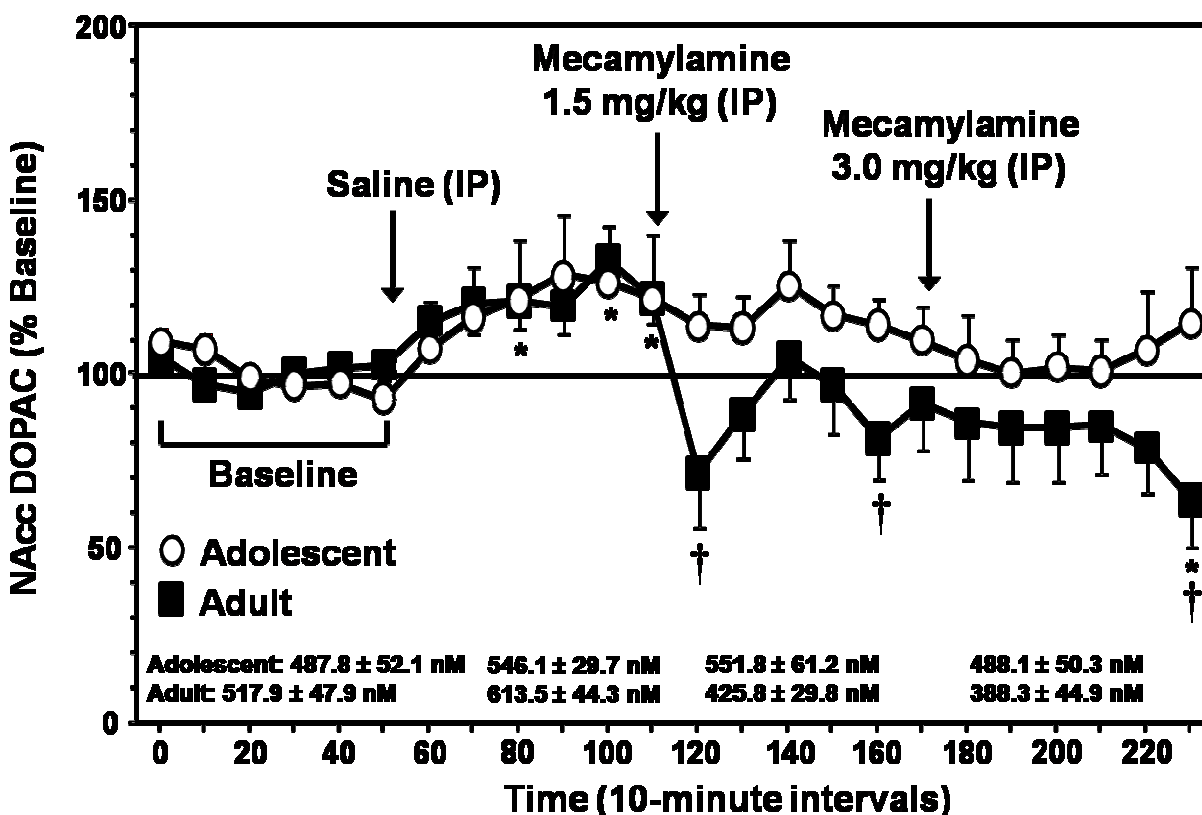


Figure 4: Data reflect % change in extracellular levels of NAcc DOPAC (\pm SEM) plotted across 10-minute sample collections during baseline and following administration of saline and then 2 doses of mecamylamine to precipitate withdrawal in adolescent and adult rats ($n=7-8$ per group). The average DOPAC nM concentration (\pm SEM) is noted below each treatment condition collapsed across the 1 hour of sample collection. The arrows indicate the onset of drug administration. Asterisks (*) denote significant differences from baseline levels ($P_s < 0.05$), and daggers (†) denote significant differences between age groups ($P_s < 0.05$).

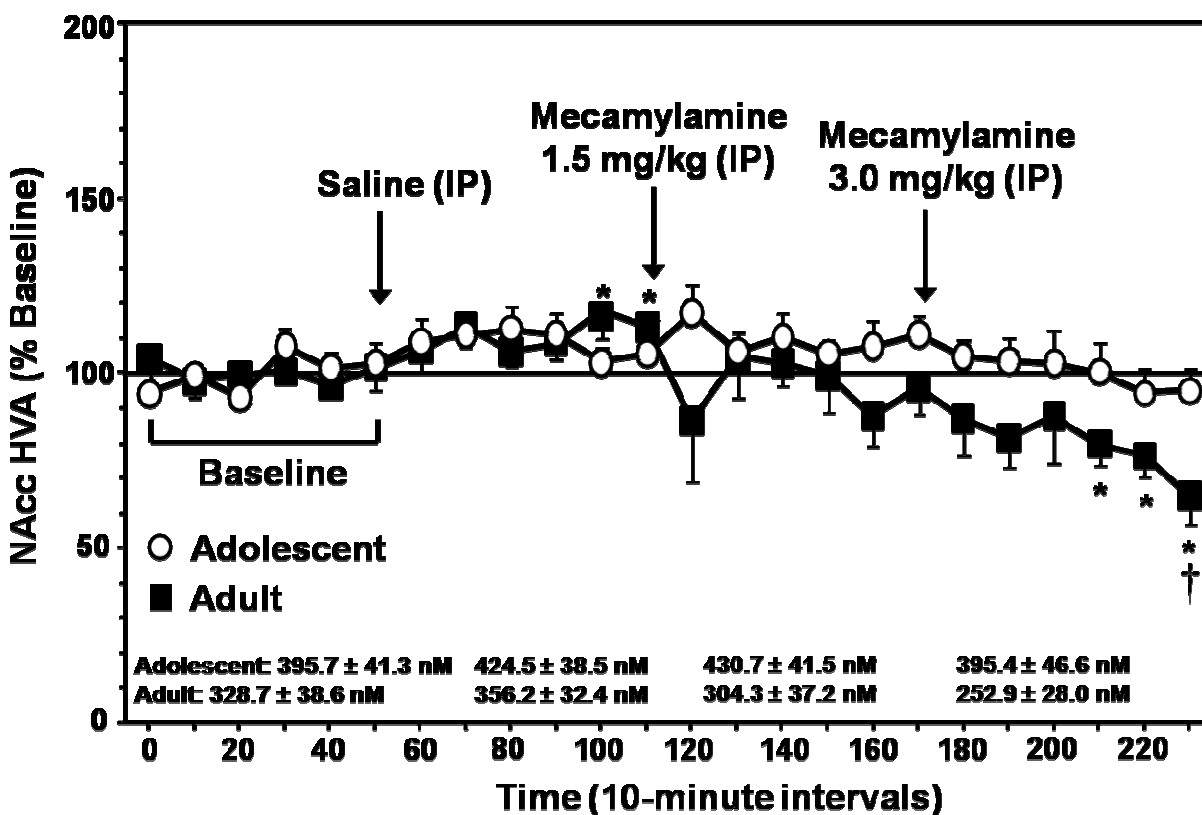


Figure 5: Data reflect % change in extracellular levels of NAcc HVA (\pm SEM) plotted across 10-minute sample collections during baseline and following administration of saline and then 2 doses of mecamylamine to precipitate withdrawal in adolescent and adult rats ($n=7-8$ per group). The average HVA nM concentration (\pm SEM) is noted below each treatment condition collapsed across the 1 hour of sample collection. The arrows indicate the onset of drug administration. Asterisks (*) denote significant differences from baseline levels ($P < 0.05$), and daggers (†) denote significant differences between age groups ($P < 0.05$).

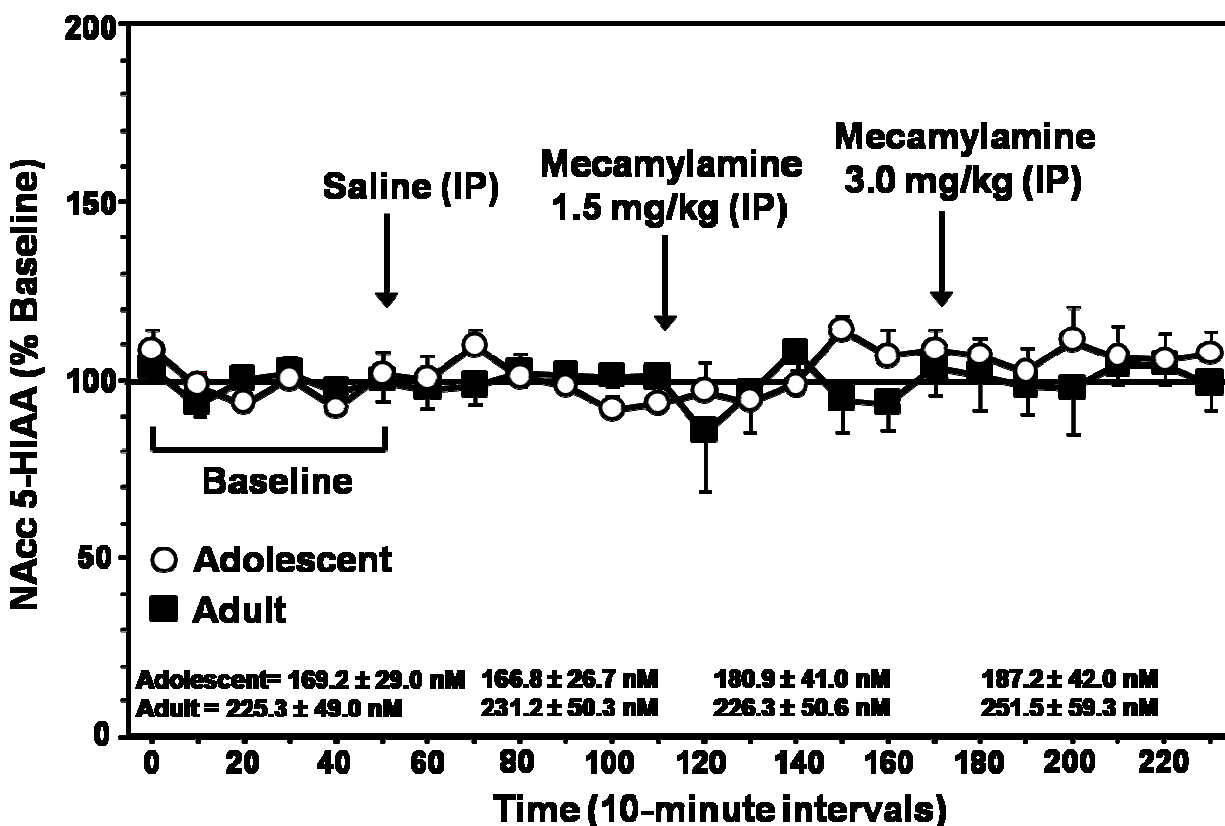


Figure 6: Data reflect % change in extracellular levels of NAcc 5-HIAA (\pm SEM) plotted across 10-minute sample collections during baseline and following administration of saline and then 2 doses of mecamylamine to precipitate withdrawal in adolescent and adult rats ($n=7-8$ per group). The average 5-HIAA nM concentration (\pm SEM) is noted below each treatment condition collapsed across the 1 hour of sample collection. The arrows indicate the onset of drug administration.

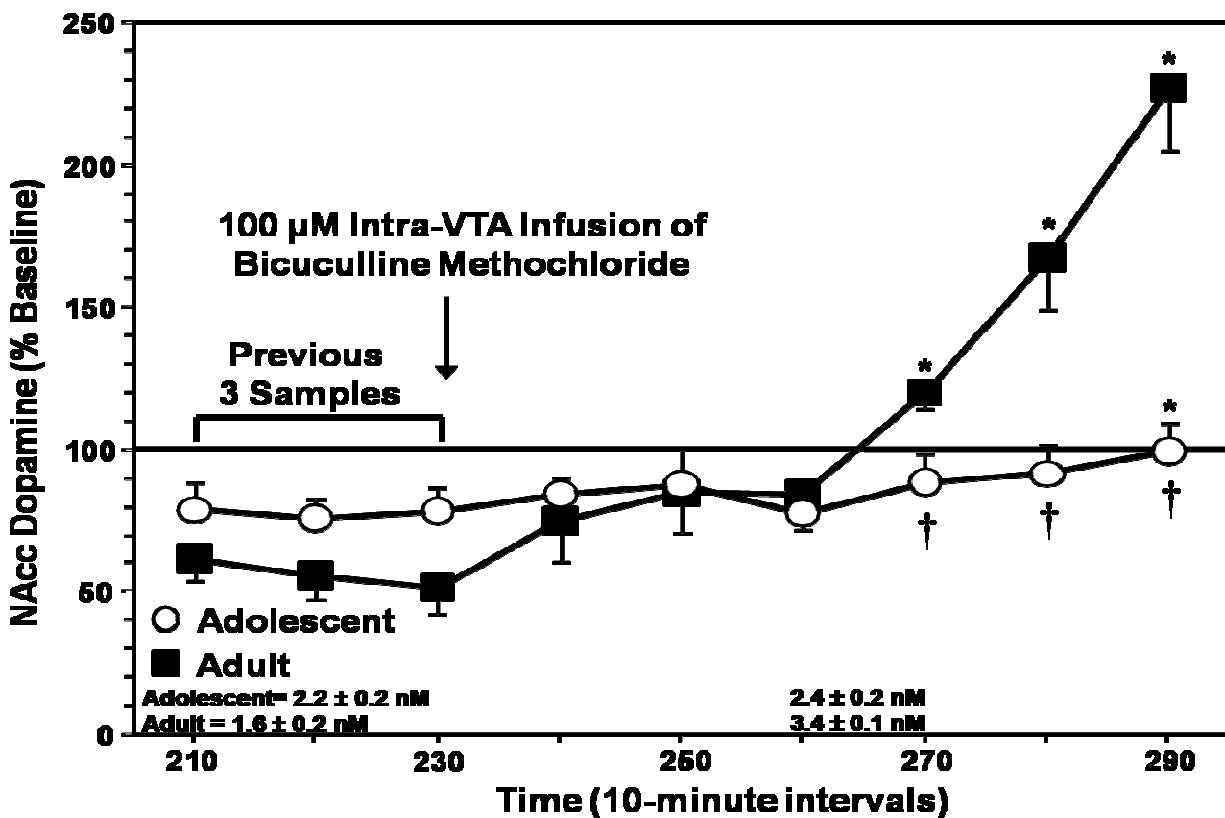


Figure 7: Data reflect % change in extracellular levels of NAcc dopamine plotted across 10-minute sample collections during the 3 samples prior to and the 6 samples following intra-VTA bicuculline administration in adolescent and adult rats ($n=7-8$ per group). The average dopamine nM concentration (\pm SEM) is noted below prior to and following bicuculline administration. Asterisks (*) denote significant differences relative to the 3 samples collected prior to bicuculline administration ($P_s < 0.05$), and daggers (†) denote significant differences between age groups ($P_s < 0.05$).

Curriculum Vitae

Luis Alberto Natividad was born to Pedro and Margarita Natividad in El Paso, Texas on January 9th, 1980. He graduated from Stephen F. Austin High School in El Paso, Texas in May, 1998 and entered The University of Texas at El Paso (UTEP) the following semester. During this time, Luis was an Americorp National Service member where he mentored underprivileged Hispanic students. Thereafter, he entered the University of Texas at Austin in August, 2000 to continue his undergraduate studies in Psychology. He became interested in Neuroscience and soon began research work with Dr. Adriana Alcantara who studied the mechanisms of alcohol addiction in the brain. Luis received his Bachelor of Arts degree in May, 2002 and obtained an internship as a drug addiction counselor. He became interested in the patterns of drug abuse as they differed between adolescents and adults. This inspired him to explore the neural basis of adolescent vulnerability to drug addiction in a laboratory setting. He entered the Social, Cognition, and Neuroscience program at UTEP in August, 2005 where he received training from Dr. Laura E. O'Dell. Her laboratory combines behavioral and biochemical tools to study the mechanisms of nicotine and alcohol addiction in rats. Luis has presented his research work in 20 conferences focused on drug abuse. He is co-author on 2 published articles in *Neurotoxicology and Teratology* and *Pharmacology, Biochemistry and Behavior*. He has received fellowships from the American Psychological Association- Diversity Program in Neuroscience sponsored by the National Institute of Mental Health, and the National Research Service Award- Ruth L. Kirschstein Predoctoral Fellowship sponsored by the National Institute on Drug Abuse. He received his Master's of Arts degree in May, 2009 and is currently working to obtain his Ph.D.

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