RAPIDLY EMERGING ADOLESCENT NICOTINE-INDUCED DENDRITIC REMODELING IS D1-DOPAMINE RECEPTOR DEPENDENT

by

Daniel G. Ehlinger

A Dissertation
Submitted to the
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of
Doctor of Philosophy
Psychology

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Daniel G. Ehlinger
Master of Arts
George Mason University, 2011

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DEDICATION

This dissertation is dedicated to my loving wife Kathy, my two beautiful children Julia and Maeble, and to my parents Richard and Linda Ehlinger, for their unwavering support during my graduate school career.

ACKNOWLEDGEMENTS

I would like to give special acknowledgement to Julian Burke, Hadley Bergstrom, Gina Fernandez, and my committee members, Robert Smith, Craig McDonald, and Daniel Cox for their extensive support with the design, implementation, and analysis of this dissertation. I would also like to acknowledge the Virginia Foundation for Healthy Youth (VHFY) for their generous funding. Finally, I would like to acknowledge all students, faculty, and staff within the Psychology department, and especially the Cognitive and Behavioral Neuroscience program, at George Mason University for their support throughout this project.

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LIST OF ABBREVIATIONS

(-)-Nicotine hydrogen tartrate ..... Nicotine
D1-dopamine receptor D1DR
D2-dopamine receptor D2DR
Dopamine ..... DA
Medium spiny neuron ..... MSN
Nucleus accumbens ..... NAcc
Postnatal day ..... P
R(+)-SCH-23390 hydrochloride SCH-23390
Subcutaneous ..... SC

ABSTRACT

RAPIDLY EMERGING ADOLESCENT NICOTINE-INDUCED DENDRITIC REMODELING IS D1-DOPAMINE RECEPTOR DEPENDENT

Daniel G. Ehlinger, PhD

George Mason University, 2014

Dissertation Director: Dr. Robert F. Smith

Chronic nicotine exposure during adolescence induces dendritic remodeling of medium spiny neurons (MSNs) in the nucleus accumbens (NAcc) shell of the rat. While nicotine-induced dendritic remodeling has frequently been described as persistent, no time-course studies have assessed whether dendritic remodeling is present immediately following nicotine exposure or if dendritic remodeling develops over an extended drug-abstinent period. Furthermore, the specific neuropharmacological mechanisms through which nicotine exposure may alter dendrite morphology is relatively unknown. To address these questions, Sprague-Dawley rats were co-administered subcutaneous injections of either saline or the D1-dopamine receptor (D1DR) antagonist SCH-23390 (0.05mg/kg) 20 minutes prior to the subcutaneous injection of either saline or nicotine (0.5 mg/kg) beginning on postnatal day (P) 28. Injections were administered every other day for 8 total injection days ending on P42. Brains were then processed for Golgi-Cox

staining either 1-day (P43) or 21-days (P63) following drug exposure. Dendrites from MSNs were digitally reconstructed in three-dimensions, and dendritic spine density was assessed on ~40µm segments of terminal branches. Our results show that (1) chronic adolescent nicotine exposure rapidly induces the formation of new dendritic branches and dendritic spines on NAcc Shell MSNs by P43, (2) nicotine-induced dendritic remodeling, but not increased spine density, is maintained through an extended abstinent period until at least P63, (3) the co-administration of SCH-23390 prior to each nicotine injection blocked nicotine-induced dendritic remodeling of MSNs when measured at both P43 and P63, suggesting that nicotine-induced formation of new dendritic branches is D1DR-dependent, and (4) SCH-23390 failed to block nicotine-induced increases in spine density. Collectively, these results suggest that chronic adolescent nicotine-induced dendritic remodeling of NAcc Shell MSNs rapidly develops during the course of nicotine exposure, and that nicotine-induced D1DR activation has a specific influence on the formation of new dendritic branches, but not dendritic spines. These data highlight the influence of chronic nicotine on adolescent brain development and show that long-lasting, nicotine-induced, dendritic remodeling is D1DR dependent in the NAcc shell.

CHAPTER ONE: INTRODUCTION

Adolescence is a time period in which the majority of smokers initiate tobacco use, and the rate of successfully quitting is substantially lower among users that begin smoking during adolescence (NIDA, 2012). Adolescence represents both a critical stage in normal brain development, and a unique period of vulnerability to the effects of nicotine exposure in both human and animal models (Smith, 2003; O'Dell, 2009; Baler & Volkow, 2011). One of these unique vulnerabilities is nicotine-induced dendritic remodeling, which is considerably more pronounced in the nucleus accumbens (NAcc) shell when drug exposure occurs during adolescence compared to adulthood (McDonald et al., 2005; McDonald et al., 2007). In other areas of the brain's reward circuitry, a unique pattern of nicotine-induced dendritic remodeling is exhibited following adolescent versus adult exposure (Bergstrom et al., 2008; Bergstrom et al., 2010). Importantly, dendritic remodeling has the potential to greatly alter the overall pattern of synaptic connectivity in or with these brain regions (Chklovskii, 2004), and may play a role in the emergence or maintenance of the neurobehavioral effects of adolescent nicotine exposure.

While adolescent nicotine-induced dendritic remodeling has been established, its underlying neuropharmacological mechanisms are unknown. Although nicotine's direct neuropharmacological action is at the nicotinic acetylcholine (ACh) receptor (Picciotto et

al., 1998), its influence on the NAcc shell is largely through increased ventral tegmental area (VTA) dopaminergic transmission onto the medium spiny neurons (MSNs) (Di Chiara, 2000; Zhou, Liang, & Dani, 2001; Picciotto, 2003). Both D1- and D2-type dopamine (DA) receptors are located on MSN cell bodies and dendritic arbors (Surmeier et al., 1996), directly alter MSN function following nicotinic ACh receptor activation, and are involved in the neurobehavioral effects of chronic nicotine exposure (Pierce & Kumaresan, 2006). Interestingly, MSNs are the cell-type that is selectively prone to nicotine-induced dendritic remodeling NAcc shell (McDonald et al, 2005) yet the influence of DA signaling on nicotine-induced dendritic remodeling of MSNs is unknown. DA signaling at D1-type DA receptors (D1DR) might be a particularly important neuropharmacological mechanism for nicotine-induced dendritic remodeling in the NAcc shell due to positive coupling of these receptors with the cAMP-PKA pathway and their ability to upregulate intracellular signaling pathways in the NAcc that influence dendritic plasticity (Lachowicz & Sibley, 1997; Self 2004; Arikkath, 2012; Nestler, 2013). In the present study, we tested this hypothesis by co-administering the selective D1DR antagonist R(+)-SCH-23390 hydrochloride (SCH-23390) during the chronic administration of nicotine in adolescent rats. Our data show that co-administration of SCH-23390 during chronic adolescent nicotine exposure prevents nicotine-induced dendritic remodeling, and that this effect is specific to remodeling of the dendritic arbor rather than to nicotine-induced increases of dendritic spine density.

Additionally, we sought to characterize the time-course of nicotine-induced dendritic remodeling. While this form of plasticity has often been described as persistent

(Robinson & Kolb, 1999; Nestler, 2001) current research has yet to assess drug-induced dendritic remodeling at multiple time-points following chronic nicotine exposure. Therefore, current research has left open the possibility that nicotine-induced dendritic remodeling develops over time during abstinence, rather than as a direct result of nicotine exposure. Following cessation of chronic nicotine exposure, several neurochemical alterations are present that could potentially influence dendritic structure, including a sharp decrease in NAcc DA levels associated with both natural (Rahman et al., 2004) and drug-induced withdrawal (Rada et al., 2001), a persistently blunted DA response throughout an extended abstinent period (Trauth et al., 2001), and persistently altered intracellular and extracellular signals related to prior dopaminergic activation of MSNs such as deltaFosB (Nestler, 2001; Marttila, Raattamaa, & Ahtee, 2006) and BDNF expression (Yeom et al., 2005; Kim et al., 2007). Therefore, we analyzed adolescent nicotine-induced dendritic remodeling and spine density of MSNs both immediately following chronic adolescent nicotine exposure (early abstinence) and three-weeks following exposure (extended abstinence). Our results show a nicotine-induced increase in dendritic branching and spine density present immediately following nicotine exposure, and that this increase in dendritic branching, but not spine density, persists into adulthood.

CHAPTER TWO: MATERIALS AND METHODS

Subjects

A total of 64 male Sprague-Dawley rats (Harlan, IN) were used in this study. Rats arrived on postnatal day (P) 21, were randomly assigned to experimental groups and were group-housed 3 to 4 rats per cage. The animal colony was temperature, humidity, and light controlled (12 hour/12 hour light/dark cycle, lights on 07:00), and rats were given access to food and water *ad libitum*. All experimental procedures were completed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (eighth edition; http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf) and the George Mason University Institutional Animal Care and Use Committee.

Drugs used

R(+)-SCH-23390 hydrochloride (SCH-23390; Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and administered subcutaneously (SC) at a dose of 0.05mg/kg at volume of 1ml/kg. This dose of SCH-23390 has previously been shown to disrupt behavioral responses to nicotine while limiting extrapyramidal side-effects (Acquas et al., 1989; Zarrindast, Sadegh, & Shafaghi, 1996). (-)-Nicotine hydrogen tartrate (Nicotine; Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and pH was adjusted to 7.0. Nicotine was administered SC at a dose of 0.5mg/kg at volume of 1ml/kg. This dose of nicotine administered chronically during adolescence has previously been shown to be

rewarding during adolescence (Brielmaier et al., 2007; Brielmaier et al., 2008), alter behavioral responses measured in adulthood (Bracken et al., 2011), and produce dendritic remodeling (Bergstrom et al., 2010). Physiological saline (0.9% NaCl solution - control) was administered SC at volume of 1ml/kg.

Experimental protocol

The first goal of this study was to assess the influence of the D1DR on adolescent nicotine-induced dendritic remodeling and spine density in the NAcc shell. To address this, animals were repeatedly injected with nicotine or saline (control) during adolescence (P28-42), and were co-administered pretreatment injections of either the D1DR antagonist SCH-23390 or saline (control). Animals were then sacrificed for assessment of dendritic remodeling and spine density. The age range chosen for drug administration in this study represents a conservative estimate for adolescence (Spear & Blake, 1983; Spear, 2000), although indices of adolescent brain development in the rat may extend to as late as P60 (Odell, 1990; Ojeda & Urbanski, 1994). Upon arrival at our facility, animals were randomly assigned to one of four groups that differed on pretreatment drug (saline or SCH-23390) and treatment drug (saline or nicotine) administered. Therefore, our four groups consisted of (1) saline (pretreatment) - saline (treatment), (2) SCH-23390 - saline, (3) saline - nicotine, and (4) SCH-23390 - nicotine. Pretreatment drug was administered (SC) precisely 20 minutes prior to treatment drug administration (SC). Animals were dosed in their home-cage with pretreatment and treatment drug every other day from P28-P42, for a total of 8 injection days within the two-week period (**Fig. 1**).

This pattern of repeated nicotine exposure has been previously shown to produce dendritic remodeling in the NAcc shell (Brown & Kolb, 2001).

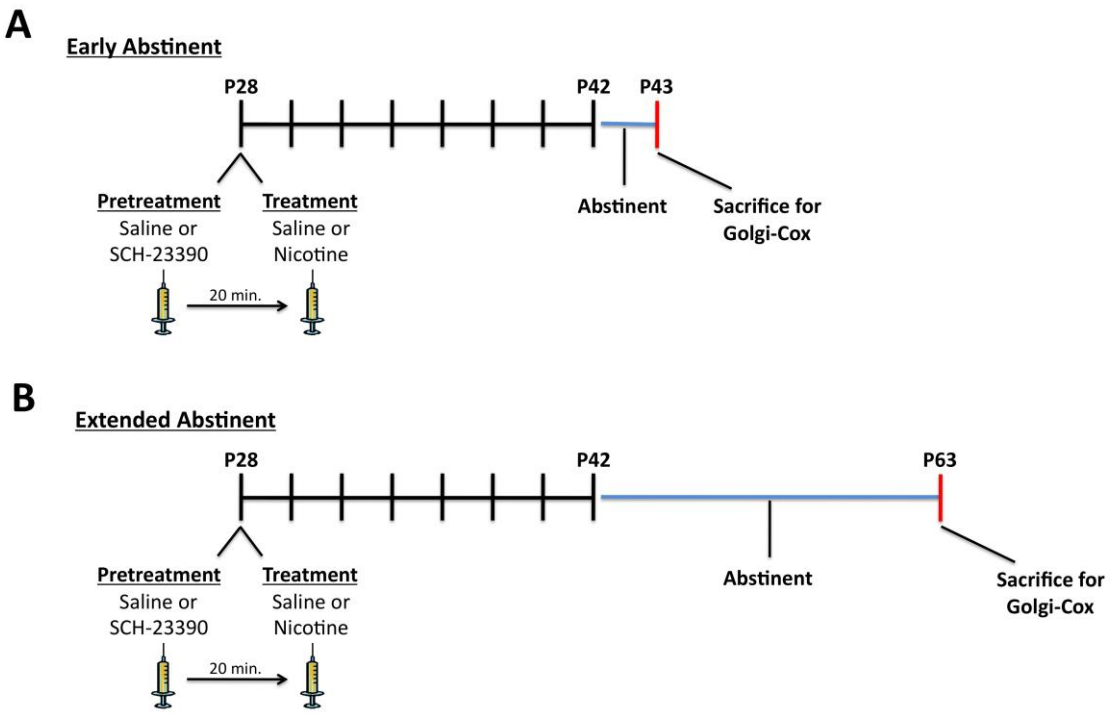

Figure 1. Dosing protocol and experimental design.
Black color represents time period of nicotine exposure, with each vertical line representing a single pretreatment/treatment injection. Blue color represents length of abstinence for either early (***A***) or extended (***B***) abstinence. Red color represents day of sacrifice for tissue staining.

At the cessation of drug dosing, animals were sacrificed for Golgi-Cox tissue staining and analysis of dendritic structure. However, a second goal of this study was to determine whether or not adolescent nicotine-induced dendritic remodeling of NAcc Shell MSNs is present immediately following the cessation of nicotine exposure and whether or not these changes are persistent into adulthood. To assess this, half of the

animal subjects (n = 32) were sacrificed for Golgi-Cox tissue staining one-day following the cessation of drug administration (P43; early abstinence)(**Fig. 1A**), while the other half of the animal subjects were sacrificed three-weeks following the cessation of drug administration (P63; extended abstinence)(**Fig. 1B**).

Sacrifice and Golgi-Cox tissue staining

On either P43 (early abstinence) or P63 (extended abstinence), all animals were deeply anesthetized with a ketamine/xylazine cocktail injected intraperitoneally (IP) and perfused intracardially with 0.9% NaCl. Brains were immediately extracted and placed into a Golgi-Cox solution, following the recipe of Glaser & Van der Loos (1981), to allow for fixation and impregnation of tissue. After 2-days, brains were placed into fresh Golgi-Cox solution for an additional 12-days. Following Golgi-Cox immersion, brains were stored in a 30% sucrose solution until vibratome sectioning (200μm sections). Sections containing the entire rostral-caudal extent of the NAcc shell were placed onto gelatinized slides. Sections were then stained using the protocol of Gibb and Kolb (1998). Briefly, sections were alkalinized in ammonium hydroxide, developed and fixed using Kodak Rapid Fix, dehydrated through a series of ethanols, and cleared in a solution of 1/3 xylene, 1/3 100% alcohol, and 1/3 chloroform. Slides were then cover-slipped and stored in the dark for the remainder of the experiment (**Appendix B**).

Analysis of dendritic structure and spine density

Golgi-Cox impregnated sections containing NAcc shell MSNs were visualized using light-microscopy and MSNs were manually reconstructed in 3-dimensions (3D) using Neurolucida software (Microbrightfield Biosciences, Williston, VT) under 60x

objective by experimenters blind to pretreatment, treatment, and abstinence group. NAcc shell MSNs were selected based on the anatomical boundaries of the NAcc shell as defined in Paxinos and Watson (2007) (**Fig. 2A**) and identified by soma size (approx. 10-20μm diameter), multi-polar shape with the presence of 3 or more primary dendrites attached to the soma, and the presence of dendritic spines on late branch orders (≥ 3rd order). Only well-stained MSNs that possessed dendrites unobstructed by neighboring cells or blood vessels and that could be followed from soma to terminal tip without interruption were chosen for reconstruction (**Fig. 2B & C**). 4-6 MSNs were reconstructed from each animal, and were sampled equally between hemispheres within each animal, for a total of 319 reconstructed neurons (early abstinence, n = 168; extended abstinence, n = 151). Additionally, spine density was assessed on 6 neurons per animal, sampled equally between hemispheres, by manual labeling of spines on a reconstructed 40-50μm segment of a distal branch (≥ 3rd order branch, at least 60μm from the soma) ending in a terminal tip (Brown & Kolb, 2001). This technique ensures that the diameter of each segment chosen for analysis of spine density is similar and places analysis of spine density at the same location of the dendritic arbor at which nicotine-induced dendritic remodeling occurs on MSNs (McDonald et al., 2005; 2007).

Morphometrics on reconstructed MSNs were obtained using Neuroexplorer (Microbrightfield Biosciences, Williston, VT). Morphometric parameters obtained included total dendritic length, total number of bifurcations, and branch-order analysis (centrifugal method; total dendritic length, total number of branches, and average length as a function of branch order). Spine density was assessed as the total number of spines ÷

length of dendritic segment reconstructed. Finally, the distribution of dendritic material was assessed using 3D Sholl analyses (20µm increments) with parameters of dendritic length and number of bifurcations.

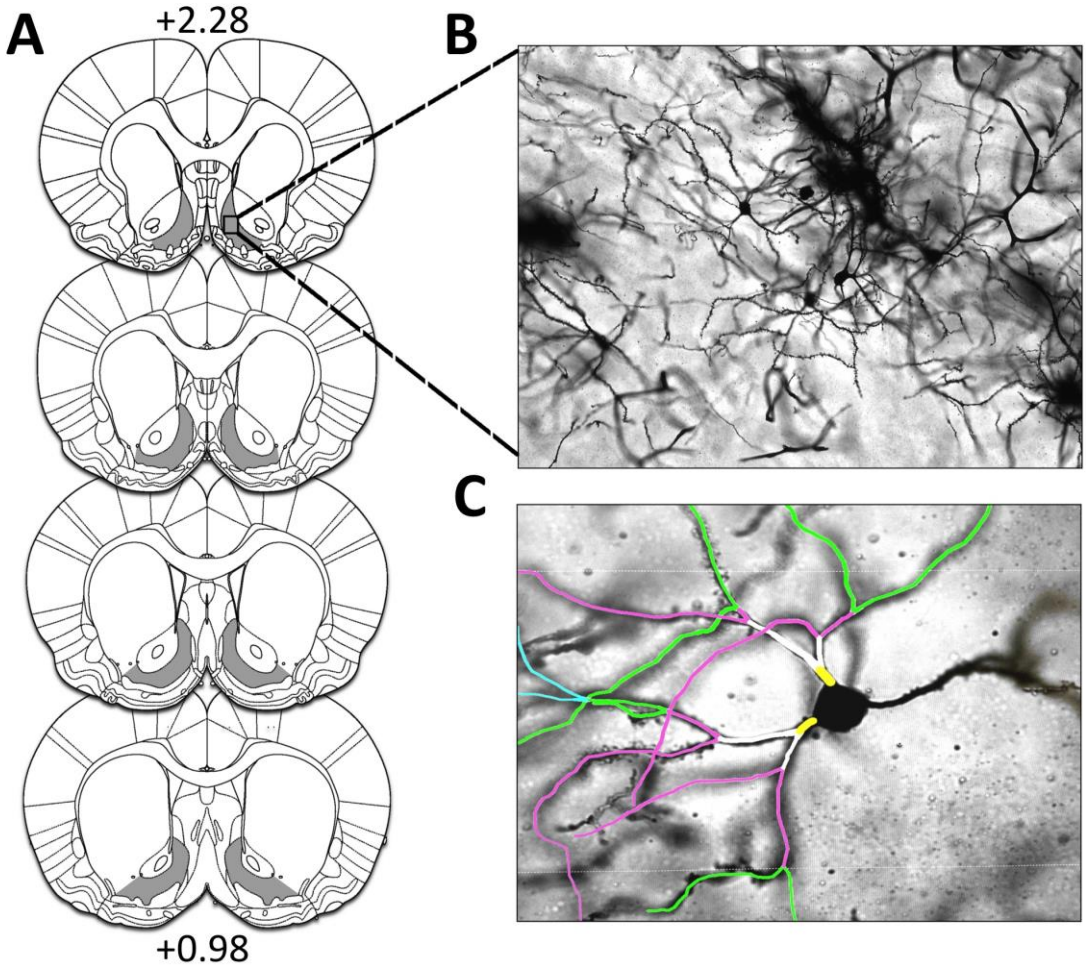

Figure 2. Location of NAcc shell and MSNs.
A, Brain atlas images representing the extent of the NAcc shell (grey highlight) from which MSNs were sampled (Paxinos & Watson, 2007). Coordinates are relative to bregma. ***B***, Micrograph of Golgi-Cox stained MSNs image under 20x objective. ***C***, Micrograph of a partially reconstructed MSN imaged under 60x objective, colored by branch order.

Data analysis

Prior to statistical analysis, a mean value per animal was obtained for each of the parameters assessed, rather than treating each individual neuron as an independent measure. For each morphometric parameter, mixed-ANOVA with within-subject factor of distance from the soma (Sholl analyses) or branch-order (branch-order analyses; **Appendix A**), and between-subject factors of length of abstinence (early vs. extended), pretreatment drug (saline vs. SCH-23390), and treatment drug (saline vs. nicotine) were conducted. Following a significant interaction, separate mixed-ANOVAs were conducted within the early and extended abstinence groups followed by planned-comparisons (Mixed-ANOVA, *bonferonni* correction) to test the following hypotheses: (1) nicotine administered alone will induce dendritic-remodeling (saline-saline vs. saline-nicotine), (2) SCH-23390 co-administered with nicotine will prevent nicotine-induced dendritic remodeling (saline-nicotine vs. SCH-23390-nicotine), and (3) SCH-23390 alone will not induce dendritic remodeling (saline-saline vs. SCH-23390-saline). Violation of the assumption of sphericity for repeated measures was corrected using the Greenhouse–Geisser correction for degrees of freedom (superscripted letter "a" proceeding an *F* value indicates Greenhouse–Geisser-corrected value for degrees of freedom). To further assess the distances from the soma at which the greatest dendritic remodeling occurs, comparisons between groups were made at specific radii from the soma (*t*-test) with significance determined as $p < .05$ at consecutive radii (Bergstrom et al., 2010; Ehlinger et al., 2012).

For spine density, three-way ANOVA with between-subjects factors of length of abstinence (early vs. extended), pretreatment drug (saline vs. SCH-23390), and treatment

drug (saline vs. nicotine) were conducted. Following significant interactions, separate two-way ANOVAs were conducted within the early and extended abstinence groups, followed by planned comparisons (*t*-test with *bonferonni* correction) to test the previously described hypotheses.

CHAPTER THREE: RESULTS

Nicotine-induced dendritic remodeling of MSNs

Representative MSN reconstructions are presented for early abstinent (P43)(**Fig. 3A**) and extended abstinent (P63)(**Fig. 3B**) groups. Statistical analyses revealed a significant interaction between radius, pretreatment drug, and treatment drug on both dendritic length (${}^{a}F_{(2.9,160.9)} = 8.2$, $p < .001$) and bifurcations (${}^{a}F_{(4.6,258.4)} = 6.1$, $p < .001$). Statistical analyses also revealed an interaction between treatment and abstinence on dendritic length ($F_{(1,56)} = 5.5$, $p < .05$), and a trend toward an interaction between radius, treatment drug, and abstinence on bifurcations (${}^{a}F_{(2.9,160.9)} = 2.5$, $p = .06$).

For animals co-administered saline (pretreatment) during adolescent nicotine treatment, nicotine significantly induced dendritic remodeling of MSNs, as MSNs from saline-nicotine animals display increased total dendritic length when measured at both P43 ($F_{(1,14)} = 12.5$; $p < 0.01$) and P63 ($F_{(1,14)} = 25.4$, $p < .001$) compared to saline-saline (control) animals (**Fig. 4A**). These nicotine-induced increases in total dendritic length were accompanied by a significant nicotine-induced increase in the total number of bifurcations when measured at both P43 ($F_{(1,14)} = 20.0$, $p < .01$) and P63 ($F_{(1,14)} = 30.0$, $p < .001$) (**Fig. 4B**), suggesting that the reported increases in total dendritic length are the result of new branch formation and that this dendritic remodeling is persistent through an extended abstinent period. Specifically, Sholl analyses indicate that nicotine-induced

dendritic remodeling occurs at radii distal to the soma. At P43, significantly increased dendritic length of MSNs from saline-nicotine animals is present at radii between 40-

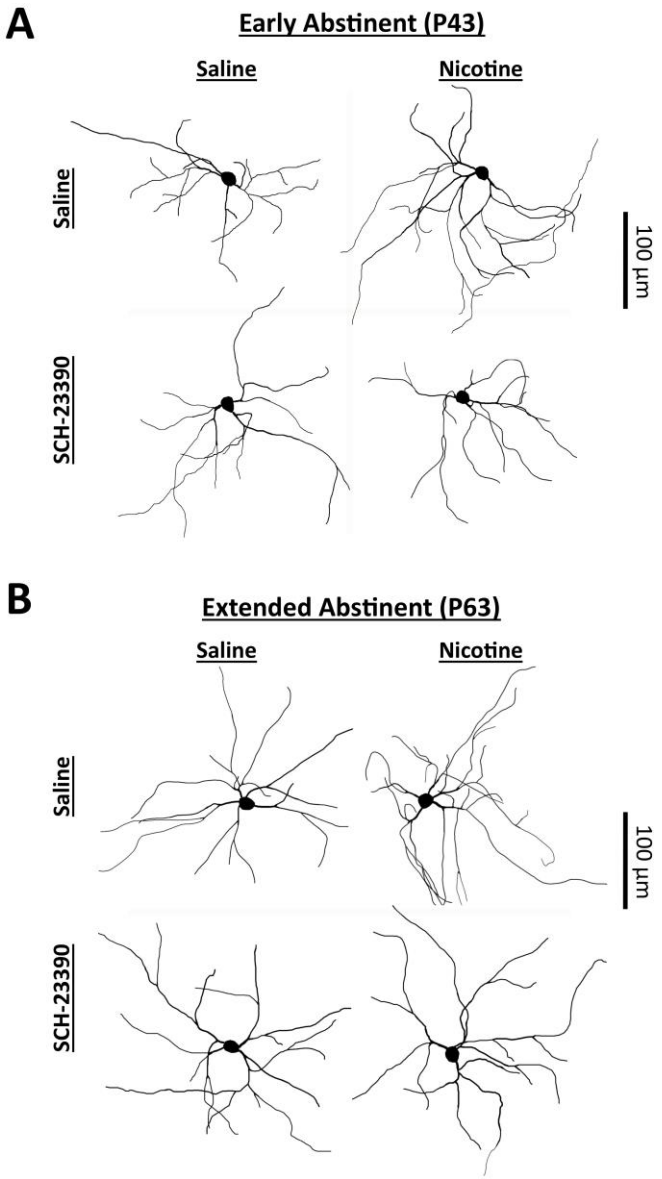

Figure 3. Representative MSN reconstructions.
A, MSNs from animals sacrificed at P43 (early abstinence). ***B***, MSNs from animals sacrificed at P63 (extended abstinence). Y-axes, pretreatment drug. X-axes, treatment drug. All MSNs are within group means (± SEM) for both total length and bifurcations.

240µm from the soma ($^{a}F_{(1.8,25.3)} = 8.1$, $p < .01$) (**Fig. 5A**) and bifurcations at radii between 40-160µm from the soma ($^{a}F_{(3.5,49.4)} = 5.2$, $p < .01$) (**Fig. 5B**). At P63, significantly increased dendritic length is still present at radii between 40-140µm from the soma ($^{a}F_{(3.7,51.8)} = 6.9$, $p < .001$) (**Fig. 5C**) and bifurcations at radii between 80-100µm from the soma ($^{a}F_{(3.3,45.4)} = 2.8$, $p < .05$) (**Fig. 5D**).

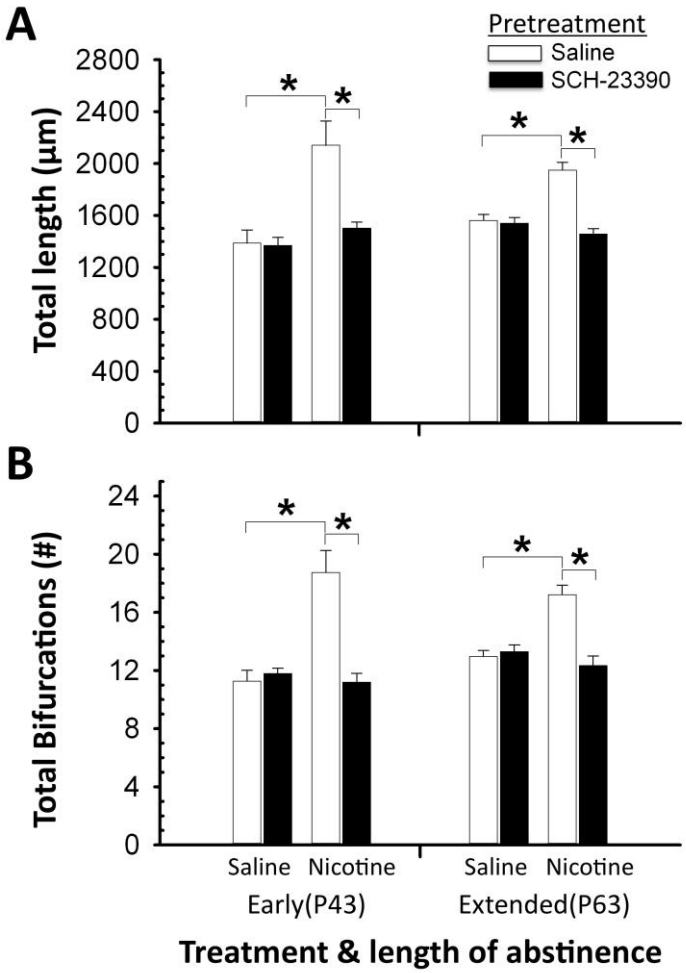

Figure 4. Influence of chronic nicotine and D1DRs on dendritic morphology (totals).
A, Mean (±SEM) total dendritic length of MSNs from early abstinent and extended abstinence animals. ***B***, Mean (±SEM) total number of bifurcations on MSNs from early abstinent and extended abstinence animals. *****, Significant difference between pretreatment-treatment groups ($p < .01$).

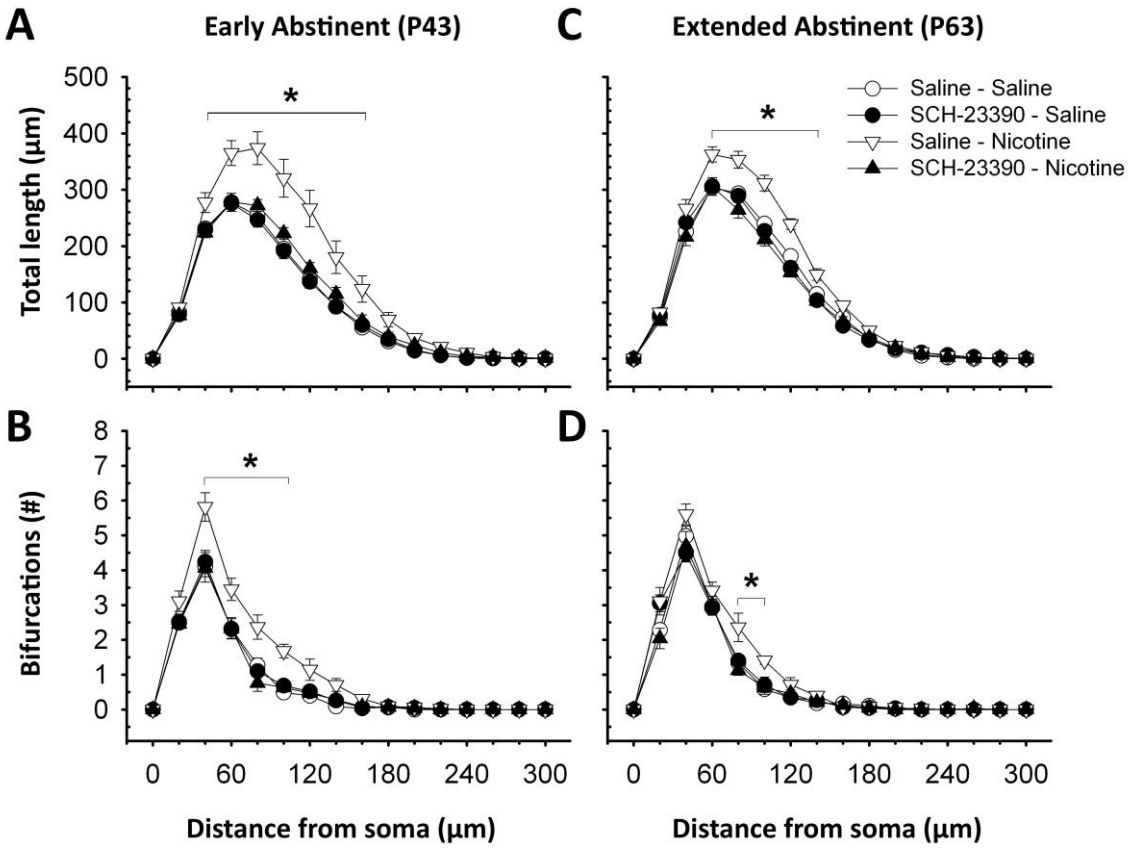

Figure 5. Influence of chronic nicotine and D1DRs on dendritic morphology as a function of distance from the soma. Total dendritic length (Mean ± SEM)(***A***,***C***) and number of bifurcations (Mean ± SEM)(***B***,***D***) within each 20µm spherical radius from the soma, in early abstinence (***A***,***B***) and extended abstinence (***C***,***D***) animals. *****, Significant difference between saline-nicotine group and all other pretreatment-treatment groups ($p < .05$ at consecutive radii).

Influence of D1DRs on nicotine-induced dendritic remodeling of MSNs

For animals co-administered SCH-23390 (pretreatment) during adolescent nicotine treatment, nicotine failed to induce the previously observed dendritic remodeling of MSNs when compared to animals co-administered saline (pretreatment) during nicotine treatment, as MSNs from SCH-23390-nicotine animals display significantly reduced total dendritic length measured at both P43 ($F_{(1,14)} = 11.0$, $p < .01$) and P63 ($F_{(1,14)} = 43.9$, $p < .001$) (**Fig. 4A**), as well as significantly reduced total number of

bifurcations measured at both P43 ($F_{(1,14)} = 21.8$, $p < .001$) and P63 ($F_{(1,14)} = 27.3$, $p < .001$) (**Fig. 4B**), compared to those from saline-nicotine animals. Furthermore, MSNs from SCH-23390-nicotine animals display no difference to those from saline-saline (control) animals. Sholl analyses suggest that these reductions in nicotine-induced dendritic remodeling of MSNs occur at radii distal to the soma. At P43, reduced dendritic length in MSNs of SCH-23390-nicotine animals compared to saline-nicotine animals is exhibited at radii between 40-180µm from the soma (${}^{a}F_{(2.0,28.3)} = 6.0$, $p < .01$) (**Fig. 5A**), and reduced number of bifurcations at radii between 40-100µm from the soma (${}^{a}F_{(3.8,53.1)} = 6.9$, $p < .001$) (**Fig. 5B**). At P63, reduced dendritic length is exhibited at radii between 40-160µm from the soma (${}^{a}F_{(2.6,36.3)} = 9.5$, $p < .001$) (**Fig. 5C**), and reduced number of bifurcations at radii between 80-100µm from the soma (${}^{a}F_{(3.8,52.6)} = 3.1$, $p < .05$) (**Fig. 5D**). Finally, no difference in total dendritic length nor total number of bifurcations was observed between MSNs from SCH-23390-saline and saline-saline (control) animals, nor were there any differences in MSN morphology at any radial distance from the soma between these two groups (**Fig. 4 & 5**). This suggests that D1DR antagonism prevents nicotine-induced dendritic remodeling rather than producing a general reduction in dendritic length or bifurcations.

Between P43 and P63, significantly increased dendritic length was observed at radii distal to the soma in saline-saline and SCH-23390-saline animals, between 100-120µm from the soma in saline-saline animals (${}^{a}F_{(2.9,41.0)} = 3.6$, $p < .05$) and 80-100µm from the soma in SCH-23390-saline animals (${}^{a}F_{(3.2,45.0)} = 2.7$, $p = .05$). These increases in dendritic length are accompanied by a trend toward increased total bifurcations in saline-

saline animals ($F_{(1,14)} = 4.0$, $p = .064$), and a significant increase in total bifurcations in SCH-23390 animals ($F_{(1,14)} = 6.9$, $p < .05$), although not dependent on radial distance from the soma (**Fig. 4 & 5**). Collectively, this suggests an increase in the formation of new dendritic branches in saline treatment (control) animals between P43 and P63. No differences in dendritic length or bifurcations between P43 and P63 were observed in nicotine treatment groups. Additional differences in dendritic morphology and spine density between P43 and P63, and within pretreatment-treatment groups, are reported in **Table 1 (Appendix A)**.

Nicotine-induced alterations in spine density and influence of D1DRs

Representative micrographs of MSN dendritic segments containing dendritic spines are presented for early abstinent (P43) (**Fig. 6A**, *left*) and extended abstinent (P63) (**Fig. 6B**, *left*) groups. Statistical analyses revealed a significant interaction between treatment drug and abstinence on spine density ($F_{(1,56)} = 13.1$, $p < .01$), with no main effects nor interactions related to pretreatment drug. When measured at P43, nicotine treatment induced a significant increase in spine density on MSNs ($F_{(1,28)} = 45.2$, $p < .001$). However, in contrast to findings on dendritic remodeling, spine density was increased on MSNs from both saline-nicotine ($F_{(1,14)} = 25.4$, $p < .001$) and SCH-23390-nicotine ($F_{(1,14)} = 15.0$, $p < .01$) animals compared to saline-saline (control) animals (**Fig. 6A**, *right*). No effect was observed on MSNs from animals co-administered SCH-23390 (pretreatment) with saline (control) treatment, as SCH-23390-saline animals did not display any observable difference in spine density from saline-saline (control) animals (**Fig. 6A & B**). Collectively, this suggests that SCH-23390 does not influence spine

density. Furthermore, nicotine's influence on spine density does not persist through the extended abstinent period, as no observable differences in spine density are shown at P63 between any of the pretreatment and treatment groups (**Fig. 6B**, *right*).

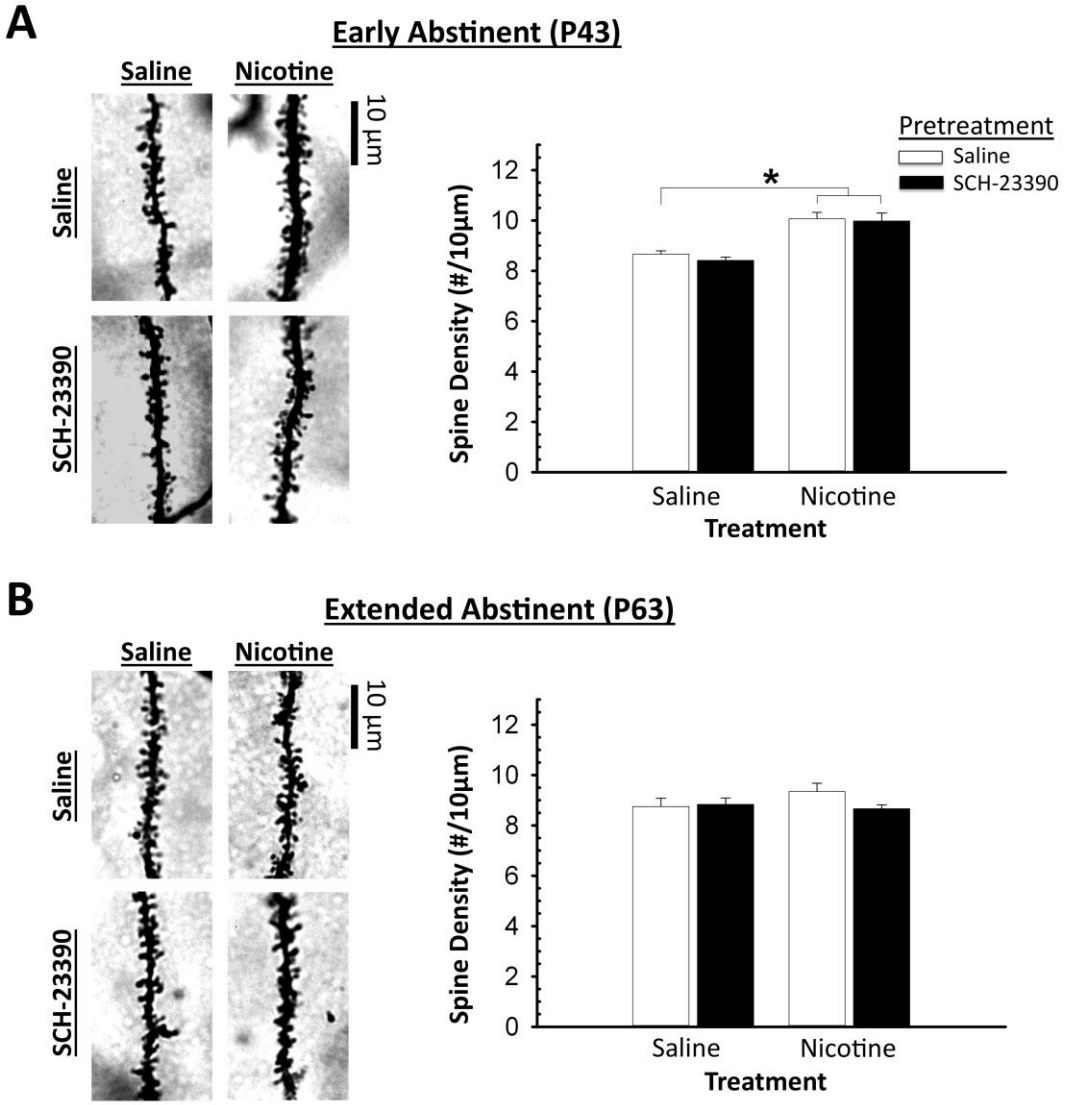

Figure 6. Influence of chronic nicotine and D1DRs on spine density.
Left, representative micrographs of distal dendritic segments on MSNs from early abstinence (***A***) and extended abstinence (***B)*** animals. Dendritic segments are within group means ± SEM for spine density. *Right*, Mean (± SEM) spine density of dendritic segments from early abstinence (***A***) and extended abstinence (***B)*** animals. *****, Significant difference between nicotine both nicotine (treatment) groups and saline-saline group ($p < .01$).

CHAPTER FOUR: DISCUSSION

This study aimed to answer two major questions regarding adolescent-nicotine induced dendritic remodeling in the NAcc shell. First, we sought to determine whether this type of neuroplasticity is dependent on D1DR activation by co-administering a D1DR antagonist during chronic adolescent nicotine exposure. Second, we sought to better characterize the time course of adolescent nicotine-induced dendritic remodeling by determining whether remodeling is present after both acute and extended abstinence from nicotine exposure.

Adolescent nicotine rapidly induces dendritic remodeling and increases spine density of NAcc shell MSNs

While the finding that chronic nicotine exposure results in the formation of new dendritic branches and increased spine density on MSNs in the NAcc shell is in agreement with previous literature (Brown & Kolb, 2001; McDonald et al., 2005; McDonald et al., 2007), the presence of this dendritic remodeling at an early abstinent period is a novel finding. Immediately following 2-weeks of chronic adolescent nicotine exposure, a substantial (54%) increase in new dendritic branches and total dendritic material was observed over a large portion of the distal dendritic arbor and was accompanied by an increase in spine density on distal dendritic branches. Therefore, our results suggest that the nicotine-induced formation of new dendritic branches and increased spine density of NAcc shell MSNs are the direct result of nicotine exposure

rather than an effect that develops over drug abstinence. Collectively, these alterations to dendritic structure and spine density can be expected to substantially increase the amount and/or alter the pattern of synaptic contact onto these neurons (Stepanyants, Hof, & Chklovskii, 2002; Chklovskii, 2004).

Importantly, this nicotine-induced formation of new dendritic branches is persistent, as increased dendritic branching and total dendritic material is maintained for at least 3-weeks following cessation of nicotine exposure, supporting the hypothesis that nicotine-induced dendritic remodeling is a long-lasting form of neuroplasticity. Although the magnitude of the nicotine-induced increase in total dendritic length (25%) observed at this time point is lower than that observed during early abstinence, it should be noted that this appears to reflect continued growth of MSNs in control animals rather than a reduction in dendritic length of MSNs from nicotine-treated animals, consistent with the presence of continued neural development during adolescence (Ojeda & Urbanski, 1994). Combined with observations that the magnitude of nicotine-induced dendritic remodeling in the NAcc shell is greater during adolescent than adult exposure (McDonald et al., 2005; McDonald et al, 2007; Brown & Kolb, 2001), this continued developmental pattern in NAcc MSNs might be an important factor mediating the enhanced vulnerability of the adolescent brain to nicotine-induced dendritic remodeling and other neurobehavioral effects of nicotine exposure (O'Dell et al., 2009).

Our findings that the nicotine-induced formation of new dendritic branches is present following both early and extended abstinent periods can provide insight into the potential cause and effects of this dendritic remodeling. The dendritic remodeling we

observed likely occurs during the time-course of repeated nicotine exposure itself and is persistent into adulthood rather than developing over abstinence. As will be further discussed below, we suggest that one specific neurochemical alteration occurring during drug exposure that is responsible for nicotine-induced dendritic remodeling is repeated nicotine-induced DA signaling at D1DRs. In contrast, any additional neurochemical changes that take place during the extended abstinent period are unlikely to be an important influence on nicotine-induced dendritic remodeling. Therefore, the specific neurobehavioral effects that correlate with this pattern of nicotine-induced dendritic remodeling are likely to be those that develop during the course of nicotine exposure itself and are maintained throughout drug abstinence, such as sensitization (Clark & Kumar, 1983; Benwell & Balfour, 1992) or tolerance (Stolerman, Fink, & Jarvik, 1973; Stolerman, Buncker, & Jarvik, 1974), rather than those that develop over abstinence, such as enhancement of drug craving (Pickens et al., 2011). However, further research is needed to better dissect the time-course of adolescent nicotine-induced dendritic remodeling and alteration in spine density, particularly the analysis of dendritic remodeling following different lengths of drug exposure and over different lengths of abstinence.

While the nicotine-induced formation of new dendritic branches is persistent, the nicotine-induced increase in spine density returned to control levels by the end of the extended abstinent period. Previous research examining nicotine-induced alterations in spine density following extended abstinent periods has yielded mixed results (Brown & Kolb, 2001; Hamilton & Kolb, 2005; Gipson et al., 2013), and could reflect differences

related to the nicotine administration protocol, the age of the animal at the initiation of nicotine exposure, or the length of abstinence following nicotine dosing. However, it is important to note that despite a return of spine density to control levels in the present study we would still expect a persistent elevation in the total number of spines per MSN even after the extended abstinent period, as total number of spines per neuron is a function of both spine density and the total amount of dendritic material (Feldman & Peters, 1979). Furthermore, it has been suggested that when assessing the impact of dendritic remodeling, the number of synapses gained or lost may be less important than the overall change in the pattern of synaptic contact onto the neuron that occurs with altered dendritic morphology and spine location (Chen & Nedivi, 2010). Therefore, the persistence of adolescent nicotine-induced formation of new dendritic branches reported in this study is consistent with an altered pattern of synaptic connectivity onto NAcc shell MSNs maintained into adulthood.

Adolescent-nicotine induced dendritic remodeling is D1DR-dependent

When the D1DR antagonist SCH-23390 was co-administered during chronic adolescent nicotine exposure, the dendritic arbors of NAcc shell MSNs displayed reduced dendritic length and branching compared to animals receiving nicotine without antagonist, and MSNs were indistinguishable from those of control animals. Furthermore, there was no observable dendritic remodeling of MSNs in animals receiving SCH-23390 alone, suggesting that D1DR antagonism blocks the nicotine-induced formation of new dendritic branches rather than simply causing a general reduction in dendritic length or branching. Finally, this inhibition of nicotine-induced dendritic remodeling was present

after both early and extended abstinent periods, suggesting that blocking D1DRs during chronic adolescent nicotine exposure produces a persistent prevention of nicotine-induced dendritic remodeling. Collectively, these results suggest that nicotine-induced activation of D1DRs during the time of drug exposure is critical to long-lasting dendritic remodeling in the NAcc shell. As drug-induced dendritic remodeling is believed to be an important correlate of long-term behavioral alterations following exposure to drugs of abuse (Robinson & Kolb, 2004), the present findings suggest that nicotine-induced dendritic remodeling might best correlate with neurobehavioral indices of nicotine-exposure that are both long-lasting and specifically D1DR dependent, such as increased impulsivity (van Gaalen et al., 2006), conditioned place preference (Spina et al., 2006; Sershen, Hashim, & Lajtha, 2010), enhanced self-administration (Corrigall & Coen, 1991; David et al., 2006), and/or reinstatement of nicotine-seeking (Liu et al., 2010).

While nicotine-induced dendritic remodeling in the NAcc shell is dependent on D1DR activation, the specific mechanisms downstream of D1DR activation responsible for this remodeling are currently unknown. Consistent with the present results, Ren et al. (2010) have shown that cocaine-induced dendritic remodeling in the NAcc is dependent on D1DR and ERK activation, suggesting that intracellular mechanisms similar to cocaine may underlie the effects of nicotine despite the significantly different pharmacological action of these two substances. Given that the dendritic cytoskeleton is quite stable during the adolescent time-period (Koleske, 2013), intracellular signaling or transcription pathways downstream of the D1DR that can destabilize and/or reorganize dendritic structure may be particularly important. Through positive coupling of the D1DR

with the cAMP-PKA pathway, nicotine up-regulates numerous intracellular signaling and transcription pathways in the NAcc, including CREB (McCarthy et al., 2012), ERK (Valjent et al., 2004), ARC (Schiltz, Kelley, & Landry, 2005), deltaFosB (Marttilla, Raattamaa, & Ahtee, 2006; Soderstrom et al., 2007), and BDNF expression (Perna & Brown, 2013). Importantly, each of these intracellular mechanisms has the potential to regulate dendritic plasticity (Redmond, Kashani, & Ghosh, 2002; Wayman et al., 2006; Schubert & Dotti, 2007; Urbanska, Blazejczyk, & Jaworski, 2008; Pitchers et al., 2013) and is up-regulated following exposure to other drugs of abuse in which drug-induced dendritic remodeling has been previously observed (Nestler, 2001; Nestler, 2004; Robinson & Kolb, 2004). Moreover, several of these pathways are up-regulated to a greater extent following adolescent nicotine-exposure compared to adult exposure (Schochet, Kelley, & Landry, 2005; Shram, Funk, & Li, 2007). Collectively, these results highlight the need to examine the intracellular signaling and transcription pathways downstream of drug-induced D1DR activation that might specifically induce dendritic remodeling, and whether adolescents versus adults are more susceptible to the effects of nicotine-induced neuroplasticity via D1DR activation.

It is important to note that in the NAcc, MSNs are largely segregated into primarily D1DR containing and primarily D2DR containing neurons (Lu, Ghasemzadeh, & Kalivas, 1998; Aubert et al., 2000), and it is possible that the pattern or presence of nicotine-induced dendritic remodeling is different for these two cell populations. In the present study, we were unable to separate these two cell types using the Golgi-Cox stain, and our results likely reflect a random sampling from both cell types. While nicotine-

induced dendritic remodeling has yet to be compared between D1DR versus D2DR MSNs, cocaine-induced dendritic remodeling appears in both subtypes (Li et al., 2012). However, Lobo et al. (2013) recently showed that increased deltaFosB following chronic treatment with cocaine is present in only D1DR MSNs, and Lee et al. (2006) report that cocaine-induced increases in dendritic spine density is maintained through an extended abstinent period only in D1DR MSNs, suggesting that for some neuroadaptations psychostimulant drug exposure may preferentially affect one MSN subtype over the other. Therefore, the specific pattern of drug-induced structural plasticity in the NAcc across MSN subtypes is an intriguing area for future study.

Our results do not exclude the possibility that other neuropharmacological manipulations could also alter adolescent nicotine-induced dendritic remodeling within the NAcc shell or in other brain regions where nicotine-induced dendritic remodeling has previously been observed. Milstein et al. (2013) have shown that treatment with the atypical anti-psychotic olanzapine during adolescence alters the developmental pattern of dendrites extending into adulthood in similar regions influenced by adolescent nicotine exposure, including the NAcc (McDonald et al., 2005; McDonald et al, 2007) and medial prefrontal cortex (Bergstrom et al., 2008). Bessa et al. (2009) have shown that chronic stress decreases dendritic length and branching in the hippocampus and medial prefrontal cortex, and that multiple anti-depressant drugs can reverse this effect. We found that antagonism of the D1DR during chronic adolescent nicotine exposure prevents nicotine-induced dendritic remodeling in the NAcc shell. The importance of D1DR activation for dendritic remodeling in other brain regions warrants further study.

Adolescent nicotine-induced increases in spine density are not D1DR-dependent

In the present study, while co-administration of the D1DR antagonist SCH-23390 prevented nicotine-induced increases in dendritic branching in the NAcc shell, the antagonist failed to block nicotine-induced increases in spine density that were observed following an acute abstinent period. Therefore, while repeated D1DR activation during chronic adolescent nicotine exposure is necessary for the formation of new dendritic branches, non-D1DR mediated mechanisms may underlie the nicotine-induced increase in spine density. Although this has yet to be examined in relation to nicotine, our results are in contrast to a number of recent studies linking intracellular pathways downstream of D1DR activation with cocaine-induced increases in spine density (Lee et al., 2006; Maze & Russo, 2010; Ren et al., 2010; Grueter et al., 2013). This suggests that, despite general similarities between nicotine and cocaine in their ability to alter dopaminergic function in the NAcc (Pierce & Kumaresan, 2006), differences between these two drugs in their specific mechanism of dopamine release, D1DR activation, or drug-induced activity at non-D1DR synapses may contribute to how each drug specifically alters dendritic spine density in the NAcc shell.

The observed differences in D1DR dependent nicotine-induced dendritic remodeling versus alteration in spine density likely reflect differences between two uniquely structured neuronal components that differ substantially in both cytoskeletal and synaptic make-up, and it is likely that both intracellular and extracellular signals mediating the formation of new dendritic spines differ from those that mediate formation of new dendritic branches (Harris & Kater, 1994; Harris, 1999). In particular, dendritic

spines are unique functional compartments and the primary sites of excitatory synaptic contact, devoid of dendritic microtubules, and enriched in *F*-actin, which provides for both stability and plasticity of spine shape (Smith et al, 1998; Hering & Sheng, 2003; Tada & Sheng, 2006; Urbanska, Swiech, & Jaworski, 2012). In contrast, dendrites contain points of contact onto non-excitatory neurotransmitter receptors, including D1DRs (Hersch et al., 1995), and their shape or plasticity is likely maintained by networks of microtubules and alterations to microtubule associated proteins (MAPs) (Sánchez, Díaz-Nido, & Avila, 2000; Georges et al., 2008; Urbanska, Swiech, & Jaworski, 2012). As the formation and remodeling of new dendritic spines and branches largely depends on the restructuring of these different cytoskeletal elements (Dillon & Goda, 2005; Newey et al., 2005; Szebenyi et al., 2005; Penzes P, Srivastava DP, Woolfrey, 2009), it is possible that in the present study antagonism of the D1DR preferentially affected only those processes related to dendritic remodeling while leaving those responsible for spine formation unopposed. In support of this, previous studies have shown that alteration in spine density and *F*-actin stability can be regulated through activity at AMPA and NMDA-type glutamate receptors (Halpain, Hipolito, & Saffer, 1998; Hering & Sheng, 2003; Passafaro et al., 2003; Ultanir et al., 2007), which were not blocked during nicotine-exposure in the present study, and that D1DR activation can alter MAP expression and dendritic outgrowth (Reinoso, Undie, & Levitt, 1996; Song et al., 2002). While more research is needed to better identify the specific mechanisms regulating plasticity of dendritic spines versus the dendritic arbor, our results certainly

highlight the importance of analyzing both spine density and dendritic remodeling in studies of drug-induced dendritic plasticity.

In conclusion, we provide evidence that adolescent nicotine-induced dendritic remodeling in the NAcc shell is D1DR dependent. Through nicotine-induced activation of D1DRs during the time course of drug exposure, there is a substantial increase in the formation of new, distally located dendritic branches and increased spine density on MSNs in the NAcc shell. The effect of nicotine on dendritic remodeling, but not spine density, is persistent as increased dendritic branching is still present following an extended abstinent period. Finally, the influence of nicotine-induced D1DR activation on dendritic structure is specific to the formation of new dendritic branches, as nicotine-induced D1DR activation does not influence nicotine-induced increases in spine density. These results provide important insight into the specific neuropharmacological mechanisms that are responsible for drug-induced dendritic remodeling, and provide further evidence that this is a long-lasting form of drug-induced neuroplasticity. Given that adolescence is a critical period of brain development, particularly of the dopaminergic system (Tarazi & Baldessarini, 2000; Wahlstrom, White, & Luciana, 2010), and a time-period of enhanced vulnerability to the neurobehavioral effects of drug exposure (Baler & Volkow, 2011; O'Dell, 2009; Smith, 2003), we suggest that this D1DR dependent and long-lasting type of neuroplasticity is an important correlate for the neurobehavioral effects that develop from chronic adolescent nicotine exposure.

APPENDIX A: ADDITIONAL FIGURES AND DATA ANALYSIS

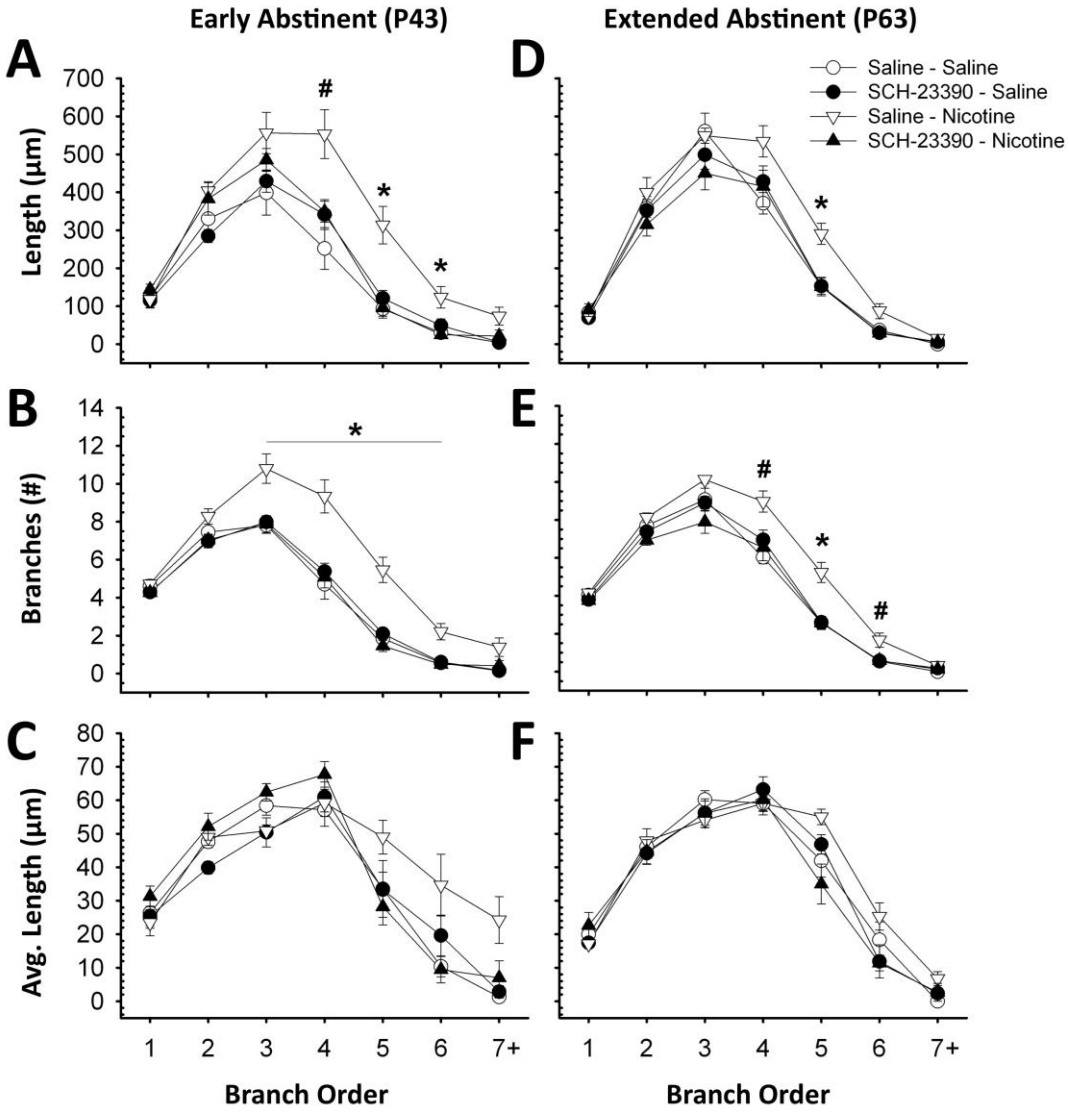

Figure 7. Influence of chronic nicotine and D1DRs on dendritic morphology as a function of branch order Branch order number labeled centrifugally. Total dendritic length (Mean ± SEM)(***A***,***D***) number of branches (Mean ± SEM)(***B***,***E***) and average length (Mean ± SEM) (***C***,***F***) within each branch order, in early abstinence (***A***,***B***,***C***) and extended abstinence (***D***,***E***,***F***) animals. *****, Significant difference between saline-nicotine group and all other pretreatment-treatment groups (*Bonferroni corrected*, $p < .007$). #, statistical trend towards difference between saline-nicotine group and all other pretreatment-treatment groups ($p < .05$).

Table 1. Changes in dendritic morphology and spine density across abstinence (P63-P43)

	Pretreatment - Treatment			
Parameter	Saline-Saline	SCH23390-Saline	Saline-Nicotine	SCH-23390-Nicotine
Branch order analyses				
Total length (μm)	174.4 ± 111.9	169.7 ± 76.1^	-191.7 ± 231.6	-45.9 ± 62.2
First order	-39.2 ± 27.4	-45.9 ± 19.4*	-43.0 ± 23.4	-53.8 ± 22.2*
Second order	27.3 ± 49.2	67.5 ± 41.1	-4.4 ± 46.6	-67.7 ± 52.2
≥ Third order	344.0 ± 137.2*	170.8 ± 70.1*	-144.4 ± 196.7	75.6 ± 85.6
Branches (#)	2.8 ± 1.7^	2.9 ± 1.3*	-3.6 ± 3.3	1.8 ± 1.8
First order	-.58 ± .19**	-.49 ± .31	-.59 ± .26*	-.51 ± .17**
Second order	.25 ± .43	.40 ± .5	.17 ± .47	-.11 ± .41
≥ Third order	3.2 ± 1.6^	3.0 ± 1.2*	2.9 ± 3.0	2.4 ± 1.7
Average length (μm)				
First order	-6.4 ± 4.8	-8.0 ± 3.3*	-6.3 ± 4.1	-8.7 ± 5.0
Second order	-1.5 ± 2.6	4.3 ± 3.8	-1.1 ± 4.4	-7.5 ± 5.4
≥ Third order	18.8 ± 12.9	13.3 ± 13.5	17.8 ±19.1	-9.4 ± 10.0
Spine Density (#/10μm)	.09 ± .3	.42 ± .29	-.72 ± .42	-1.3 ± .35 **

All values reflect mean difference between P63 and P43 ± SED. ^, $p < .07$. *, $p < .05$. **, $p < .01$

APPENDIX B: GOLGI-COX STAINING PROTOCOL

Step 1: Prepare the Golgi-Cox solution

This requires the preparation of three solutions outlined below.
Mix solutions A, B, & C separately.
Combine solutions A & B
Mix Solution C with 400 mL of dH20
Slowly pour solution A+B into C+dH20

Solution A: 5% Potassium Dichromate in distilled H2O (orange color)
200ml distilled H2O + 10 grams Potassium Dichromate
(Mix in a glass beaker using a glass rod - takes a long time to get into solution)
(best to do under fume hood)
[Sigma Aldrich #209244-500G]

Solution B: 5% Mercuric Chloride (sublimate) in distilled H2O (silver/greycolor)
200ml distilled H2O + 10 grams Mercuric Chloride
(Mix in a glass beaker using a glass rod)
(Mix solution on top of hotplate, stirring until dissolved at 55 degrees Celcius – takes a long time to get into solution)
(Must be done under fume hood)
[Sigma Aldrich#215465-100G]

Solution C: 5% Solution of Potassium Chromate in distilled H2O (yellow color)
160ml distilled H2O + 8 grams Potassium Chromate
(Mix in a glass beaker using a glass rod)
(best to do under fume hood)
[Sigma Aldrich #216615-100G]

Mix Solution A and Solution B into a 500ml glass beaker.

Mix Solution C and 400ml of distilled H2O into a 1,000ml + glass beaker.

Slowly pour the AB Solution into the C Solution while stirring continuously with a glass rod.

Store in a glass stoppered bottle for 5 days in the dark (cover stoppered bottle

with aluminum foil).

Note: You can easily manipulate the quantity of solution. Just make sure to follow these ratios:

5 Volume parts of 5% Potassium Dichromate solution
5 Volume parts of 5% Mercuric Chloride solution
4 Volume parts of 5% Potassium Chromate solution
10 Volume parts of H20 (to add to PC solution)

Step 2: Transfer Golgi-Cox solution into small glass bottles.

Use a plastic syringe to remove the GC solution from the large glass bottle(s).

Be sure to avoid the reddish precipitate that formed on the top and bottom of the bottle.

Glass bottles should be filled about ¾ full (to save room for 1 rat brain).

STORE IN THE DARK AT ROOM TEMPERATURE
(DO NOT PLACE BRAINS IN GOLGI SOLUTION IN THE REFRIDGERATOR – THEY WILL NOT STAIN)

Step 3: Perfuse animals with 0.9% saline solution and collect brains.

For normal Golgi-Cox staining, the brains do not need to be fixed with anything else. If you are running a different protocol, check to make sure.

Saline solution: 9g NaCl in 1000 ml distilled H2O = 0.9% NaCl.

Prepare empty breeder box with wire top to allow blood to drain.

Deeply anaesthetize the animal.
(Ketamine/xyline solution: 87/13 mg/kg)

Place on an empty breeder box with wire top (to allow blood to drain into box)

Open chest cavity to expose heart

Use 20G needles (tape needle so that only ~1cm enters heart) and 60 ml syringe.

Insert 60 ml syringe filled with 0.9% saline into the animals bottom left chamber of the heart.
(This would be on YOUR right)

Using small surgical scissors, cut a small nick in the animal's bottom right chamber of the heart.
(This would be on YOUR left)

Begin to slowly push saline through the animal's system until the blood leaving the left chamber is clear.
(~120 mL saline per animal. Depends on size of animal. Use more if needed.)

When fluids are clear, decapitate and remove brain.

Drop whole brain into prepared bottle(s) of Golgi-Cox solution.

Place IN THE DARK for 14 days, refreshing the solution after 2 days.
(recover bottles in aluminum foil)

Step 4: Transfer Brains into 30.0% Sucrose Solution.

Sucrose Solution: 300 grams of Sucrose into 1000ml of distilled H2O.

Place Beaker over hotplate and stir (using stir bar) until dissolved.

Cool in refrigerator.
(Once cool, ready to use.)

Empty GC solution from jar and place brain on chem. wipe paper.

Slightly blot.

Rinse jar in distilled H2O, and refill with Sucrose Solution ¾ full (In order to save room for brain.)

Place brain in jar with Sucrose Solution.
(brain may float)

Place jar(s) in refrigerator for at least 3 days.
(Brains should be sectioned within 2 months (at most) of transfer into sucrose.)

Step 5: Section using a Vibratome.

For this section of the protocol, it is important that you only use no more than 100 slides. This takes up two large racks and can fit into one large bucket for staining.

Most important however, is that 100 slides can be cover-slipped in a relatively short amount of time (< 2hrs). If slides stay in CXA solution for too long during cover-slipping, the tissue begins to degrade. If multiple people can cover-slip, then it is OK to use more slides.

Prepare razor blade by immersion in xylene for 5 minutes to remove any traces of oil. Wipe blade dry.
(Done under the fume hood.)

Prepare a 6.0% sucrose solution.
(6 grams sucrose in 100ml distilled H2O.)
(Mix well and make sure it is at room temperature or below before using.)

Fill the vibratome reservoir with the 6% Sucrose solution until the blade is covered. Place ice cubes/chips around reservoir to keep sucrose solution cold.

Mount brain section (up to ½ a full brain) onto vibratome platform using superglue.
(Make sure tissue is adhered well before sectioning, 5-7 minutes or more.)

Insert platform (with adhered brain section) into reservoir.

Set the vibratome speed and amplitude around the midpoint for sectioning
(adjust as necessary for your specific machine and comfort level.)

Section at 200 µm, or desired thickness.
(Sections over 400µm thick may be difficult to analyze.)

Using a small paintbrush coax the section onto a gelatinized slide.

Place slide on flat surface covered with bibulous paper. Cover with small (slide-sized) piece of parafilm. Using a finger, press down lightly and evenly over the entire strip of parafilm, being careful not to movement brain sections.
(Your goal is to press the section into the gelatin on the slide so it will adhere to slide during staining)

Place slides in humidity chamber until ready for staining.
(Do not keep the slides in storage (humidity chamber or water incubator) for more than 4 days)

Step 6: Staining

Prepare fresh solutions of the following in plastic or glass bins (enough to cover all slides during staining):

Distilled H2O (3 total) (keep under fume hood)

Ammonium Hydroxide (1 total) (keep under fume hood)

Kodak Fix (1 total)

(DO NOT MIX YET. Keep the following components under fume hood, in separate beakers, until ready to mix during staining. Keep a large, empty, plastic or glass container to mix this solution in under the fume hood, so that you can place slides into this solution once it is mixed):

Proportions for 1639 ml (total). These will be mixed IN THIS ORDER & IN THE DARK:

1. Distilled H20 (505ml)
2. Kodak Fix solution A (125ml)
3. Kodak Fix solution B (14ml)
4. Distilled H20 (1010ml)

50% alcohol (1 total)
70% alcohol (1 total)
95% alcohol (1 total)
100% alcohol (3 total)

CXA solution (1 total) (keep under fume hood)

Proportions for 1500ml total (you can manipulate amount of total solution by using equal parts of each):

1. Chloroform (500ml)
2. Xylene (500ml)
3. 100% Alcohol (500ml)

Remove parafilm from slides if necessary and place in slide rack (can be metal).

Set up a dark-room light under the fume hood. You can turn on the dark-room light when moving slide racks from one bin to the next, and when mixing the Kodak Fix solution.

Dip slide racks in this order according to the process below:

1. Distilled H20 #1 (for 1 minute)
2. Ammonium Hydroxide (IN THE DARK for 30 minutes)
3. Distilled H20 #2 (IN THE DARK for 1 minute)
   (MIX KODAK FIX SOLUTION NOW)

4. Kodak Fix solution (IN THE DARK for 30 minutes)
5. Distilled H20 #3 (for 1 minute)
   (Once slides are in this H20, lights can be turned back on)
6. 50% alcohol (for 1 minute)
7. 70% alcohol (for 1 minute)
8. 95% alcohol (for 1 minute)
9. 100% alcohol #1 (for 5 minutes)
10. 100% alcohol #2 (for 5 minutes)
11. 100% alcohol #3 (for 5 minutes)
12. CXA solution (for 15 minutes)

Step 7: Coverslip with permount and lie out flat to dry.

Slides can be kept in CXA solution while cover-slipping. Only pull one slide out at a time, and change gloves often.

If possible all cover slipping should be done under fume hood. Pull one slide out of CXA at a time. (Sections dry quickly, do not remove slide from CXA and allow to sit in air for more than 20 seconds.)

Lie slide flat. Using a glass dropper, place enough permount to fully cover the tissue on top of the tissue.

(Too little permount could allow tissue to dry out, too muchwill cause coverslip to slide off – monitor your work for these problems)

Place glass coverslip over section, being careful to avoid trapping air bubbles. Note:

Place slide on absorbent paper (we use white tray liners)

Allow slides to lie flat for at least 24 hours.

Begin tracing as soon as permount is dry enough. Neurons are stained best soon after processing.

APPENDIX C: EXTENDED INTRODUCTION

Nicotine remains one of the most heavily abused addictive substances, despite a current decline in the prevalence of smoking (NIDA, 2012). For nicotine, the rate of successful quitting is extremely low, further highlighting the power of nicotine as an addictive substance. This particularly underscores the need for continuing research on the biological basis behind the development and maintenance of nicotine addiction, as may be assessed in both human and animal models.

While the behavioral effects of nicotine can be attributed to a variety of neurotransmitter systems, the reinforcing and addictive properties of nicotine have been largely attributed to increased dopamine (DA) release from the ventral tegmental area (VTA) DA containing neurons projecting to the nucleus accumbens (NAcc), amygdala, and prefrontal cortex (i.e. the mesolimbic and mesocortical DA pathways) (Pierce and Kumaresan, 2006). Nicotine increases DA release to these regions primarily via direct stimulation of nicotinic acetylcholine receptors located on DA neuron cell bodies in the VTA (Di Chiara, 2000; Picciotto et al., 1998; Picciotto, 2003), but as well as through activation of presynaptic nAChRs located on DA terminals within targets of the VTA (Wonnacott, 1997; Wonnacott et al., 2000). However, in order for nicotine to produce long-term changes in behavior that are associated with drug addiction, such as sensitization, tolerance, and dependence, it has been suggested that alterations in genetic

expression and long-lasting neuroanatomical changes must also occur within structures of the mesolimbic and mesocortical DA pathways (Everitt & Wolf, 2002; Self & Nestler, 1998).

In animal models, nicotine-induced dendritic remodeling that occurs following chronic nicotine exposure represents one potentially long-lasting form of neuroplasticity related to the neurobehavioral effects of nicotine (Brown & Kolb, 2001). Nicotine induced dendritic remodeling has been demonstrated in a number of brain regions implicated in nicotine addiction, including the NAcc shell (Brown and Kolb, 2001; McDonald et al. 2005, 2007), cingulate cortex (Brown & Kolb, 2001), prelimbic cortex (PL) (Bergstrom et al, 2008; Robinson & Kolb, 2004), insular cortex (Ehlinger et al., 2012), and the amygdala (lateral and basal subnuclei; BLA) (Bergstrom et al, 2010). In each of these brain regions, an increased complexity of dendritic structure, such as increased branching, dendritic length, and spine density, has been shown in animals previously exposed to nicotine when compared to saline pretreated controls. Alterations in the complexity of dendrites in each of these brain regions would be expected to modify their pattern of synaptic connectivity (Chklovskii, 2004), and may therefore represent an important neuroanatomical correlate of nicotine dependence.

In particular, dendritic remodeling within the NAcc shell, a brain region that is widely considered the most critical target of DA from the VTA and the most important region for reward processing, could have a particularly strong influence on addiction-related behaviors during and following nicotine administration. Nicotine-induced dendritic remodeling has been observed in this region selectively on the medium spiny

neurons (MSNs) (Brown & Kolb, 2001; McDonald et al., 2005; 2007), which are GABAergic neurons projecting primarily to the substantia nigra and ventral pallidum, and are considered critical to behavioral output following confrontation with a rewarding substance such as nicotine. As the MSNs represent the primary output from the NAcc, alterations in the MSN function following nicotine administration would also be expected to alter behaviors associated with nicotine dependence in animal models, including enhanced drug-seeking behavior, conditioned-place preference, and locomotor sensitization (Rothwell, Kourrich, & Thomas, 2011; Pascoli, Turiault, & Luscher, 2011).

There are strong indications in animal models that nicotine-induced dendritic remodeling in the NAcc is significantly more pronounced when nicotine exposure occurs during the developmental period of adolescence. These indications are in line with human nicotine consumption, which typically begins during adolescence and is associated with a significantly higher rates of nicotine dependence and relapse (NIDA, 2012). While adolescence in the rat is difficult to define, a conservative estimate limits the adolescence as beginning around postnatal day (P) 28 when gonadal cycling is first detected and the achievement of reproductive maturity around P60 in the male rat (Smith, 2003). McDonald et al. (2007) showed that continuous nicotine exposure during an early adolescent period from P32-P42 via subcutaneously implanted osmotic pump selectively increases dendritic length and branching of MSNs in the NAcc shell when measured thirty days following cessation of nicotine exposure, and is associated with a sensitized locomotor response to a challenge nicotine injection. However, a similar dosing regimen during adulthood does not result in these neuroanatomical changes, nor does it result in a

sensitized locomotor response to a nicotine challenge. These results suggest that during adolescent brain development, nicotine induced dendritic remodeling may be particularly pronounced. Furthermore, these results correlate an important behavioral response to repeated nicotine exposure with nicotine-induced dendritic remodeling in the NAcc shell.

To date, research on nicotine-induced dendritic remodeling has focused on identifying which drugs of abuse are capable of producing these alterations (Robinson & Kolb, 1997; Robinson & Kolb, 1999; Robinson & Kolb, 2004), localizing the brain regions in which remodeling either does or does not occur, and determining selectivity within brain structures based on age of drug-exposure (McDonald et al, 2007), cell-type (McDonald et al, 2005; Bergstrom et al., 2008) or hemisphere (Bergstrom et al, 2010). However, there has been surprisingly little research aimed at determining the more precise neuropharmacological and molecular mechanisms responsible for drug-induced dendritic remodeling. Although the direct neuropharmacological actions of nicotine are at the nicotinic ACh receptor, nicotine binding on these receptors has primarily ionotropic action and would be unlikely to directly regulate cytoskeletal elements that confer dendritic morphology. However, one common characteristic of the previously mentioned brain regions in which nicotine induced dendritic remodeling has been observed is that each of these regions receives significant DA enervation from the ventral tegmental area (VTA), and that DA release in these structures is increased upon nicotine exposure (Pierce & Kumaresan, 2006). Unlike the nicotinic ACh receptor, DA receptors have well characterized metabotropic function, and have been shown to directly alter enzymatic activity, transcription factors, and gene expression that could potentially influence

neuronal cytoskeletal structure either during or following repeated and excessive DA binding (Nestler, 2001). This warrants the question of whether or not increased DA transmission in these brain regions during repeated drug exposure is a critical substrate of nicotine-induced dendritic remodeling.

Although little research has been aimed at determining whether DA transmission is critical for the structural plasticity of dendrites in the NAcc, prior research has shown that DA transmission is required for some forms of synaptic plasticity on MSNs in the striatum. For example, both corticostriatal long-term potentiation (LTP) and long-term depression (LTD) onto MSNs requires either high or low DA release, respectively (Calabresi et al., 2007). It has been hypothesized that high DA influx to the striatum preferentially activates D1-type dopamine receptors (D1DRs), while lower DA influx preferentially activates D2-type dopamine receptors (D2DRs), which have a higher affinity for DA (Jaber et al, 1996). Therefore, high frequency stimulation of NMDA-type glutamate receptors located on MSNs of the striatum, via corticostriatal afferents, may elicit LTP in these neurons only in the presence of a high dopaminergic tone, while low frequency stimulation of NMDA receptors located on MSNs of the striatum might elicit LTD in these neurons only in the presence of a weak dopaminergic tone. This form of DA-dependent synaptic plasticity could be one key element of the long-term effects of drugs of abuse such as nicotine, as it provides a learning mechanism by which drug-related stimuli could become abnormally coupled with drug-induced increases in DA release (Calabresi et al., 2007; Surmeier, Plotkin, & Shen, 2009).

Research on the influence of DA signalling specifically on dendritic remodeling within the mesocortical and mesolimbic DA pathways has yielded mixed results. Solis et al. (2007) reported that a unilateral 6-hydroxydopamine lesion of the substantia nigra reduced the length, branching, and spine density of MSNs of the caudate-putamen on the ipsilateral side of lesion, while only reducing the spine density on NAcc MSNs and PFC pyramidal neurons. Importantly, this study did not selectively lesion the VTA, which would be of greater relevance for MSN dendritic morphology in the NAcc. Ramkumar et al. (2012) found that long-term stress induced dendritic atrophy (e.g. decreased branch length, number of branches, and spine density) in the prefrontal cortex was reversed by 10 consecutive days of intracranial self-stimulation (ICSS) of the VTA, suggesting that increased DA efflux to the PFC could potentially reverse an already present atrophy of dendritic morphology. However, whether or not ICSS is capable of inducing increases in dendritic length, branching, or spine density in stress-naïve animals is unknown. Finally, Selemon et al. (2007) reported that a 6-week amphetamine sensitization regimen in rhesus monkeys resulted in dendritic atrophy across all cortical layers of PFC that was present long after (more than one year) the conclusion of amphetamine exposure, and was correlated with long-term cognitive impairments. Furthermore, the chronic administration of a selective D1DR antagonist (SCH39166) to these same monkeys >1 year after the amphetamine sensitization regimen produced an increase in dendritic length and spine density of PFC neurons, and was correlated with improved cognitive performance only when tested following the cessation of chronic D1DR antagonist treatment (Selemon et al., 2010). Again, this study suggests that alteration of dopaminergic function can reverse

already-present dendritic atrophy in the PFC. It should be noted that it was the administration of a D1DR antagonist, and not agonist, which resulted in dendritic growth, though an improvement in the cognitive impairment of these animals was only present following the cessation of D1DR antagonist treatment. Therefore, it is possible that a compensatory increase in DA receptor sensitivity or DA efflux, either during or following the cessation of chronic D1DR antagonist treatment, is responsible for the increase in dendritic growth.

Importantly, while these previous studies do suggest a general role for DA on dendritic remodeling, none of these previous studies have specifically targeted drug-induced dendritic remodeling in the NAcc. The NAcc shell is a particularly important target to look for the influence of DA on nicotine-induced dendritic remodeling, as the MSNs in this region contain a particularly high DA receptor density and are the primary recipient of DA terminals from VTA neurons (Self & Nestler, 1998). Furthermore, nicotine administration has been shown to directly elevate NAcc shell DA levels (Nisell et al, 1994) as well as increase VTA DA neuron burst-firing to the NAcc shell (Rice & Cragg, 2004). Lastly, nicotine-induced dendritic remodeling in the NAcc shell has at least been correlated with one long-term behavioral alteration resulting from nicotine exposure: locomotor-sensitization, a behavior in which repeated drug-exposure produces an enhanced, or sensitized, behavioral response upon subsequent drug challenge (McDonald et al., 2007; Vezina et al., 2007). Importantly, locomotor sensitization has also been been correlated with alteration of DA transmission in the NAcc (Cadoni & Di Chiara, 2000). Therefore, there is a potential connection between nicotine exposure, DA

release in the NAcc, locomotor sensitization to nicotine exposure, and dendritic remodeling in the NAcc shell.

On MSNs of the striatum, the two major DA receptor subtypes (D1DR and D2DR) are largely isolated to separate groups of MSNs that are distinguishable by chemical, electrophysiological, and anatomical properties, although there is at least a small proportion of MSNs that display both subtypes (Gerfen & Young, 1988; Le Moine, Normand, & Bloch, 1991; Gertler, Chan, & Surmeier, 2008). D1DR expressing MSNs co-express GABA, substance P, and dynorphin, project primarily to the substantia nigra pars reticulata, are less electrically excitable than D2DR expressing MSNs, and possess an increased number of primary dendrites. In contrast, D2DR expressing MSNs co-express GABA and enkephalin, project primarily to the globus pallidus, are more electrically excitable, and possess a smaller number of primary dendrites. However, D1DRs and D2DRs are also located on interneurons within NAcc, as well as on dopaminergic neuron presynaptic terminals located within the striatum, and therefore activation of either receptor subtype has the ability to influence the activity of both classes of MSN (Calabresi et al., 2007).

Importantly, the D1DR and D2DR have opposing effects on intracellular signaling within MSNs, and these differences in intracellular signaling may be critical to both behavioral alterations associated with prolonged nicotine exposure and to potential nicotine-induced alterations in dendritic morphology. Specifically, while D1DRs are positively coupled to adenylyl cyclase (AC), stimulate cyclic AMP (cAMP) formation, and increase protein kinase A (PKA) activity, D2DRs are negatively coupled to AC,

inhibit cAMP formation, and reduced PKA activity (Lachowicz & Sibley, 1997; Self 2004). During prolonged or repeated exposure to drugs of abuse including nicotine, MSNs within the NAcc display a persistent upregulation of the cAMP pathway that is indicative of repeated D1DR activation, and is accompanied by persistently increased levels of transcription factors such as deltaFosB and cAMP response-element binding protein (CREB) (Nestler, 2001). This is a major mechanism by which D1DR activation during nicotine exposure could potently regulate genetic expression within MSNs, and lead to stable changes within these neurons.

Behavioral alterations associated with repeated nicotine exposure have been correlated with an up-regulation of the D1DR intracellular pathway. As mentioned previously, DA binding to D1DRs (rather than D2DRs) requires a high level of DA influx to the NAcc, such as that which occurs following an acute nicotine exposure (Jaber et al., 1996). Self (2004) has hypothesized that in a non-addicted state, the length of D1DR activation following drug exposure is prolonged and can limit overall drug consumption. However, during repeated nicotine exposure, a sustained upregulation of cAMP and PKA activity results in a compensatory desensitization of D1DRs, through either direct phosphorylation of D1DRs or CREB mediated processes, and a rightward shift in the dose-response curve. For example, experimentally increasing PKA activity in the nucleus accumbens increases both the accumulation of CREB and the amount of drug that is self-administered, suggesting a decreased reward value in the drug with increased CREB accumulation (Self et al., 1998). Collectively, these findings suggest that an upregulation of CREB, as the result of repeated D1DR activation, is associated with the

presence of a long-lasting behavioral alteration following repeated exposure to drugs of abuse. Tolerance develops to D1DR activation and the drug's rewarding effects, and leads to a failure to regulate (i.e. escalated) drug intake.

Simultaneously, cAMP and PKA activity is also up-regulated in D2DR containing MSNs, and rather than desensitizing D2DRs these receptors appear to become sensitized, again via direct phosphorylation of the receptor and/or CREB mediated processes (Self, 2004). It has been shown that in animals that are repeatedly exposed to a psychostimulant drug and given a withdrawal period, the administration of a D2DR agonist (but not a D1DR agonist) or a PKA inhibitor (but not PKA activator) reinstates drug-seeking behavior (Caine & Koob, 1994). These findings suggest that although D1DR activation and a prolonged CREB up-regulation during prolonged periods of drug exposure is associated with the development of tolerance to the rewarding effects of drugs of abuse, D2DR activation (either drug or cue-induced) and an acute PKA/CREB down-regulation is associated with relapse and drug-seeking behavior (Self et al., 1996).

Therefore, two critical behaviors related to drug addiction (i.e. inability to regulate drug intake and enhanced drug seeking) can be explained through long-term alterations in dopamine receptor function. Importantly, it appears that the initiating factor in both of these addiction related behaviors is the initial and repeated activation of D1DRs, rather than D2DRs, and their intracellular pathways. During the development of nicotine dependence, repeated and excessive D1DR activation is perhaps the largest contributor to nicotine dependence, and following the development of nicotine dependence the acute activation of D2DRs obtains prominence (Self et al., 2004).

Therefore, when assessing the role of DA on nicotine-induced dendritic remodeling in the NAcc, it is of greatest interest to specifically assess the influence of D1DR activation or inactivation during drug exposure on dendritic morphology.

Not only is D1DR activation the most likely contributor in the developmental stages of nicotine dependence, but the repeated activation of this DA receptor subtype is perhaps the most likely to influence dendritic remodeling. As mentioned previously, drug-induced D1DR activation through repeated drug exposure does lead to tolerance to the rewarding effects of drugs of abuse, however, CREB levels following drug cessation normalize within one week of the final drug exposure despite the continued presence of tolerance to the drug's rewarding effects (Nestler, 2004). Similarly, although D2DR activation can result in a reinstatement of drug-seeking behaviors following withdrawal, the reduction in CREB is even more transient following D2DR activation (Nestler, 2004). Therefore, it is likely that the long-lasting neurobehavioral alterations are not simply due to accumulation of CREB in neurons containing DA receptors, but most likely reflect other long-term changes to neuronal structure and/or function. It has been suggested that during repeated drug exposure, the up-regulation of CREB and subsequent transcription of genes containing a CRE region could alter cytoskeletal elements and provide a much longer-lasting alteration in neuronal function supportive of these long-term neurobehavioral alterations (Nestler, 2004). In partial support of this, the activation of CREB has been shown to mediate morphological changes in the structure of dendritic spines in some brain regions (Murphy & Segal, 1997), although this has not been directly tested in the NAcc or other regions of the mesocortical or mesolimbic DA pathways.

Although CREB is a relatively transiently expressed transcription factor, other transcription factors, such as the fos family of transcription factor proteins, can be significantly longer lasting in their expression. One result of CREB regulated gene transcription in the NAcc is the increased synthesis of the particularly stable fos isoform deltaFosB. This protein has been linked to numerous aspects of drug addiction, and is induced in the mesolimbic and mesocortical DA pathways in response to all drugs of abuse (Perrotti et al., 2008), is significantly elevated long after the cessation of drug exposure (Kelz et al., 1999), and regulates the transcription of numerous genes that are directly related to the regulation of cytoskeletal structure, including actin-related proteins (ARP) and activity-regulated cytoskeletal protein (Arc) (McClung & Nestler, 2003). Furthermore, deltaFosB appears to be selectively expressed in the D1DR subclass of NAcc MSNs, and its overexpression in these neurons has been shown to enhance the locomotor and rewarding responses to both cocaine and morphine (Kelz et al., 1999), enhance cocaine self-administration (Colby et al., 2003), and increase cocaine-seeking behavior in an animal model of relapse (Bachtell et al., 2005). Although these findings have yet to be replicated for nicotine, these results suggest that the up-regulation of a transcription factor within the mesolimbic DA pathway is associated with both the regulation of cytoskeletal structure and long-lasting behavioral indices of addiction, and therefore the D1DR-CREB-deltaFosB signaling cascade is hypothesized to be a critical aspect of neuroplasticity underlying the development of increased drug-intake and drug-seeking behaviors (Nestler, 2001; Kalivas & O'Brien, 2008).

Recently, Lee et al. (2006) have provided an important link between DA receptor activation, the accumulation of deltaFosB, and alterations in dendrite morphology following repeated drug exposure. These researchers analyzed both the accumulation of deltaFosB and changes in spine density following repeated cocaine exposure in D1DR and D2DR MSNs. After a 2-day withdrawal period, spine density and deltaFosB levels were significantly increased in both MSN subclasses. However, following a 28-day withdrawal period both spine density and deltaFosB levels were only maintained in the D1DR containing MSNs. These results show a correlation between D1DR activation, expression of deltaFosB, and long-lasting structural changes in dendrite morphology in the form of increased spine density. However, it is important to note that other measures of dendrite morphology, such as dendritic length and branching pattern, were not examined in this study.

A final DA-mediated neurochemical mechanism that could potentially influence dendritic remodeling is the induction of brain-derived neurotrophic factor (BDNF) in neurons that receive DA enervation. BDNF levels rise in the VTA, NAcc, and prefrontal cortex following exposure to most drugs of abuse, and CREB is a major activator of BDNF gene transcription (Kalivas & O'Brien, 2008). In support of a role for BDNF in dendritic remodeling, one unique aspect of BDNF expression is that BDNF mRNA is mainly induced and transported into the dendrites of neurons, and is also known to induce dendritic spine formation (Bramham & Messaoudi, 2005). Therefore, the induction and synthesis of BDNF in response to repeated drug exposure is localized in the area of interest for the altering of dendrite morphology.

In the present study, it was hypothesized that D1DR-mediated mechanisms during chronic nicotine exposure would be responsible for nicotine-induced dendritic remodeling. However, another important and related question concerns the time-course of nicotine-induced dendritic remodeling. Due to the persistent up-regulation of PKA, CREB, and deltaFosB that has been shown during nicotine exposure, one might expect that dendritic remodeling occurs over the actual time-course of nicotine exposure itself. However, current research on drug-induced dendritic remodeling has analyzed dendrite morphology only following a prolonged withdrawal period of 14 to 30 days (Bergstrom et al., 2010; McDonald et al 2005, 2007; Robinson & Kolb 2001). Therefore, current research has left open the possibility that nicotine induced dendritic remodeling either occurs during, or is the result of, the removal of nicotine exposure, rather than the result of nicotine exposure itself. For example, it is possible that nicotine-induced dendritic remodeling represents a compensatory alteration in dendrite morphology following the removal of nicotine and/or the cessation of D1DR activation.

Dynamic changes in neurotransmitter release, receptor expression, and levels of intracellular messengers and transcription factors have been shown during the withdrawal period from exposure to nicotine and other psychostimulants. Immediately following chronic nicotine exposure, it has been shown that there is a sharp decrease in DA signaling to the NAcc and within the VTA that is associated with both somatic and motivational (conditioned-place aversion) signs of withdrawal (Hildebrand et al., 1998; Liu & Jin, 2004; Rada, Jensen, & Hoebel, 2001). This decrease in DA levels within the NAcc has been shown following both naturally occurring (Rahman et al., 2004) and

mecamylamine-induced withdrawal (Rada et al., 2001). Although these studies have only assessed DA levels following a short withdrawal period, it has also been shown that DA turnover in the striatum of rats exposed to nicotine during adolescence is still blunted at both 12 and 30 days of withdrawal, although this effect is not seen in adult rats (Trauth et al., 2001). However, whether alterations in dendritic morphology are related to or caused by this blunted DA response has yet to be determined.

Despite these decreases in dopaminergic function during abstinence from chronic nicotine exposure, some intracellular signals of dopaminergic activation remain elevated following cessation of nicotine exposure. For example, CREB levels in the striatum are still elevated immediately (24-48 hours) following chronic nicotine exposure, while elevation of deltaFosB has been shown at least 30 days following nicotine-withdrawal (Nestler, 2004). Therefore, if D1DR-mediated intracellular mechanisms are responsible for nicotine-induced dendritic remodeling, it is still possible that this remodeling occurs post-drug exposure.

It is also extremely likely that in addition to DA, other neurotransmitter systems play a role in nicotine-induced neuroplasticity, and it is also known other neurotransmitter systems show alterations in function that are either present during nicotine exposure or occur following cessation of nicotine-exposure. For example, both serotonin (5-HT) (Slotkin & Seidler, 2007) and norepinephrine (Trauth et al., 2001) levels and turnover in the striatum is reduced following chronic nicotine exposure during adolescence, and these effects persist at least 12 days post nicotine exposure (Slotkin & Seidler, 2007). In contrast, nACh receptor upregulation in the midbrain persists for at

least 30 days post-nicotine exposure (Abreau-Villaca et al., 2003), suggesting that acetylcholinergic function could remain elevated or maintained following cessation of nicotine exposure. Therefore, these other neurotransmitter systems may play a role in nicotine-induced neuroplasticity and may have substantial influence during the withdrawal or abstinent period following chronic nicotine exposure.

Finally, BDNF expression actually increases with increasing periods of abstinence from psychostimulant drugs (Grimm et al., 2003), although this effect has not been specifically examined in response to nicotine exposure. Although BDNF elevation has been related to activation of the dopaminergic system, BDNF elevation is certainly not unique to dopamine activation and could involve alterations in other neurotransmitter systems as well. As mentioned previously, BDNF mRNA is mainly induced and transported into the dendrites of neurons, and has been shown to induce the formation of new dendritic spines (Bramham & Messaoudi, 2005). Therefore, increases in BDNF expression post nicotine exposure could be an important aspect of nicotine-induced neuroplasticity.

Although current research has not assessed full-scale dendritic remodeling at different time-points following nicotine exposure, Lee et al. (2006) have provided an interesting link between drug withdrawal effects, DA-mediated intracellular signalling, and dendritic spine expression. These researchers analyzed both the accumulation of deltaFosB and changes in spine density following repeated cocaine exposure in NAcc D1DR and D2DR containing MSNs. After a 2-day withdrawal period, spine density and deltaFosB levels were significantly increased in both subclasses of MSN's. However,

following a 28 day withdrawal period both spine density and deltaFosB levels were only maintained in the D1DR containing subclass of MSN's. Importantly, these results suggest that at least one measure of dendritic morphology shows persistence over a long withdrawal and drug-free period, as alterations in spine density were displayed both immediately and 28-days post drug exposure. Furthermore, these results suggest that altered spine density occurs either during or immediately after nicotine exposure, suggesting a strong correlation between D1DR activation, expression of deltaFosB, and long-lasting structural changes in dendrite morphology (in the form of increased spine density). Finally, these results also provide an important correlation between D1DR activation, deltaFosB expression, and alterations in dendrite morphology. Again, it is important to note that other measures of dendrite morphology, such as the length of dendritic branches and branching pattern, were not examined in this study.

It is now widely accepted that nicotine induced dendritic remodeling occurs in multiple brain regions interconnected with the mesolimbic and mesocortical dopamine pathways. However, only indirect evidence exists to suggest a role for DA on nicotine induced dendritic remodeling, or to suggest that dendritic remodeling occurs early during nicotine exposure. Therefore, we sought to study the influence of DA transmission and receptor activation on dendritic remodeling more directly, as well as to study the time-course for nicotine-induced dendritic remodeling. Specifically, we analyzed nicotine-induced dendritic remodeling in the NAcc, as this region has extremely high DA enervation from the VTA and has been widely associated with multiple aspects of nicotine dependence. Furthermore, as animals exposed to nicotine during adolescence are

most vulnerable to nicotine dependence and nicotine-induced dendritic remodeling, the study of these processes during adolescent brain-development is most warranted in this research. To examine the role of DA-mediated mechanisms on nicotine-induced dendritic remodeling, we co-administered the D1DR antagonist SCH-23390 during repeated, chronic nicotine exposure, then analyzed dendritic remodeling in the NAcc shell. hypothesized that blocking D1DRs, using a selective D1DR antagonist, during repeated nicotine exposure could prevent nicotine induced dendritic remodeling in the NAcc. Our data show that co-administration of SCH-23390 during chronic adolescent nicotine exposure prevents nicotine-induced dendritic remodeling, and that this effect is specific to remodeling of the dendritic arbor rather than to nicotine-induced increases of dendritic spine density.

We also sought to better determine the time-course of nicotine-induced dendritic remodeling, in combination with the co-administration of a D1DR antagonist. As mentioned previously, current studies of nicotine induced dendritic remodeling have only examined morphology following a 2-4 week withdrawal period (McDonald et al., 2005; McDonald et al, 2007; Bergstrom et al. 2008 ; Bergstrom et al, 2010; Robinson & Kolb, 2004). However, it is known that D1DR activation is dominant during the initial stage of drug exposure rather than during the withdrawal or abstinent period during which D2DR activation may become most important. As intracellular mechanisms related to D1DR activation, such as CREB and deltaFosB expression, are upregulated during initial drug exposure, and only some of which continue during a sustained abstinent period, we hypothesized that nicotine-induced dendritic remodeling would be present immediately

following nicotine exposure, rather than be produced as a compensatory effect following removal of nicotine exposure during an extended drug abstinent period. Therefore, we analyzed adolescent nicotine-induced dendritic remodeling and spine density of MSNs both immediately following chronic adolescent nicotine exposure (early abstinence) and three-weeks following exposure (extended abstinence). Our results suggest that dendritic remodeling is present immediately following nicotine exposure, and confirm that adolescent nicotine-induced dendritic remodeling is persistent through an extended abstinent period.

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