UC Irvine

UC Irvine Electronic Theses and Dissertations

Title

Microglial activation increases cocaine self-administration following adolescent nicotine exposure by a D2 receptor mechanism

Permalink

https://escholarship.org/uc/item/9kr272g1

Author

Linker, Kay Elizabet

Publication Date

2019

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, IRVINE

Microglial activation increases cocaine self-administration following adolescent nicotine exposure by a D2 receptor mechanism

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

Submitted by

Kay E. Linker

Dissertation Committee:

Professor Frances Leslie

Professor Marcelo Wood

Associate Professor Kim Green

Professor Christine Gall

Associate Professor David Lyon

© 2019 Kay Linker

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

CURRICULUM VITAE

ABSTRACT OF DISSERTATION

INTRODUCTION

i. Significance 1

ii. The Adolescent Period 3

iii. Nicotine has distinct effects on adolescent neurobiology and behavior 7

iv. Role of Microglia in Development and Drug-Associated Behaviors 10

CHAPTER 2: Adolescent, but not adult, nicotine exposure increases cocaine self-administration and microglial activation 19

CHAPTER 3: Nicotine-induces increases to adolescent cocaine self-administration are microglia dependent 35

CHAPTER 4: D2 Receptors are a critical interface for nicotine-induced changes to microglia and cocaine self-administration 52

CHAPTER 5: Nicotine-induced changes to synaptic plasticity and architecture are microglial and D2 dependent 65

DISCUSSION

i. Significance 86

ii. Microglia are activated by adolescent nicotine exposure and increase cocaine-seeking 87

iii. The Role of D2 Receptors 89

iv. Neuroinflammation and D2 Receptors are Required for Nicotine-Induced Enhancement of Synaptic Plasticity 90

v. Limitations and future directions 91

REFERENCES 95

LIST OF FIGURES

Figure 1.1 Toll-like receptor 4 (TLR4) is a central intermediary between abused drugs and glia. 14

Figure 1.2 Inflammatory cytokines alter dopamine synthesis. 15

Figure 2.1 Schematic of nicotine pretreatment paradigm. 21

Figure 2.2 Nicotine pretreatment increases adolescent, but not adult cocaine responding. 24

Figure 2.3 Nicotine suppresses adult IBA1 expression, but increases adolescent IBA1 expression. 25

Figure 2.4 Brain wide IBA1 protein expression 26

Figure 2.5 Adolescents have differential astrocyte marker expression within the NAc. 27

Figure 2.6 Nicotine promotes ramified adult microglial morphology, and reactive adolescent microglial morphology. 29

Figure 2.7 Pathway Scores derived from Nanostring multiplex gene expression in panel 31

Figure 2.8 Analysis of specific inflammatory genes, expressed as percentage of adult saline 32

Figure 3.1 Schematic of paradigm with nicotine pretreatment and minocycline water bottle administration 39

Figure 3.2 Schematic of paradigm with nicotine pretreatment and PLX3397 chow administration 40

Figure 3.3 Minocycline blocks nicotine-induced increase in microglial activation 43

Figure 3.4 Minocycline blocked nicotine-induced increases in cocaine responding 44

Figure 3.5 Antibiotic cocktail blocked nicotine-induced increases in cocaine reinforcement. 45

Figure 3.6 Preliminary results suggest nicotine increased cocaine responding and this was unaffected with vancomycin administration 46

Figure 3.7 Preliminary results suggest nicotine increased cocaine responding and this was unaffected with vancomycin administration. 46
Figure 3.8 Microglia are necessary for nicotine-induced increases in cocaine seeking 47
Figure 3.9 PLX3397 did not affect reward or stress behaviors 48
Figure 4.1 D2 Receptor protein and mRNA levels are increased after adolescent nicotine exposure 56
Figure 4.2 Nicotine-induced increases in cocaine seeking and microglial activation are blocked with raclopride administration. 57
Figure 4.3 Neurons express D2 receptors while microglia do not 60
Figure 4.4 RNAi of D2 receptors blocks nicotine-induced increases in cocaine-seeking 61
Figure 4.5 Validation of D2 knockdown and demonstration that knockdown of D2 receptors is predominantly in neurons. 62
Figure 4.6 Neuronal D2 knockdown blocks nicotine-induced increases in microglial activated morphology 63
Figure 5.1 Nicotine alters synaptic genes in an age-dependent manner. 72
Figure 5.2 Nicotine suppresses synaptophysin levels in the NAc and BLA 73
Figure 5.3 Adolescent nicotine exposure has region-specific effects on VGlut1 and VGlut1 expression 74
Figure 5.4 Nicotine induces a decrease in synaptic density that is microglia and D2 dependent. 76
Figure 5.5 Nicotine suppresses BLA synaptophysin levels, which is blocked with PLX3397, but not raclopride or minocycline. 77
Figure 5.6 Nicotine increases fractalkine signaling in the adolescent NAc 78
Figure 5.7 Nicotine causes a microglia-dependent increase in NAc Arc+ cells 79
Figure 5.8 Nicotine-induced enhancement in LTP in the NAc core is microglia and D2 dependent 81

ACKNOWLEDGEMENTS

To the many people who helped me through my graduate career, I would like to thank you from the bottom of my heart. It has been an amazing journey, and this dissertation would not have been possible without everyone who supported me along the way.

I would like to thank Dr. Frances Leslie for her guidance, and support throughout my graduate career. She had the patience to allow me to pursue all of my scientific curiosities, as long as I could provide rational for a particular pursuit. Her mentorship fueled my love for science, and I am very thankful for that. Dr. Frances Leslie is a remarkable advisor, and a true mentor that has shaped my view of science.

I would like to thank Dr. Marcelo Wood for his guidance, encouragement, and advice throughout my graduate career. Dr. Wood has taught me (very patiently) how to form a well written grant, and the art of composing a high-impact paper. His excitement for excellent and innovative research will continue to inspire me. Even with a full lab, mentoring younger faculty and running a department, Dr. Wood always found the time to help me and I am very grateful for that.

I would also like to thank Dr. Kim Green for his help with my many, many questions about experimental design and microglial research. Dr. Green was very generous with his time and I could not have completed this thesis without his guidance.

To my thesis committee members, Drs. Chris Gall and David Lyon, thank you for the insight, time, and encouragement throughout these years.

I would like to thank Dr. Belluzzi for his help with statistical design, as well as Dr. Loughlin for her guidance in neuroanatomy and neurochemical techniques. I would like to thank Dr. Eniko Kramar for providing electrophysiology data in Figure 5.8.

Most importantly, to my friends and family, thank you for believing in me! I am deeply grateful for the love and encouragement you have given me over these last few years.

Kay Linker

(760) 274-4388
klinker@uci.edu | kaylinker@gmail.com vi

Current Position
Graduate Research Student in the Department of Anatomy and Neurobiology at the University of California, Irvine
Co-Advisors: Dr. Marcelo Wood and Dr. Frances Leslie

Research Interests

I am interested in how microglia and astrocytes modulate neural circuits and behavior. Throughout my undergraduate and graduate research I have had an emphasis on molecular and cellular mechanisms underlying different behaviors. My undergraduate training with Dr. Kate Wassum gave me a strong background in reward-associated behavior. My current work under Dr. Frances Leslie at UC Irvine has extended my understanding of complex behaviors, as I have learned drug self-administration tasks in adolescent and adult rats. My work with Dr. Marcelo Wood investigates the unique cellular and molecular changes after adolescent and adult drug exposure. I specifically investigate how adolescent nicotine exposure has distinct effects on microglia, how adolescent nicotine exposure alters microglial-dependent synaptic pruning and how these nicotine-induced changes to microglia alter future cocaine self-administration. In the future I would like to explore how microglia and astrocytes modulate behavior in health and disease.

Education

2014 – Present Graduate Research Student, Anatomy and Neurobiology
University of California, Irvine, CA
GPA – 3.9
Advisor: Frances Leslie Co-advisor: Marcelo Wood

2014 B.S. Neuroscience with departmental honors
University of California, Los Angeles, CA
GPA – 3.3

Academic Positions

2014 – Present Graduate Research Student, Anatomy and Neurobiology
UC Irvine, CA
Advisor: Marcelo Wood Co-advisor: Frances Leslie

Skills Learned

Behavioral:

- Conditioned place preference
- Self-administration
- Object location and object recognition memory tasks
- Locomotor and stereotypy

Surgical:

- Jugular vein catheterization surgeries
- Stereotaxic surgeries for viral and RNAi infusion surgeries

Molecular:

- Western blotting, immunohistochemistry

- RT-qPCR, In situ hybridization
- Radioligand receptor binding assays
- Confocal Microscopy
- IMARIS analysis

2012 – 2014 Undergraduate Student Researcher, UC Los Angeles
Supervisor: Dr. Kate Wassum
Skills Learned:

- Seek-Take natural reward instrumental tasks
- General and specific Pavlovian to instrumental transfer (PIT) tasks
- Systemic (intra peritoneal, sub cutaneous) and local (intra-cannula) pharmacological manipulations
- Fast scan cyclic voltammetry dopamine transient analysis

Awards and Honors

2019 Public Impact Fellowship
2018 Ten Tianqiao and Chrissy Chen Fellowship to attend *Brains & Behavior: Order and Disorder in the Nervous System*
2018 UCI School of Medicine Travel Grant
2017 ARCS Fellowship Finalist
2017 Travel award to attend Cold Spring Harbor, Cellular Biology of Addiction Course
2017 UC Irvine Center for Addiction Research First Prize Data Blitz Presentation
2017 Brews and Brains highlighted speaker, Orange County, CA
2015 Effective Mentor for Undergraduate Students
2014 Graduate Dean Recruitment Fellowship
2014 UCLA Dean's Prize for excellence in undergraduate research

Abstracts & Publications

1. Linker KE, Sarah SJ, Leslie FM. Glial Mechanisms of Drug of Use and Dependence. *European Journal of Neuroscience* (2018).
2. Linker KE, Wood MA, Tawadrous P, Leslie FM. Nicotine increases limbic microglial expression and cocaine self-administration in the adolescent rat by a D2 receptor mechanism. Poster presented at: Society for Neuroscience; 2017 Nov 11-15, Washington DC.
3. Cross SJ, Linker KE, Leslie FM. Sex-dependent effects of nicotine on the developing brain. *Journal of Neuroscience Research* (2017).
4. Collins AL, Greenfield VY, Bye JK, Linker KE, Wang AS, Wassum KM. Dynamic mesolimbic dopamine signaling during action sequence learning and expectation violation. *Nature Scientific Report*s (2016).
5. Malvaez M, Greenfield VY, Wang AS, Yorita AM, Feng L, Linker KE, Monbouquette HG, Wassum KM. Basolateral amygdala rapid glutamate release encodes an outcome-specific representation vital for reward-predictive cues to selectively invigorate reward-seeking actions. *Nature Scientific Reports*. (2015).
6. Wassum KM, Greenfield VY, Linker KE, Maidement NT, Ostlund SB. Inflated reward value in early opiate withdrawal. *Addiction Biology.* 2014: Jul 31.

Invited Talks

May 2018	Brains and Behavior: Order and Disorder Cold Spring Harbor Laboratory, New York
June 2018, 2017, 2016	Grad Day, Anatomy and Neurobiology University of California, Irvine
January 2017	Irvine Center for Addiction Research Annual Symposium University of California, Irvine
March 2017	Brews and Brains Orange County, CA
April 2017	Irvine Center for the Neurobiology of Learning and Memory Annual Symposium University of California, Irvine

Professional Affiliations

2016 – Present	ICAN, Irvine Center for Addiction Research Graduate Student member
2015 – Present	Recreational Science Founding Member
2014 – Present	CNLM, Center for the Neurobiology of Learning and Memory Graduate Student member
2013 – Present	Society for Neuroscience Graduate Student Member

Leadership and Mentorship

Honors

UC Irvine Interdepartmental Neuroscience, Incoming Graduate Advisor Panel	2016, 2017
UROP financial Award for 2 undergraduate students (Peter Tawadrous, Monica Bekhit)	2017

Certificates

Mentoring for Excellence Program	2015

Undergraduates Student Mentorship

Student	Major	Institution
Peter Tawadrous	Pharmacology	UCI
John Sedaros	Biology	UCI
Junior Su	Biology	UCI
Monica Bekhit	Pharmacology	UCI
Mario Elabd	Pharmacology	UCI

ABSTRACT OF THE DISSERTATION

Microglial activation increases cocaine self-administration following adolescent nicotine exposure by a D2 receptor mechanism

By Kay Linker

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2019

Professor Frances M. Leslie, Chair

Professor Marcelo A. Wood, Co-Advisor

With the increasing use of e-cigarettes, teen nicotine exposure is becoming more widespread. This is a growing public health crisis, given findings from both clinical and preclinical studies that show adolescent brain to be uniquely sensitive to nicotine. Animal studies have shown that adolescent nicotine exposure results in increased sensitivity to cocaine and other drugs, although the mechanisms that underlie this are poorly understood. We now show microglia to be critical regulators of nicotine-induced increases in adolescent cocaine self-administration. Nicotine has age-dependent effects on microglial morphology and immune transcript profiles. Moreover, blocking D2 receptors eliminates both nicotine-induced increases in cocaine self-administration and microglial activation. Finally, we show nicotine causes microglial-dependent synaptic pruning and plasticity. Taken together, we demonstrate that adolescent microglia are uniquely sensitive to perturbations by drugs of abuse, crucial for nicotine-induced increases in cocaine-seeking, and D2 receptors play a mechanistic role in these phenomena.

CHAPTER 1

Introduction

i. Significance

Adolescence is a tumultuous transition period that marks the final developmental stage before adulthood. It comprises the formative years during which individuals reach sexual maturity and develop social, emotional, and cognitive skills (Spear, 2000). It is a time of increased exploration, which often includes increased sensation seeking and the initiation of drug use in both rodents and humans (Steinberg, 2008; Spear 2000). During this developmental window, the brain is sensitive to novel experiences, including drugs of abuse, with major experience-dependent plasticity occurring in executive control and decision-making regions (Bernheim et al., 2013). Research into the neurobiological underpinnings of adolescence is important for basic understanding of normative social, emotional, and cognitive development as well as for understanding, prevention and treatment of health risks and disorders that emerge during this developmental period.

Tobacco and Drug Addiction as a World Heath Crisis

Tobacco is the leading cause of preventable death in the US and 90% of smokers start before the age of 18 (WHO, 2011; SAMHSA, 2011). Teens that smoke become dependent faster, and experience symptoms of withdrawal even before establishing regular smoking habits (Chen and Millar, 1998; Colby et al., 2000; DiFranza et al., 2007). Furthermore, early initiation of smoking increases the likelihood of tobacco dependence and decreases the likelihood of cessation (Cengelli et al., 2012; Kendler et

al., 2014). Smoking causes immediate adverse effects on both physical and mental health of adolescents (USDHHS, 2012).

Teen smokers have disproportionately high rates of future substance abuse compared to their nonsmoking peers (Lai et al., 2000; Hanna et al., 2001; Biederman et al., 2006). Smoking before 15 years of age significantly raises the risk of further substance abuse by 80%, suggesting that tobacco acts as a "gateway" to future illicit drug use (Kandel et al., 1992; Degenhardt et al., 2010). Whereas causal proof is difficult to obtain in clinical studies, preclinical data demonstrate that adolescent nicotine increases the rewarding effects of other abused drugs. Brief treatment of adolescent rats with nicotine enhances acquisition of self-administration for cocaine, methamphetamine and alcohol, and induces cocaine locomotor sensitization and place preference (McQuown et al. 2007; McQuown et al. 2009; Dao et al. 2011, Alajaji et al., 2016). Compounded with epidemiological data, this preclinical evidence demonstrates adolescent nicotine exposure significantly increases risk for future substance abuse.

The Rising Problem of E-Cigarettes

Electronic cigarettes (e-cigarettes) were first introduced in the United States market in 2007, and since then their use has escalated exponentially (CDC, 2013; Camenga et al., 2014; Bunnell et al., 2015). E-cigarettes are now the most commonly used tobacco product among teenagers and have the lowest perceived health risk (Dutra and Glantz, 2014; Wills et al., 2015). Marketing of e-cigs is effectively targeting teens, as nearly 80% of middle and high school students are exposed to e-cigarette

advertisements from at least one source (Marynak et al., 2018). Furthermore, e-cigarettes are not strictly regulated by the FDA and can be obtained by minors in some states (Paradise, 2014). In addition, there is a wide selection of flavors which is appealing to young people, and adolescents perceive that e-cigarettes with artificial flavors are less harmful than those with tobacco flavors (Grana et al., 2014; Jensenn and Boykan, 2019). A recent study showed 40% of teens who believed their e-cigs contain only flavoring, had high urine levels of nicotine metabolites (Boykan et al., 2019). Thus, adolescents are heavily exposed to e-cigarette advertising, are attracted to the flavorants, can readily access e-cigarettes, believe them to be safer, and are consuming nicotine unintentionally.

The clinical literature on the health consequences of teen e-cigarette use is still limited, but there is evidence that e-cigarettes increase the likelihood of perpetuating future tobacco use (Dutra and Glantz, 2014; Wills et al., 2015; Lee et al., 2014). Moreover, there is substantial evidence that nicotine alone exerts long-term effects at critical stages of brain development (Slotkin, 2004; Ginzel et al., 2007; Dwyer et al., 2008; 2009). Adolescent nicotine exposure triggers lasting changes in neuronal signaling. Smoking and vaping may have potentially severe consequences for teen addiction, cognition, and emotional regulation. Therefore, understanding the unique effects of nicotine during adolescence is essential for treating and preventing the adverse health consequences that follow.

ii. The Adolescent Period

Defining Adolescence

Puberty and adolescence both refer to a developmental period from childhood to adulthood, but these terms are not equivalent. Puberty is reserved for physiological and behavioral changes associated with the attainment of reproductive maturation. Characteristics of pubertal maturation include activation of the hypothalamic-pituitary-gonadal (HPG) axis, appearance of secondary sex characteristics, and onset of sexual interest and mating behaviors. Adolescence is a broader term that includes puberty as well as nonreproductive traits (e.g., social, emotional, and cognitive development).

Reproductive hormones can have widespread transitional effects, including the development of several nonreproductive adolescent traits which can be driven by activation of the HPG axis at puberty (Primus and Kellogg, 1989, 1990; Vetter-O'Hagen and Spear, 2011). The different physiological, anatomical, and temporal changes of puberty between males and females can also lead to the emergence of sex differences during adolescence (Schulz et al., 2009). There are adolescent traits, however, that develop independently of gonadal hormones and merely coincide temporally with pubertal development (e.g., social play, aggression, sex differences) (Whitsett, 1975; Smith et al., 1996; Wommack and Delville, 2007). This puberty-dependent versus puberty-independent distinction is important because many neuropsychiatric and behavioral disorders arise during adolescence, exhibit striking sex differences, and are impacted by pubertal hormones as well as nonpubertal factors (Fombonne, 2009; Graber, 2013; Trotman et al., 2013). Given this, it is important to highlight that the mesocorticolimbic dopamine pathway is sexually dimorphic (Becker, 2009) and

regulated by gonadal hormones in adults (Kuhn et al., 2010; Becker et al., 2012). Therefore, adolescent development of reward-related behaviors and circuitry can be sexually dimorphic, and this maturation and divergence may be puberty-dependent or puberty-independent.

Normative maturation of the adolescent brain

The adolescent brain undergoes dynamic structural and functional reorganization. There is asymmetry in functional development across brain regions, with earlier development occurring in posterior regions and anterior regions progressing later. This causes underdeveloped connections between ventral striatal and prefrontal circuits (Casey, Getz, & Galvan, 2008; Ernst, 2014; Gogtay et al., 2004). This imbalance enables heightened risk-taking behavior, particularly when the behavior results in immediate rewards (Defoe, Dubas, Figner, & van Aken, 2015; van Duijvenvoorde, Jansen, Visser, & Huizenga, 2010). The basolateral amygdala (BLA) is also underdeveloped and can contribute emotional intensity and avoidance, which may serve to boost or dampen impulsive decision making (Eldreth, Hardin, Pavletic, & Ernst, 2013; Ernst, 2014).

Synaptic pruning and refinement are hallmarks of adolescent development. In humans, adolescents exhibit a decrease in cortical gray matter thickness, resulting from experience-dependent loss of synapses and a concomitant strengthening of the remaining connections (Ostby et al., 2009; Gogtay and Thompson, 2010; Paus, 2010). This experience-dependent synaptic elimination is also observed in rodents and is

critical for normative development. In rodents, long-term potentiation (LTP) is more readily induced in the adolescent NAc (Schramm et al., 2002). In addition, adolescent cortex LTP does not require NMDA receptors for maintenance. (Konstantoudaki et al., 2018).

The dopamine (DA) system, in particular, experiences a profound reorganization that is critical for the development of motivated behaviors and associative learning (O'Donnell, 2010; Wahlstrom et al., 2010). One of the most dramatic brain changes occurring during adolescence is the unfolding of DA connectivity in the mPFC. In contrast to DA projections to the NAc (Coyle and Molliver, 1977; Levitt and Moore, 1979; Lidov et al., 1980; Benes et al., 2000; Diamond, 2002), DA projections to the mPFC do not fully mature until early adulthood (Kalsbeek et al., 1988; Benes et al., 2000; Manitt et al., 2011; Naneix et al., 2012). In rodent models, the number of dopaminergic fibers in the mPFC increases linearly between the juvenile period and young adulthood, with the most prominent increases occurring between early and late adolescence (Naneix et al., 2012; Willing et al., 2017). Importantly, this is not a rodent-specific phenomenon as protracted mesocortical DA development occurs in nonhumans primates and possibly in humans (Rosenberg and Lewis, 1994; Lambe et al., 2000).

Dopaminergic cells also have different firing patterns, receptor expression levels and receptor functionality. Within the ventral tegmental area (VTA) DA cells fire faster in adolescents, than adults, likely because of an underdeveloped GABAergic tone (McCutcheon et al., 2012). Changes in dopaminergic receptor expression are prevalent throughout the mesocorticolimbic system, which may underlie the altered sensitivity to

rewarding stimuli during adolescence. In rodents, DA receptor binding site expression exhibits a pattern of overproduction followed by massive pruning that varies based on region and cell type (Teicher et al., 1995; Andersen et al., 1997; 2000; Brenhouse et al., 2008; Naneix et al., 2012).

D2 receptors are functionally immature during adolescence and manipulating them has profound effects on adolescent behavior and neurobiology (Yuan et al., 2015, Dao et al., 2012). In adolescents, AMPA-evoked firing of D2 expressing cells increases the probability of their firing, while in adults it decreases the probability of their firing (Tseng and O'Donnell, 2007; O'Donnell, 2010). D2 receptor stimulation of fast-spiking interneurons in the prefrontal cortex does not emerge until late adolescence, with the recruitment and maturation of local GABAergic activity (Tseng and O'Donnell, 2007; O'Donnell, 2010). Furthermore, important D1-NMDA receptor interactions in cortical pyramidal neurons that are necessary for mature cognitive and attentional processing are still developing during this time (Tseng and O'Donnell, 2005). These differences in dopaminergic circuitry make adolescents particularly vulnerable to the neurobiological disturbances inflicted by drugs of abuse.

iii. **Nicotine has distinct effects on adolescent neurobiology and behavior**

Nicotine binds to neuronal nicotinic acetylcholine receptors (nAChRs) which exhibit distinct patterns of expression, that parallel key maturational events from prenatal development through adolescence (Dwyer et al., 2009). In rodents, α4β2* and α7 nAChR expression and binding are higher in many brain regions in adolescents than

in adults (Adriani et al. 2003). These nAChRs can regulate dopamine release within the nucleus accumbens (NAc) as well as other brain regions (Reinoud et al., 2014, Anderson et al., 2009).

In adolescents, nicotine robustly enhances neuronal activity, as measured by c-fos mRNA expression, in several reward-related regions, including the nucleus accumbens shell, basolateral amygdala, and ventral tegmental area (Shram et al., 2007; Dao et al., 2011). This pattern of immediate early gene expression is not observed in adults, suggesting nicotine more actively engages critical reward processing circuits in the adolescent brain. Nicotine also induces epigenetic remodeling, and this is associated with increased sensitivity to nicotine-associated behaviors (Mychasiuk and Metz, 2012).

Nicotine can sensitize catecholaminergic systems via upregulation of nAChRs or tyrosine hydroxylase. Indeed, acute nicotine administration increases catecholamine synthesis in the NAc (Mitchell et al., 1989), and adolescent rats are more sensitive than adults to the DA releasing effects of nicotine in the NAc (Corongiu et al., 2019). The adolescent brain appears to be exceptionally sensitive to nicotine-induced neuroplasticity.

Epidemiologic studies document that individuals who begin smoking as teens are more likely to become life-long smokers than those who start smoking in their 20's or later (Dierker et al., 2012, Volkow, 2006). Furthermore, adolescents experience symptoms of dependence at lower levels of nicotine exposure than adults (O'Loughlin et al., 2003, DiFranza et al., 2000, Kandel and Chen, 2000). Animal studies have shown

that, compared to adults, adolescents experience more rewarding properties of nicotine (O'Dell, 2009; Yuan et al., 2015; Belluzzi et al., 2004; Shram et al., 2006; Torres et al., 2008). Adolescent rats also readily acquire intravenous nicotine self-administration and take more nicotine than adults (Chen et al., 2007; Levin et al., 2007; Natividad et al., 2013). They also simultaneously evade the aversive properties of nicotine (Adriani et al., 2002; Shram et al., 2006; Torres et al., 2008; Yuan et al., 2015). Moreover, pre-exposure to nicotine during adolescence diminishes aversion to high doses nicotine later in life (Torres et al., 2008). Adolescents also display blunted withdrawal symptoms following chronic nicotine exposure, whereas adults display prominent, severe nicotine withdrawal (O'Dell et al., 2006; Shram et al., 2008). In adolescents, nicotine produces more positive and less negative psychological states, and this may cause increased vulnerability to nicotine addiction.

Nicotine is more rewarding in adolescents, and nicotine exposure is associated with increased drug use. Notably, even brief, low-dose administration of nicotine can have profound effects on adolescent behavior and neurochemistry. Nicotine pretreatment in adolescents, increases self-administration for cocaine, methamphetamine, and alcohol and increases cocaine locomotor sensitization and conditioned place preference (McQuown et al., 2007; McQuown et al., 2009; Dao et al., 2011; Alajaji et al., 2016; Thomas et al., 2018). This pretreatment paradigm also increases locomotion induced by the D2-agonist, quinpirole (Dao et al., 2011). Dao et al., demonstrated 5HT1a receptors are absolutely critical for nicotine-induced increases cocaine self-administration and D2 receptor behavioral sensitization. Independently, adolescent nicotine increases alcohol

use by suppressing VTA DA firing through heightened GABAergic signaling (Larraga et al., 2017; Thomas et al., 2018). Taken together, these studies demonstrate that nicotine has unique, age-dependent effects on behavior and neurobiology that underlie these behaviors are distinct. However, a complete understanding of the neurobiological mechanisms by which adolescent nicotine exposure produces such profound behavioral effects has yet to be achieved.

iv. Role of Microglia in Development and Drug-Associated Behaviors

Defining microglia and their development

The above pivotal studies have elucidated nicotine-induced changes in neuronal systems and their effect on subsequent cocaine self-administration, but the role of microglia in adolescent nicotine exposure remains unexplored. Microglia are the resident immune cells of the brain, and the first defenders against biological threats – including drugs of abuse. They are in constant communication with neurons and microglia are important for brain development (Stevens et al., 2007; Paolicelli et al., 2011; Bialas et al., 2013; Miyamoto et al., 2013; Wu et al., 2015; Frost et al., 2016).

Throughout development microglia transform their structure and function. In early development, microglia have larger, round morphology, consistent with their role as macrophages (Brenhouse and Schwarz, 2016). In adolescent development, microglia within certain brain regions continue to show what appears to be a more “activated” or immature morphology, with thick variegated branches, suggesting that these cells are continuing to undergo maturational changes throughout late stage development

(Schwarz et al., 2011). In addition, adolescent microglia are more sensitive to lipopolysaccharides (LPS) induced inflammation (Brenhouse and Schwarz, 2016). The sensitivity of adolescent microglia may contribute to the robust effects that inflammatory insults have on brain and behavior that do not occur if the same insult were to be experienced earlier or later in development (Schwarz and Bilbo, 2013).

Microglia regulate neuronal function

Microglia release a range of secreted factors (cytokines) that influence neurons, and microglial processes constantly palpate neurons (Wu et al., 2015). Increasing evidence demonstrates that microglia play a fundamental role in monitoring neuronal activity and in modulating neuronal structure and function. Microglia can use their secreted factors to regulate developmental pruning, synaptic plasticity and neurotransmission (Stevens et al., 2007; Paolicelli et al., 2011; Wu et al., 2015; Linker et al., 2018).

Nearly all drugs of abuse activate microglia (Coller and Hutchinson, 2012). Activated microglia exhibit stereotypical morphology of increased soma size, increased process thickness and decreased process length (Lacagnina et al., 2017). Activation of microglia promotes a pro-inflammatory state, which then alters neurocircuits associated with reward and dependence (Lacagnina et al., 2017). The neurochemical signaling cascade that transforms ramified microglia into reactive microglia is complex, but an integrative receptor is toll-like receptor 4 (TLR4). TLR4 activation promotes translocation of the transcription factor Nf-κβ to the nucleus, which then induces the

production of pro-inflammatory cytokines and chemokines (Lee & Kim, 2007; Trotta et al., 2014).

Many drugs of abuse directly interact with TLR4 (**Fig. 1.1**). Extensive studies have demonstrated that opioids can act directly on TLR4 as an agonist at a non-classical binding site, where LPS also binds, to produce neuroinflammation (Watkins et al., 2009; Coller and Hutchinson 2012). Cocaine also directly interacts with TLR4, and blocking the cocaine binding site inhibits cocaine-induced cytokine release (**Fig. 1.1**). Alcohol also induces a TLR4-dependent release of pro-inflammatory cytokines (Alfonso-Loeches et al., 2010). The mechanism by which alcohol enhances activation of TLR4 is unclear, but one possibility is through modulation of lipid rafts in astrocyte membranes, and endocytosis of active TLR4 receptors (**Fig. 1.1**; Blanco et al., 2008; Pascual-Lucas et al., 2014). In contrast to most abused drugs, nicotine has been shown to suppress microglia in adult brain (Shytle et al., 2004; De Simmone et al., 2005; Mishra et al., 2015; Noda et al., 2017). Recent studies in adult rat microglia have shown this suppression to occur via activation of the α7 nAChR (Mishra et al., 2015; Noda et al., 2017). However, it is unclear if nicotine has similar effects on adolescent microglia.

After drug exposure, proinflammatory cytokines are released into the extracellular milieu and bind to receptors expressed in microglia, astrocytes, and neurons, causing changes to intracellular signaling and cell function. The inflammatory cytokine, TNFα, regulates synaptic activity by driving the internalization of AMPA receptors and regulating astrocyte glutamate release (Santello & Volterra, 2012; Beattie et al., 2002; Pribiag & Stellwagen, 2013). Cocaine-induced TNFα production causes the

stereotypical depression of glutamatergic synaptic strength observed within in the NAc after cocaine exposure (Lewitus et al., 2016). Interestingly, dopamine stimulates TNFα release in the NAc, which is predominantly driven by D2 receptors (Lewitus et al., 2016), and without TNFα, cocaine has blunted dopamine release abilities (Northcutt et al., 2015). Many other chemokines and cytokines have been shown to critically affect dopaminergic and glutamatergic circuit dynamics (Linker et al., 2018).

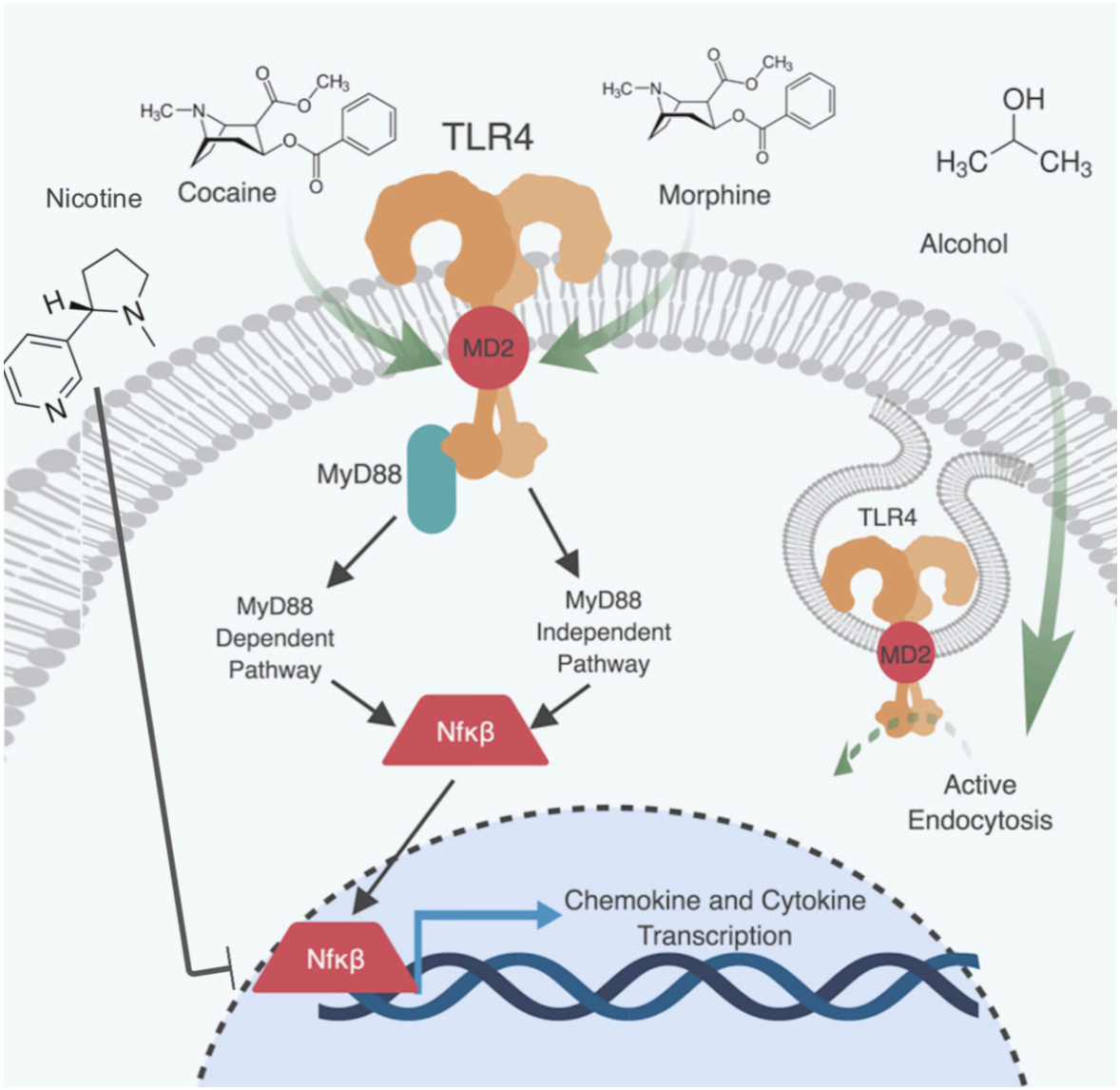


Fig. 1.1: Toll-like receptor 4 (TLR4) is a central intermediary between abused drugs and glia. Morphine is a direct agonist of TLR4 and binds to a unique binding site in the myeloid differentiation factor 2 (MD-2) domain of the TLR4 receptor. Cocaine is a direct agonist for TLR4 and binds to a domain in the MD-2 region of the receptor, this binding site is the same site of (+)-naltrexone. Alcohol promotes active endocytosis of TLR4 receptors by modulating lipid rafts. TLR4 activation causes the translocation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κβ) to the nucleus via the myeloid differentiation primary response 88 (MyD88) pathway and MyD88-independent pathways. Adapted from Linker et al., 2018.

Cytokines have also been shown to stimulate intra-neural signaling pathways that alter the synthesis of neurotransmitters essential for reward and addiction-like behaviors (Cui et al., 2014). Dopamine is synthesized by a series of chemical reactions which requires the enzymes phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH) (**Fig. 1.2**). Tetrahydrobiopterin (BH4) is an essential cofactor for both enzymes, and BH4 production is stimulated by pro-inflammatory cytokine release. Through this

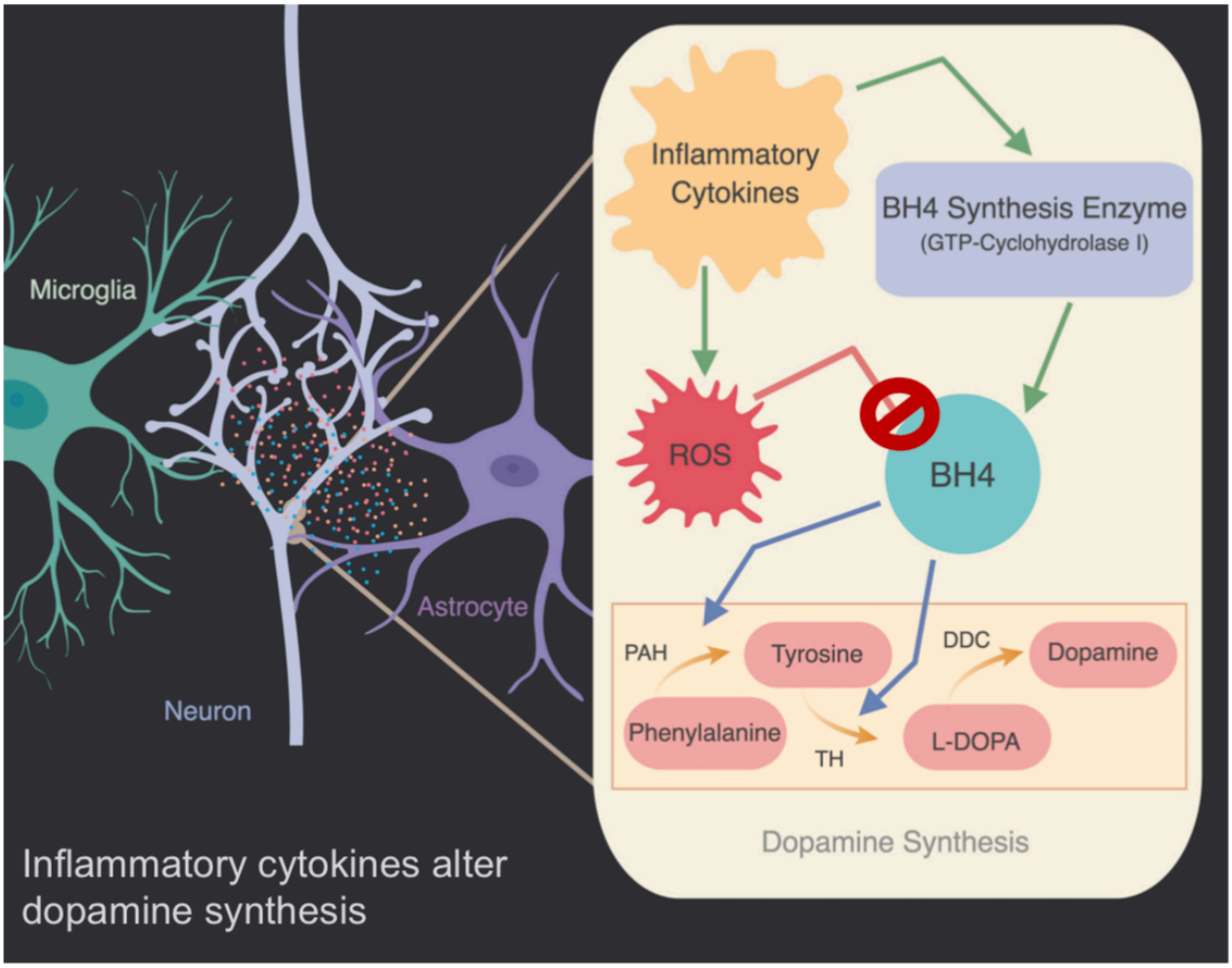


Fig. 1.2: Inflammatory cytokines alter dopamine synthesis. Dopamine synthesis relies on the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH). Phenylalanine is converted to tyrosine by phenylalanine hydroxylase (PAH). Both TH and PAH require tetrahydrobiopterin (BH4) as a cofactor. Although inflammatory cytokines induce GTP-cyclohydrolase I expression, an enzyme necessary for BH4 synthesis, they also increase free radicals. Reactive oxygen species (ROS) contribute to oxidative stress and reduction of BH4. Inflammatory cytokines can both increase and decrease dopamine synthesis through different pathways. Red arrows indicate inhibitory effects and green arrows indicate excitatory effects on enzymes. Adapted from Linker et al., 2018.

pathway, inflammatory cytokines can promote dopamine synthesis via BH4 (Cui et al., 2014; **Fig. 1.2**). However, inflammatory cytokines also increase reactive oxygen species that ultimately decrease the oxidation-labile BH4 and, in turn, decrease TH production (Neurauter et al., 2008). This complex, push-pull dynamic allows for a feedback loop that regulates dopamine synthesis based on the level of local cytokine production. Indeed, alcohol-induced TLR4 pro-inflammatory signaling has been shown to increase TH production in VTA dopaminergic neurons, which contrasts with cytokine-induced decreases in TH observed in many depression studies (Aurelian et al., 2016; Neurauter et al., 2008; Felger & Miller, 2012).

Microglia can also modulate synapse elimination and formation. Microglia prune synapses via phagocytosis of dendritic spines (Nimmerjahn et al., 2005; Wake et al., 2009; Wu et al., 2015) in an activity-dependent manner (Tremblay et al., 2010; Paolicelli et al., 2011). Several mechanisms have been elucidated for this pruning, most notably the classical complement cascade and fracktalkine-CX3CR1 signaling (Stevens et al., 2007; Paolicelli et al., 2011). Microglia can also promote synaptic formation through the anti-inflammatory cytokine IL-10 (Kucukdereli et al., 2011; Miyamoto et al., 2015). Taken together, microglial-derived cytokines and chemokines can alter neurotransmitter synthesis and release, neuronal activity, and synaptic structure (Cui et al., 2014; Linker et al., 2018). However, little is known about the role microglia play in adolescent synaptic plasticity or how drugs of abuse alter microglial synaptic elimination

Inhibiting the inflammatory state induced by drugs of abuse can inhibit the rewarding effects of addictive drugs (Lacagnina et al., 2017). Minocycline is a blood–brain barrier

permeable tetracycline antibiotic that prevents a multitude of neuroinflammatory signaling cascades. Minocycline blocks proinflammatory cytokine release and microglia activation induced by drugs of abuse (Coller and Hutchinson 2012; Lacagnina et al., 2017; Linker et al., 2018). Systemic administration of minocycline blocks stimulatory and rewarding effects of morphine, cocaine, methamphetamine and alcohol (Linker et al., 2018). Minocycline also suppresses methamphetamine and cocaine-induced dopamine release (Zhang et al., 2006; Northcutt et al., 2015). Multiple behavioral paradigms that measure drug reward, including self-administration, conditioned place preference, and locomotor sensitization, are suppressed by minocycline (Lacagnina et al., 2017; Linker et al., 2018). Moreover, site specific knockdown of neuroinflammation with infusion of minocycline into the NAc blocks acquisition and maintenance of morphine conditioned place preference (Zhang et al., 2012). Minocycline also suppresses alcohol consumption (Agrawal et al., 2011; Bell et al., 2015; Syapin et al., 2016). and methamphetamine self-administration (Snider et al., 2013). Together, this evidence demonstrates minocycline has profound inhibitory effects on reward associated behaviors across a spectrum of addictive drugs.

Nearly all drugs of abuse activate microglia and inhibiting this inflammatory state can prevent the acquisition and maintenance of drug-seeking behaviors. In contrast, nicotine suppresses microglia in the adult brain, and this suppression occurs via the α7 nicotinic receptor (Shytle et al., 2004; De Simmone et al., 2005; Mishra et al., 2015; Noda et al., 2017). Since the vast majority of smokers start during adolescence, it is critical to investigate changes that occur in the adolescent brain after nicotine exposure. In

addition, early adolescent nicotine use is associated with enhanced cocaine use in both clinical populations and in animals. Many studies have investigated changes in neurons after adolescent nicotine exposure, yet effective therapeutic targets remain elusive. In contrast, to my knowledge, no studies have yet investigated the effects of adolescent nicotine exposure on microglia and many questions remain. Does nicotine activate or suppress microglia in the adolescent brain (**Chapter 2**)? What is the behavioral significance of changes in microglia after adolescent nicotine exposure (**Chapter 3**)? What is the role of dopamine, in particular D2 receptors, in nicotine-induced changes to adolescent microglia and behavior (**Chapter 4**)? Finally, how does nicotine-induced microglial activation contribute to neuronal structure and function (**Chapter 5**)? This dissertation aims to answer these questions and address my hypothesis that adolescent reactive microglia increase subsequent cocaine-seeking by a D2 receptor mechanism.

CHAPTER 2

Adolescent, but not adult, nicotine exposure increases cocaine self-administration and microglial activation

INTRODUCTION

The adolescent period is a dynamic developmental window, where drugs of abuse induce unique perturbations (Spear, 2000; Yuan et al., 2015). Adolescents have distinct brain and behavioral responses to nicotine in comparison to adults (Dwyer et. al, 2009). Our lab has repeatedly demonstrated that adolescent, but not adult, nicotine pretreatment increases cocaine self-administration (McQuown et al., 2007, 2009; Dao et a., 2011). Adolescent nicotine exposure also has unique effects on neuronal connectivity, synaptic architecture, and gene expression (Dao et al., 2011, Yuan et al., 2015). In particular, adolescent, but not adult, nicotine exposure increases the IEG *cFos* in two regions that encode reward: the NAc and BLA (Dao et al., 2011). Prior studies have investigated changes in neurons following nicotine exposure, but the cellular and molecular mechanisms that contribute to this phenomenon remain poorly understood. To my knowledge, no study has investigated changes in glia after adolescent nicotine exposure.

Two important glial cell-types are astrocytes and microglia. Astrocytes provide metabolic support for neurons, modulate synaptic plasticity and are vital for neurotransmitter uptake and release (Linker et al., 2018). Microglia are the resident immune cells of the brain, with functions expanding beyond that of biological defense (Wu et al., 2015). Microglia are vital for proper brain maturation (Stevens et al., 2007).

They have distinct functional and morphological characteristics that are developmentally regulated. The vast majority of drugs promote a reactive microglial phenotype (Coller and Hutchinson, 2012, Linker et al., 2018). However, nicotine suppresses microglia in the adult brain. It remains unclear if this anti-inflammatory response is present in adolescent microglia. Since adolescents have unique neurobiological responses to nicotine, and microglia are developmentally regulated, I investigated whether nicotine would differentially affect adolescent microglia. I used a brief-low dose nicotine pretreatment paradigm that increases adolescent, but not adult, cocaine-seeking, and found that this suppressed adult microglia, but activated adolescent microglia.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats were group-housed in an AAALAC-accredited vivarium on a 12 hr light/dark cycle with unlimited access to food and water. Pups were weaned on P21. All experiments were performed during the light cycle and carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine.Drugs. Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2 – 7.4; doses were calculated as free base. Cocaine HCl (Sigma, St. Louis, MO) was dissolved in sterile saline.

Catheter Implantation: Rats, aged postnatal day (P) 24/25 or P82/83, were anesthetized with equithesin (0.0035 ml/g) and surgically implanted with an indwelling catheter in their jugular vein (Belluzzi et al, 2005). Throughout the 2-3 days of recovery, catheters

were flushed daily with heparinized saline solution (1000 units heparin per 30 ml bacteriostatic saline) to maintain patency. Catheter patency was verified by testing with propofol (5 mg/kg, i.v.) after drug pretreatment, and only animals showing rapid anesthesia were included in analyses.

Pretreatment: Adolescent (P28-31) and adult (P86-89) rats received an intravenous drug pretreatment of nicotine (60 μg/kg), or saline for 4 consecutive days (McQuown et al., 2007). Behavioral testing was conducted the following day on either P32 or P90 (**Fig. 2.1**).

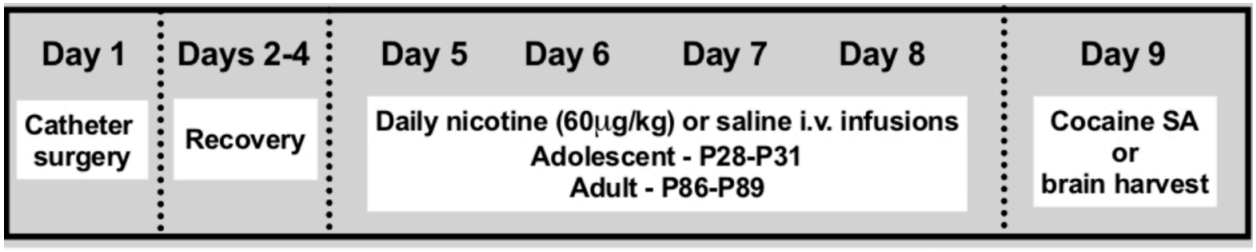


Figure 2.1: Schematic of nicotine pretreatment paradigm.

Self-Administration: One day after pretreatment, at P32 for adolescents and P90 for adults, acquisition of cocaine or sucrose self-administration was evaluated in an operant chamber containing two nose poke holes (MED Associates, Inc., St. Albans, VT). Rats self-administered cocaine (0.5 mg/kg, i.v., 20 μl, 1.1s) in a single 2 hr session on a fixed ratio 1 (FR1) schedule. During each infusion, the cue light above the reinforced hole was illuminated. Afterward the house light shut off for a 20 sec timeout where responses were counted but had no effect. To control for nonspecific activity, responses for the non-reinforced hole were recorded but had no programmed consequences.

Immunohistochemistry: All animals were perfused according to established procedures

and brains were post- fixed in 4% paraformaldehyde for 24 h to ensure proper tissue fixation. To investigate changes in protein levels, 40µm coronal sections were run through free-floating immunohistochemistry (IHC). Briefly, tissue was blocked and permeated in 0.2% Triton X-100, 5% Normal Goat Serum (NGS), in PBS. Sectioned tissue is incubated with primary antibody in incubation buffer (5% NGS, 0.2% Triton X-100 in PBS) overnight. Primary antibody used was IBA1 (1:1000; Wako-Chem; 019-1974) and GFAP (1:500; Abcam). Tissue was incubated with secondary antibody (goat-anti rabbit IgG Alexa fluor 488, Abcam ab150077) in incubation buffer. Omission of the primary antibody was a negative control.

Microscopy and Analysis: Images for absorption analysis were captured on an Olympus Scanner BX61VS and image analysis was performed using ImageJ. A Zeiss LSM 700 was used to capture images for microglial morphological analysis. Microglia morphology, cell count and soma size were analyzed by IMARIS. IHC and analysis of all experimental groups was performed in parallel. All analysis was done blind.

Nanostring: Total mRNA was extracted using an RNA Plus Universal Mini Kit (Qiagen) and was hybridized and multiplexed with NanoString probes, according to the manufacturer's instructions. Counts for target genes were normalized to house-keeping genes (Aars, asb7, Ccdc127, Csnk2a2, Fam104a, Mto1, Supt7l, Tada2b) to account for variability in the RNA content. Background signal was calculated as a mean value of the negative hybridization control probes. Pathway scores are calculated as the first principal component of the pathway genes' normalized expression

Data Analysis: Self-administration, IBA1 absorption, and IBA1 count data were analyzed with a two-way ANOVA for sex and drug, and no sex differences were found. These data were therefore collapsed across sex. Acquisition of cocaine self- administration was analyzed with a 2-way ANOVA for nicotine pretreatment × responses, with repeated measures on responses. Absorption, microglia count and nanostring data were analyzed with a two-way ANOVA for age x nicotine pretreatment. Morphological data were separated by age and analyzed with a two-way ANOVA for sex x nicotine pretreatment. All significant main effects were further analyzed with Bonferonni post hoc analysis.

RESULTS

Nicotine exposure has age-dependent effects on cocaine self-administration and microglia

In confirmation of earlier studies from our lab (McQuown et al. 2009; Dao et al., 2011), I show that nicotine pretreatment increases initial acquisition of cocaine self-administration in adolescent rats but not adults (**Fig. 2.2 b,c**). No sex differences were observed for any measure in **Fig 2.2**, as such data were collapsed across sex.

Although it is well established that nicotine suppresses microglia in the adult brain (Shytle et al., 2004; DeSimone et al., 2004), no prior studies have examined the effect of nicotine on adolescent microglia. Consistent with prior findings (Shytle et al., 2004), nicotine significantly suppressed expression of the microglial marker, IBA1, in the nucleus accumbens (NAc) of adult rats (P90, **Fig. 2.3 a,c**). In contrast, nicotine significantly increased IBA1 expression in adolescent (P32, **Fig. 2.3 a,c**). Whereas

nicotine pretreatment had no significant effect on

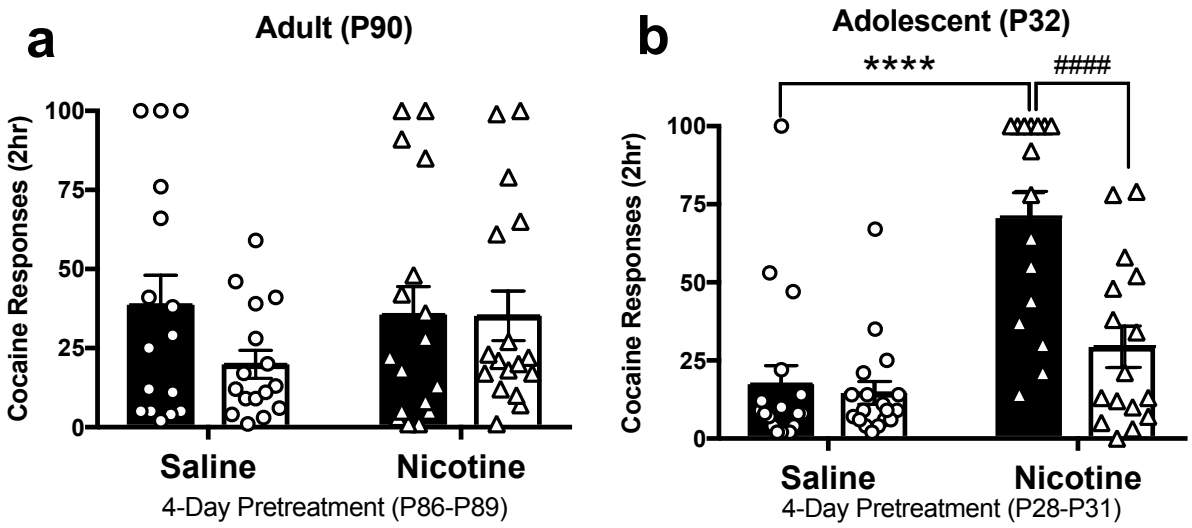


Fig. 2.2: Nicotine pretreatment increases adolescent, but not adult cocaine responding. No significant sex differences were observed in either adult ($F(1,29)=0.0225$, $p=0.8818$; n=5 female 11 male saline, 12 male nicotine) or adolescent ($F(1,30)=0.1242$, $p=0.7269$; n=5 female, 13 male saline, 11 male nicotine) cocaine responding. Behavioral data are collapsed across sex. **a**, Adult nicotine pretreatment did not significantly affect cocaine responding ($F(1,31)=0.4876$, $p=0.4902$; n=16 saline; 17 nicotine). **b**, Adolescent nicotine exposure significantly increased cocaine responding ($F(1,32)=20.75$, $p<0.0001$). A significant effect of response ($F(1,32)=24.29$, $p<0.0001$) and interaction between response and nicotine ($F(1,32) =18.48$, $p=0.0002$) was also observed. Adolescent rats pretreated with nicotine distinguished between reinforced and non-reinforced holes (###$p=0.0004$). (n=18 saline, 21 nicotine). All data analyzed with Bonferonni post hoc analysis (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$). All n is number of rats. Bars show mean +/ s.e.m

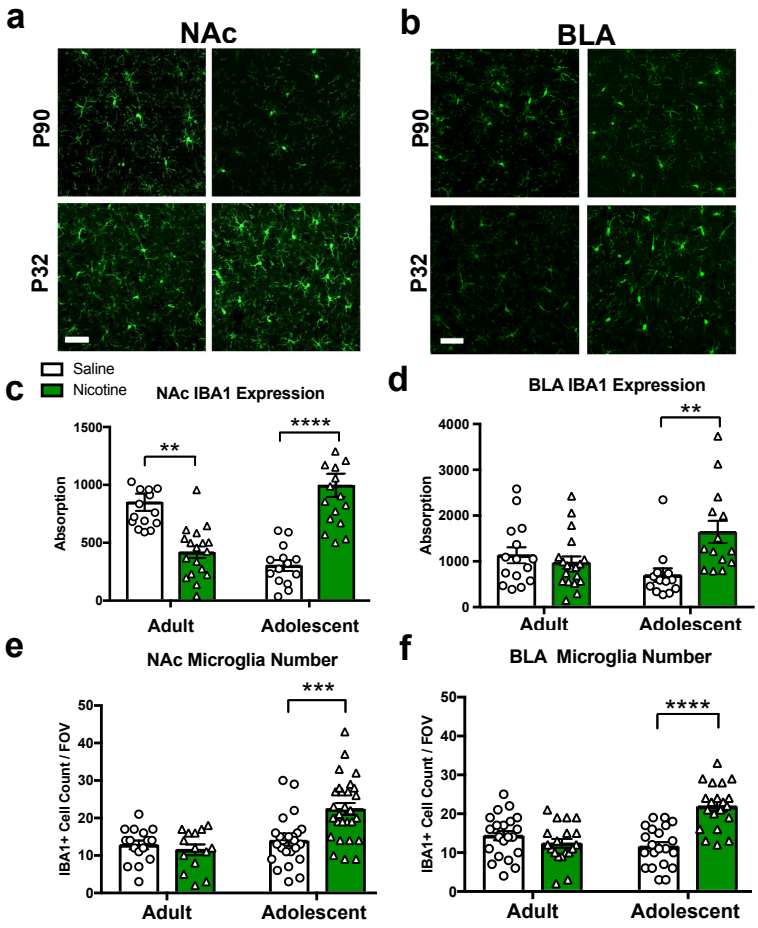


Fig. 2.3: Nicotine suppresses adult IBA1 expression, but increases adolescent IBA1 expression. No sex differences were observed in either adult NAc expression ($F(1,29)=3.247$, $p=0.0820$; n=4 female saline, 5 female nicotine, n=10 male saline, 14 male nicotine), cell count ($F(1,36)=0.09269$, $p=0.7625$; n=5 female, 16 male saline, 14 male nicotine) or BLA expression ($F(1,30)=1.061$, $p=0.3113$; n=4 female, 11 male saline, 15 male nicotine), cell count ($F(1,37)=0.5165$, $p=0.4769$; n= 5 female saline, 4 female nicotine, 18 male saline, 14 male nicotine). No adolescent sex differences were observed in NAc IBA1 expression ($F(1,40)=0.008322$, $p=0.9278$; n=5 female rats, 17 male rats), count ($F(1,49)=0.3572$, $p=0.5528$; n=11 female saline, 12 female nicotine, 14 male saline, 16 male nicotine) or BLA IBA1 expression ($F(1,23)=1.64$, $p=0.2131$; n=3 female saline, 5 female nicotine rats, 10 male saline, 9 male nicotine rats), count ($F(1,33)=3.128$, $p=0.0862$; n=5 female saline, 6 female nicotine, 13 males). As such all below data are collapsed across sex **a,b**, Representative images of IBA1 expression in the NAc (**a**) and BLA (**b)**. **c**, Nicotine significantly decreased adult NAc IBA1 expression, but increased adolescent IBA1 NAc expression, with a significant interaction between age and nicotine ($F(1,64)=25.69$, $p<0.0001$; n=10 adult saline, 14 adult nicotine, 21 adol. saline, 22 adol. nicotine). **d**, Nicotine had age-dependent effects on IBA1 BLA expression, where nicotine selectively increased IBA1 BLA expression ($F(1,57)=4.84$, $p=0.0319$), with a significant interaction between age and nicotine ($F(1,49)=9.629$, $p=0.0030$) (n=15 adult saline, 19 adult nicotine, 13 adol. saline, 16 adol. nicotine). **e**, Nicotine exposure significantly increased adolescent NAc microglia number ($F(1,66)=7.828$,$p=0.0067$). An overall effect of age ($F(1,66)=17.71$; $p<0.0001$) and interaction between age and nicotine ($F(1,66)=14.01$, $p=0.0004$) was observed (n=16 adult saline, 14 adult nicotine, 25 adol. saline, 28 adol. nicotine). **f**, Nicotine exposure increased adolescent BLA microglia number ($F(1,78)=12.21$, $p=0.0008$), with an effect of age ($F(1,78)=7.987$, $p=0.0060$) and interaction between age and nicotine ($F(1,78)=26.71$, $p<0.0001$) (n=22 adult saline, 19 adult nicotine, 21 adol. saline, 20 adol. nicotine). All data analyzed with Bonferonni post hoc analysis (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$). All n is number of rats. Scale bar is 50µm. Bars show mean +/ s.e.m

IBA1expression in adult basolateral amygdala (BLA), there was significantly increased in expression in the adolescent BLA (**Fig. 2.3 b,d**). Nicotine also increased IBA1+ cell number in both the NAc (**Fig. 2.3 e**) and BLA (**Fig. 2.3 f**) of adolescents but not adults. No other forebrain regions studied showed any nicotine-induced changes in IBA1 expression (**Fig 2.4**). These findings demonstrate that nicotine has unique, age-dependent effects on microglial expression in the NAc and BLA, two regions that encode aspects of reward, are critical for reinforcement behaviors and are actively

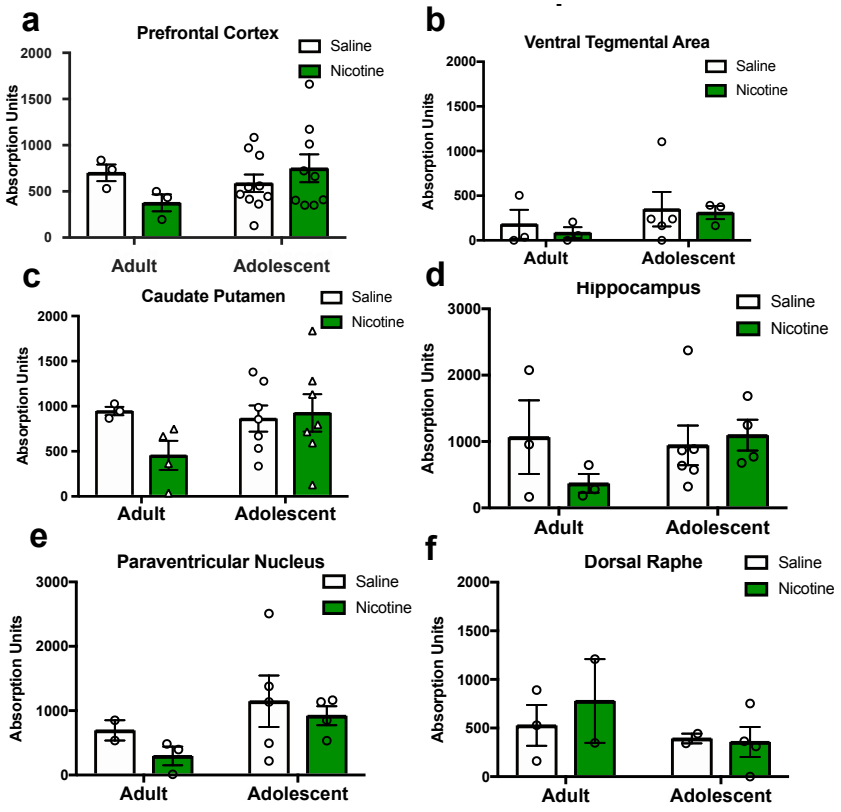


Fig. 2.4: Brain wide IBA1 protein expression. Nicotine did not significantly change IBA1 expression in **a**, PFC ($F(1,21)= 0.2478$, $p=0.6238$; n=3-9); **b**, VTA ($F(1,10)= 0.1524$, $p=0.7044$; n=4-5); **c**, caudate putamen ($F(1,17)=1.194$, $p=0.2899$; n=3-7); **d**, hippocampus ($F(1,12)=0.6186$, $p=0.4468$; n=3-6); **e**, hypothalamic paraventricular nucleus ($F(1,10)=0.8307$, $p=0.3835$; n=2-5); or **f**, dorsal raphe ($F(1,7)=0.2214$, $p=0.6523$; n=2-4).

maturing during adolescence (Yuan et al., 2015; Dwyer et al., 2009).

Nicotine has age-dependent effects on astrocyte marker expression

To investigate changes in astrocytes following nicotine exposure, we measured two astrocyte markers: S100b and GFAP. The adolescent NAc expresses higher levels of S100b irrespective of nicotine treatment (**Fig. 2.5 a**). GFAP expression was significantly increased in the NAc of adolescent rats after nicotine exposure (**Fig. 2.5 c**). No age or drug-induced differences in S100b or GFAP expression were observed in the

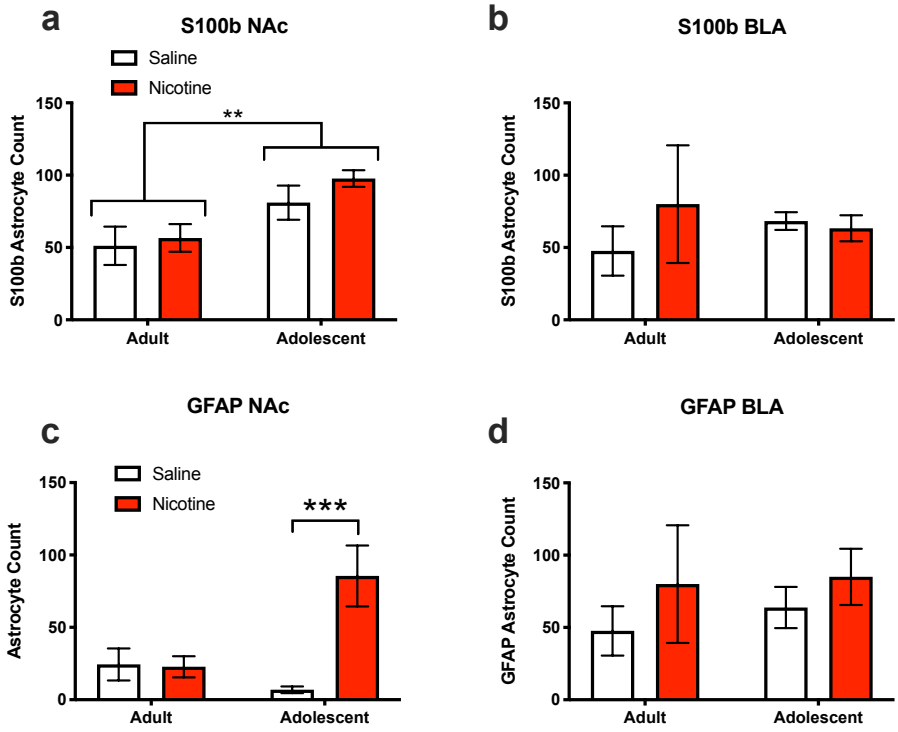


Fig. 2.5: Adolescents have differential astrocyte marker expression within the NAc. **a,** A significant effect of age demonstrates adolescents have higher S100b expression in the NAc ($F(1, 23) = 11.64$; $p=0.0024$; n=5-9). **b,** No significant differences in S100b were observed in the BLA (n=5-10). **c**, An overall significant effect of nicotine ($F(1, 24) = 6.389$; $p=0.0185$), and interaction between age and nicotine ($F(1, 24) = 6.930$; $p=0.0146$; n=5-9) demonstrated nicotine significantly increased GFAP expression only in the adolescent NAc. **d,** No significant differences in GFAP were observed in the BLA (n=5-10). All data analyzed with Bonferonni post hoc analysis (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). All n is number of rats. Bars show mean +/ s.e.m

BLA. (**Fig. 2.5 b,d**). Within the NAc, expression of astrocyte markers are

developmentally regulated and influenced by nicotine exposure.

Nicotine exposure promotes a ramified phenotype in adult microglia, and a reactive phenotype in adolescent microglia

Reactive microglia exhibit stereotypical morphology of increased soma size, increased process thickness, decreased process length and decreased microglial branching, whereas ramified, inactive microglia demonstrate the reverse (Bessis et al., 2007). To further investigate if adolescent microglia are activated by nicotine, I measured morphological changes in both adolescent and adult microglia. I observed some adult sex differences in baseline NAc and BLA microglia morphology. Most notably, adult male microglia are larger than female (**Fig. 2.6 a,b**), which reflects recent findings (Guneykaya et al., 2018). No significant adolescent sex differences were observed (**Fig. 2.6 c-j**). I also found significant age differences in soma size and process branching. In the adult NAc, nicotine exposure significantly decreased microglial soma area (**Fig. 2.6 c**), decreased microglial process diameter (**Fig. 2.6 e**), increased process lengths (**Fig. 2.6 g**), and increased microglial branching (**Fig. 2.6 i**). These morphological changes indicate an increase in a ramified microglial phenotype and replicate prior work demonstrating that nicotine suppresses microglial activation (Guan et al., 2015; Noda et al 2017). In the adolescent NAc, nicotine significantly increased microglial process diameter (**Fig. 2.6 e**), decreased process lengths (**Fig. 2.6 g**), and decreased microglial branching (**Fig. 2.6 i**), consistent with a reactive phenotype

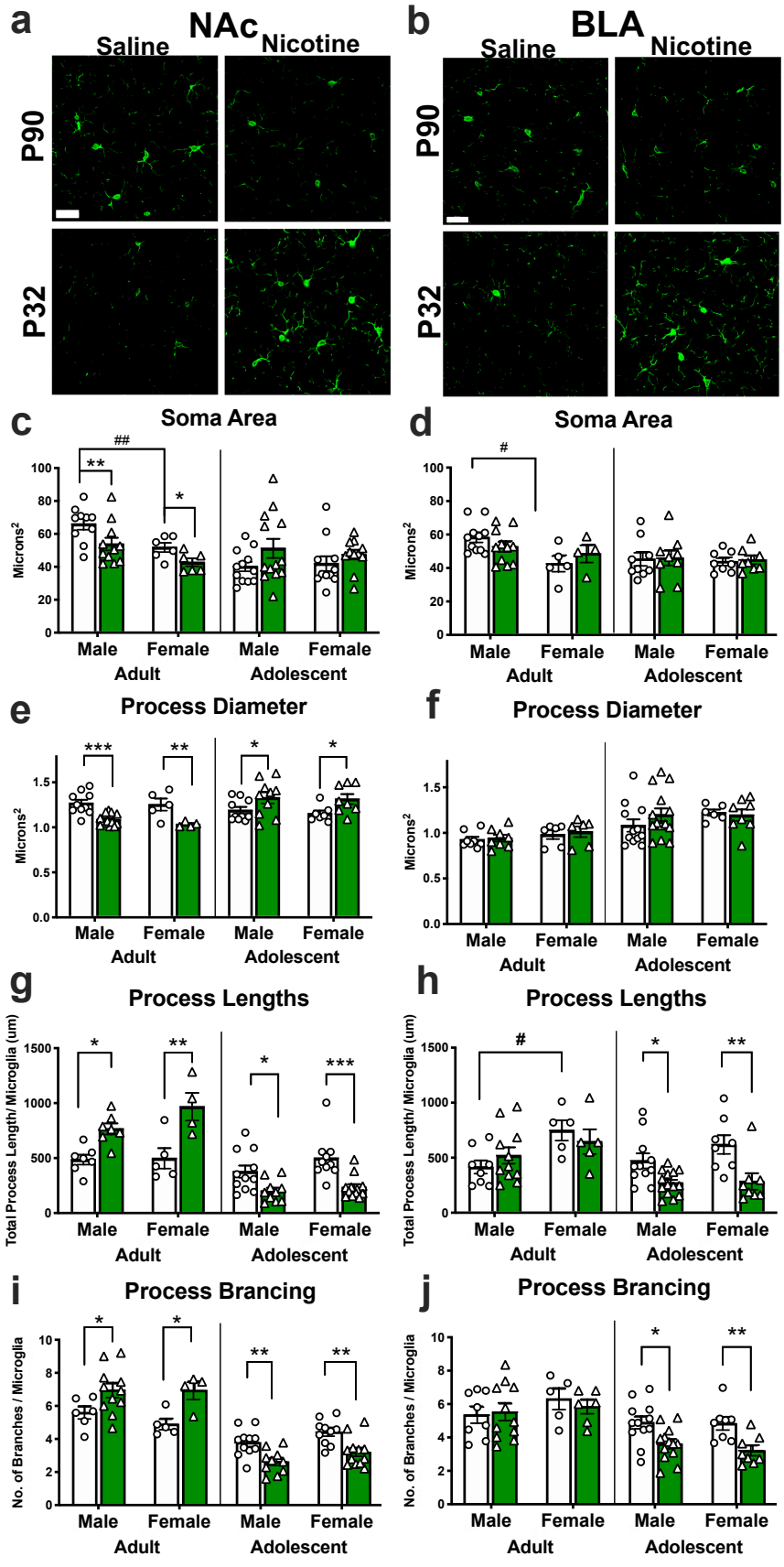

a
NAc
Saline
Nicotine
P90
P32
b
BLA
Saline
Nicotine
P90
P32
c
Soma Area
Microns²
d
Soma Area
Microns²
e
Process Diameter
Microns²
f
Process Diameter
Microns²
g
Process Lengths
Total Process Length/ Microglia (um)
h
Process Lengths
Total Process Length/ Microglia (um)
i
Process Brancing
No. of Branches / Microglia
j
Process Brancing
No. of Branches / Microglia
Male
Female
Male
Female
Adult
Adolescent

Fig 2.6: Nicotine promotes ramified adult microglial morphology, and reactive adolescent microglial morphology. **a,b**, Example confocal images of adult and adolescent NAc (**a**) and BLA (**b**). **c**, In adult soma area there was a significant effect of sex (F(1,29)=14.58, p=0.0007) and nicotine (F(1,29)=16.67, p=0.0003) (n=6 adult females, 10 adult male saline, 11 adult male nicotine). There were no significant effects of sex or nicotine on adolescent soma area (n=11 adol. female, 12 adol. male saline, 13 adol. male nicotine). **d,** Adult male BLA were significantly larger than adult female (F(1,27)=6.475, p=0.0170; n=5 adult female saline, 4 adult female nicotine, 11 males). There were no significant changes in BLA

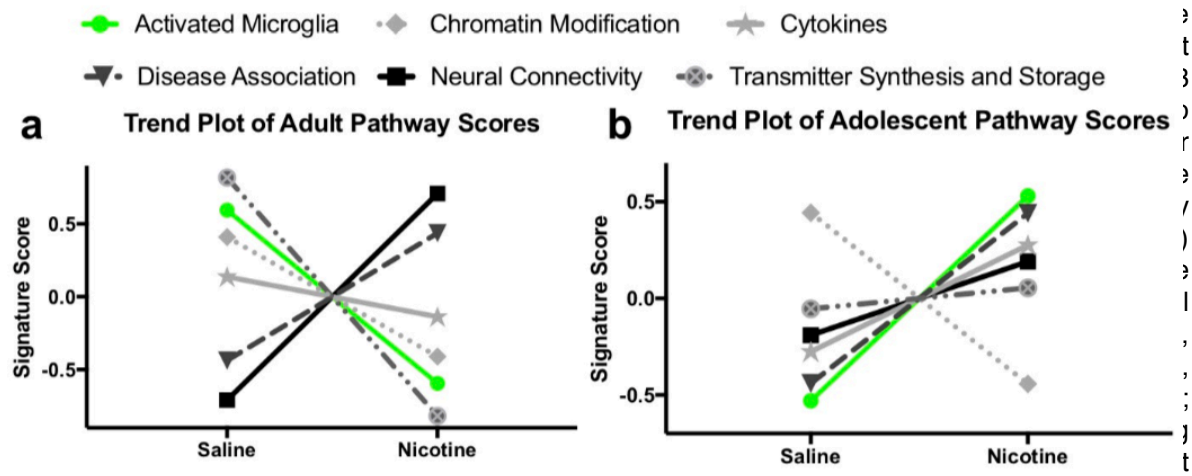


Fig 2.7: Pathway Scores derived from Nanostring multiplex gene expression in panel **a**, Adult nicotine exposure has distinct effects on pathway scores for associated with a variety of cellular functions. In particular, nicotine decreased overall expression of a pre-defined set of genes associated with microglial activation, which is shown as a lower activated microglia pathway score. **b**, Adolescent nicotine exposure increased overall expression of pre-defined genes associated with microglial activation, as well as altering other pathway scores associated with immune and neuronal function.

male nicotine) and decreased adolescent branching (F(1,29)=23.29, p<0.0001; n=10 adol. female saline, 11 adol. female nicotine, 12 adol. male saline, 10 adol. male nicotine). **i**, Nicotine did not alter adult BLA branching (n=5 adult female saline, 6 adult female nicotine, 8 adult male saline, 11 adult male nicotine), but decreased adolescent BLA branching (F(1,37)=17.84, p=0.002; n=8 adol. female, 13 adol. male). All data analyzed with a two-way ANOVA for sex and drug, with Bonferonni post hoc analysis (*=nicotine-induced differences; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). (#=sex differences, #p<0.05, ##p<0.01. Scale bar is 20µm. Bars show mean +/ s.e.m.

(Bessis et al., 2007). In the BLA, adult nicotine exposure did not alter BLA microglial morphology (**Fig. 2.6 d-j**), which is consistent with the lack of change observed in IBA1 expression (**Fig. 2.3 d,f**). In the adolescent BLA, nicotine exposure did not alter microglial process diameter (**Fig. 2.6f**), but did significantly decrease microglial process lengths (**Fig. 2.6 h**), and microglial branching (**Fig. 2.6 j**). Together, these data demonstrate that nicotine promotes reactive microglial morphology in the adolescent NAc and BLA, with a more pronounced effect on NAc microglia. This contrasts to adults, where nicotine promoted a ramified microglial phenotype in the adult NAc, but not the adult BLA.

Nicotine induces expression of anti-inflammatory transcripts in adults and pro-inflammatory transcripts in adolescents

In addition to microglial morphology, I also assessed whether nicotine induced

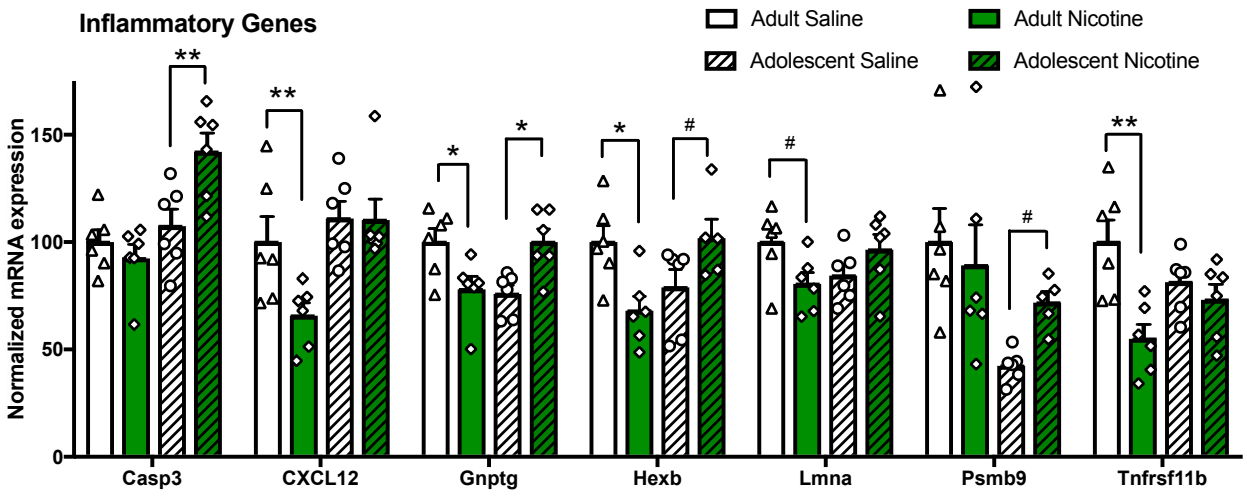


Fig. 2.8: Analysis of specific inflammatory genes, expressed as percentage of adult saline (n=6). All genes were analyzed with a two-way ANOVA for age x nicotine pretreatment. All main effects were further analyzed with Bonferonni post hoc tests (#p<0.1;*p<0.05; **p<0.01). Bars show mean +/ s.e.m.

age-specific changes in mRNA transcripts associated with microglial activation in the NAc of adult and adolescent male rats. Using a Nanostring multiplex neuropathology panel to assess changes in mRNA levels of transcripts associated with microglial activation, disease association, and neural connectivity (**Fig. 2.7-2.9),** Activated microglial pathway scores were decreased following adult nicotine exposure (**Fig. 2.7a**), but increased following adolescent nicotine exposure (**Fig. 2.7b**).

Selected transcripts associated with microglial activation are shown in **Fig. 2.8**. Nicotine exposure significantly decreased expression of microglial-associated transcripts *Gnptg* and *Hexb* in adults, while increasing the expression of these

transcripts in adolescents. Nicotine also decreased adult expression of the cytokine receptor *Tnfrs11b*, and the inflammatory chemokine *CXCL12*, and the neuroinflammatory cellular senescent marker *Lmna* with no change in adolescents (Trias et al., 2019). *Casp3*, a transcript associated with apoptosis and neuronal death, was significantly increased following adolescent nicotine exposure. Nicotine increased adolescent Psmb9, an immunoproteasome gene important for degradation if phagocytosed material within microglia (Kremer et al., 2010). Taken together, these data show that nicotine exposure has contrasting effects on adolescent and adult NAc immune activation associated transcripts.

DISCUSSION

Adolescent, but not adult, nicotine exposure increases cocaine reinforcement. Correlated with this behavioral change, is an increase microglial activation only in adolescent rats. Through extensive protein, mRNA and cellular analysis, I characterized unique age-dependent changes to IBA1 expression, activated microglia gene transcript levels, and microglial morphology after nicotine exposure.

Nicotine increased adolescent IBA1 expression and promoted a reactive microglial morphology. Nicotine thickened microglial processes, shortened process length and decreased microglial branching. These morphological changes are stereotypical of a reactive microglial state. In contrast nicotine suppressed adult IBA1 expression, promoted a ramified microglial phenotype. Nicotine reduced microglia soma size, and extended their process lengths and branching. These morphological changes indicate a more ramified state.

Nicotine did not have sex-dependent effects on cocaine responding or microglia. I did, however, observe some baseline sex differences in adult microglial morphology. I found adult male microglia are bigger than female. In addition, adult female BLA microglia have altered process morphology. This developmental delay in microglial sex differences reflects new literature (Guneykaya et al., 2018), and may indicate that pubertal brain changes influence microglia morphology and function. Whether these changes are hormonal or otherwise remains unclear. Although these microglial morphological sex differences did not contribute to the nicotine-associated behaviors studied here, they may contribute to other sex-dependent behaviors.

Nicotine increased GFAP expression only in the adolescent NAc. This robust increase in GFAP suggests astrocytes could be involved in nicotine-induced changes to reward-associated neural circuits and behaviors. In addition, the heightened S100b expression in adolescents may indicate adolescent NAc astrocytes are immature. These preliminary data highlight the need for future studies to investigate the role of astrocytes in adolescent drug associated behaviors.

My transcript analysis analyzing inflammatory and synaptic genes corroborates decades of research demonstrating nicotine is distinctly harmful to adolescents. I now provide evidence that microglia are critical regulators of these effects, as I observe contrasting effects of nicotine on adolescent and adult immune pathway scores. Notably, the transcripts *Gnptg* and *Hexb* were increased by nicotine in adolescents and decreased in adults. Both of these transcripts are associated with microglial activation, suggesting these two genes may play a role in the conflicting microglial phenotypes

observed. Importantly, this gene analysis mirrors the previous morphological data showing that nicotine induces a maladaptive activated microglial state in adolescents, while inducing a ramified, inactive microglial state in adults. Taken together, these findings extensively characterize differential changes to microglia following adolescent or adult nicotine exposure, and provides strong correlative evidence for a role for microglia in cocaine self-administration.

CHAPTER 3

Microglia increase cocaine self-administration after adolescent nicotine exposure

INTRODUCTION

Drugs of abuse significantly alter microglial marker expression, microglial activation, and inflammatory gene expression in reward encoding regions (Linker et al., 2018; Coller and Hutchinson, 2012). We have demonstrated adolescent nicotine exposure increases microglial activation, while adult nicotine exposure suppresses this activation. Decades of research has correlated reactive microglia and drug exposure, but a direct causal link between microglia and drug seeking has yet to be established (Linker et al., 2018; Lacagnina et al., 2017).

Minocycline is a broad spectrum semi-synthetic tetracycline antibiotic, that readily crosses the blood brain barrier to suppress neuroinflammation. Minocycline reduces the release of cytokines and chemokines, and has other anti-inflammatory effects including the inhibition of phospholipase A2, prostaglandin E2, oxidative stress and apoptosis, and inhibits glutamate excitotoxicity (Beardsley and Hauser, 2014; Soczynska et al., 2012). Several studies have implicated minocycline in drug-associated behaviors. Minocycline suppressed psychostimulant-induced conditioned place preference, hyperlocomotive effects and dopamine release (Fujita et al., 2012, Zhang et al., 2006). These studies provide foundational evidence that the immune system may be critical for drug-seeking.

Symbiotic bacteria in the gut contribute to microglia development, function and reactivity (Erny et al., 2015; Sampson et al., 2016). Disturbances in the gut microbiome have been associated with different body mass indices, anxiety, depression and substance use disorders (Koliada et al., 2017; Foster et al., 2017; Meckel and Kiraly, 2019). Antibiotics like minocycline alter the gut microbiome, and this may contribute to the brain and behavioral affects observed.

Interfering with the gut microbiome species with antibiotics can alter drug-associated behaviors (Meckel and Kiraly, 2019). Within the gut there are thousands of species of bacteria that are generally classified into two phyla: *Firmicutes*, which are predominantly gram-positive and *Bacteroidetes* which are gram-negative bacteria. The choice of antibiotic used affects which types of bacteria are depleted. Ampicillin depletes both gram-positive and gram-negative bacteria, while Vancomycin depletes only gram-

positive bacteria and neomycin chiefly inhibits gram-negative bacteria with partial activity against gram-positive bacteria (Heyes, 1979; Fröhlich et al., 2016). In addition, ampicillin crosses the gut and the blood brain barrier and can directly interact with the central immune system. In contrast, vancomycin and neomycin remain in the gut, and can therefore only directly interact with the peripheral immune system and microbiome (Dudley et al., 1986; Fröhlich et al., 2016). The distinct mechanism of action of these antibiotics allows the experimental dissection of the contribution of the peripheral versus central immune system, and of gram-positive versus gram-negative bacteria to drug associated behaviors.

While minocycline and ampicillin can directly suppress microglial activation, its pharmacological actions are not only through this cell type. Minocycline also directly affects astrocytes and neurons (Garwood et al., 2011; Alvarez, et al., 2011). Since minocycline is an indiscriminate drug these studies lack mechanistic specificity and do not determine if microglia alone contribute to psychostimulant associated behaviors.

In the brain, the colony-stimulating factor 1 receptor (CSF1R) is expressed only on microglia (Erblich et al., 2011; Nandi et al., 2012). CSF1R ligand, CSF1, regulates proliferation, differentiation and survival of macrophages. Mice and humans lacking CSF1R are devoid of microglia, and die before adulthood (Erblich et al., 2011; Ginhoux et al., 2010, Oosterhof et al., 2019). However, elimination of microglia after development with the CSF1R antagonist, PLX3397, has no outright, measurable effects on memory, motor or stress behaviors (Elmore et al., 2014; Elmore et al., 2015). To circumvent the

experimental confounds of minocycline, we used PLX3397, to clear microglia from the brain.

In this chapter we used minocycline or an antibiotic cocktail (containing ampicillin, vancomycin and neomycin) throughout the nicotine pretreatment, and blocked nicotine-induced increases in cocaine self-administration. However, administration of vanocymycin or neomycin alone did not. We found elimination of microglia with PLX3397 blocked nicotine-induced increases in cocaine self-administration. Together, these data demonstrate microglia are critical regulators of this increase in cocaine responding after adolescent nicotine exposure.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats were group-housed in an AAALAC-accredited vivarium on a 12 hr light/dark cycle with unlimited access to food and water. Pups were weaned on P21. All experiments were performed during the light cycle and carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine.Drugs. Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2 – 7.4; doses were calculated as free base. Cocaine HCl (Sigma, St. Louis, MO) was dissolved in sterile saline.

Catheter Implantation: Rats, aged P24/25 or P82/83, were anesthetized with equithesin (0.0035 ml/g) and surgically implanted with an indwelling catheter in their jugular vein (Belluzzi et al, 2005). Throughout the 2-3 days of recovery, catheters were flushed daily with heparinized saline solution (1000 units heparin per 30 ml bacteriostatic saline) to

maintain patency. Catheter patency was verified by testing with propofol (5 mg/kg, i.v.) after drug pretreatment, and only animals showing rapid anesthesia were included in analyses.

Pretreatment: Adolescent (P28-31) and adult (P86-89) rats received an intravenous drug pretreatment of nicotine (60 μg/kg), or saline for 4 consecutive days (McQuown et al., 2007).

Minocycline: Minocycline was administered (0.533mg/ml) in water bottles from catheterization (P24) to cocaine self-administration (P32). Standard water was

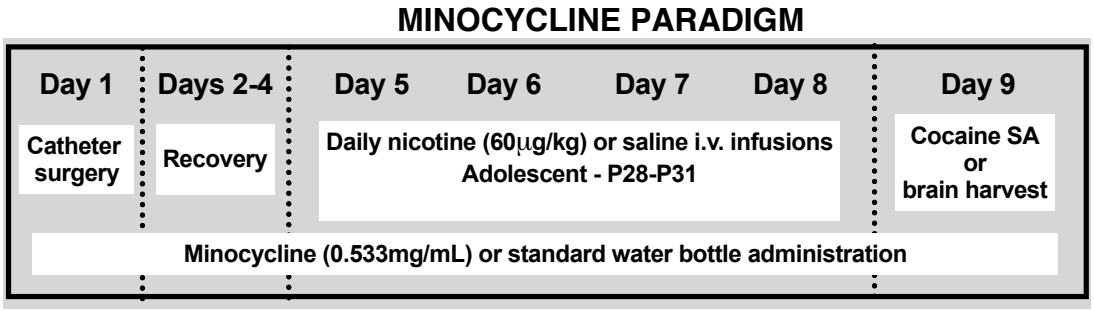


Fig. 3.1: Schematic of paradigm with nicotine pretreatment and minocycline water bottle administration

administered to control rats (**Fig 3.1**).

Antibiotic cocktail, neomycin, vancomycin: Antibiotic-cocktail treated animals were provided ampicillin (1 g/l; Sigma Aldrich, St.Louis, MO), vancomycin (0.5 g/l; Chem-Impex, Wood Dale, IL), and neomycin (100 mg/L; Chem-Impex) in drinking water for 4 days prior to nicotine/saline pretreatment. This cocktail of antibiotics was selected to significantly deplete the microbiome, and does not mirror clinical antibiotic doses. Animals given neomycin (1.5g/l) and vancomycin (1.5g/l) alone treated were treated with antibiotic for 4 days prior to nicotine/saline pretreatment.

These individual antibiotic treatments were given because they do not cross the gut (neomycin/vancomycin) or blood brain barrier (neomycin).

PLX3397: PLX3397 was administered (600ppm) from weaning (P21) to cocaine self-administration (P32). Vehicle chow was administered to control groups (**Fig. 3.2**). Both chows have equivalent nutritional profiles. Behavioral testing was conducted following the last day of pretreatment on P32.

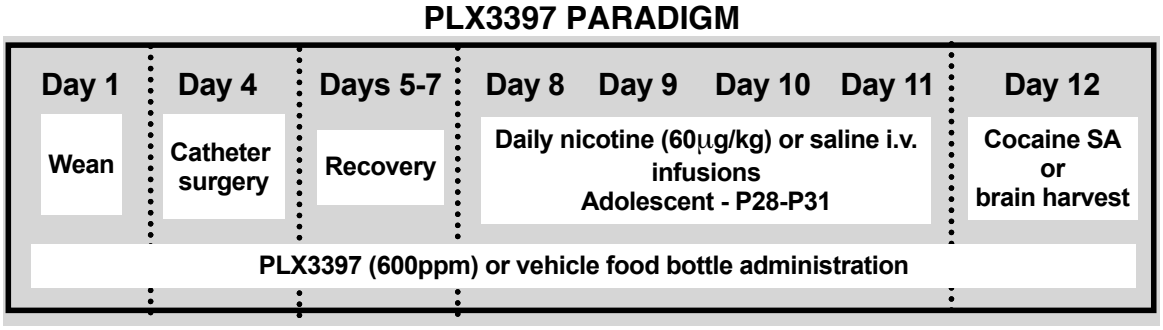


Fig. 3.2: Schematic of paradigm with nicotine pretreatment and PLX3397 chow administration

Intravenous Self-Administration: Animals were placed into an operant chamber equipped with two nose poke holes and were tested in a single 2 h session on a fixed ratio 1 schedule to deliver a 0.02 ml intravenous infusion of cocaine (0.5 mg/kg). During each 1.1s infusion, a signal light above the reinforced hole illuminated, after which the house light shut off for a 20-s time-out period. To control for nonspecific activating effects of drugs, activity on a non- reinforced hole, whose activation had no programmed consequences, was recorded. A maximum of 100 infusions was allowed.

Open Field and Light Dark Box Test: For open field tests adolescent rats were habituated for 30 minutes in their home cage prior to testing. Adolescent rats were placed into the middle of a locomotion box. Time spent in the center area of the

apparatus was measured over 30 min using an open- field activity system measuring 43.2 × 43.2 × 30.5 cm (MED Associates). For light dark box test adolescent rats were habituated for 10 minutes prior to testing. Inserts were placed into locomotor box to create a dark and light area. Animals were placed into the dark side of the chamber and activity was measured for 5 minutes.

Food Restriction: Beginning 3 days prior to food training adolescent rats were food restricted to 15g a day. Rats were weighed every day to ensure proper growth. Food was given 15 min after each experimental session, and any remaining chow was removed an hour before the next test session.

Food Acquisition Training: Adolescent rats were trained to lever-press for food pellets (45 mg rodent purified diet; Bio-Serv) under a fixed ratio 1 schedule with a 1 s timeout period (FR1TO1), followed by FR1TO10, and completed with FR1TO20. Rats progressed to the next timeout period when they earned at least 30 reinforcers in the daily 30-min session.

Immunohistochemistry: All animals were perfused according to established procedures and brains were post- fixed in 4% paraformaldehyde for 24 h to ensure proper tissue fixation. To investigate changes in protein levels, 40µm coronal sections were run through free-floating immunohistochemistry (IHC). Briefly, tissue was blocked and permeated in 0.2% Triton X-100, 5% Normal Goat Serum (NGS), in PBS. Sectioned tissue is incubated with primary antibody in incubation buffer (5% NGS, 0.2% Triton X-100 in PBS) overnight. Primary antibody used was IBA1 (1:1000; Wako-Chem; 019-

1974). Tissue was incubated with secondary antibody (goat-anti rabbit IgG Alexafluor 488, Abcam ab150077) in incubation buffer. Omission of the primary antibody was a negative control.

*Microscopy and Analysis***:** Images for absorption analysis were captured on an Olympus Scanner BX61VS and image analysis was performed using ImageJ. A Zeiss LSM 700 was used to capture images for microglial morphological analysis. Microglia morphology, cell count and soma size were analyzed by IMARIS. IHC and analysis of all experimental groups was performed in parallel. All analysis was done blind.

*Statistics***:** All data are expressed as mean ± SEM. For behavioral data, non-reinforced responses had a normal data distribution, while reinforced responses did not. This is likely due to the imposed maximum of 100 response, which creates an artificial ceiling effect. Kolmogorov-Smirnov normality test demonstrated normal distribution for all morphology measures but the BLA Soma area and NAc mean diameter. Minocycline, antibiotic and PLX3397 self-administration data were analyzed by three-way ANOVA for drug x pretreatment x response. Immunohistochemistry was analyzed with a two- way ANOVA for nicotine pretreatment x PLX3397 exposure or nicotine pretreatment x minocycline exposure. Morphological analyses were analyzed with a three-way ANOVA for age x sex x drug. All significant main or interaction effects were further analyzed with Bonferroni post hoc comparisons.

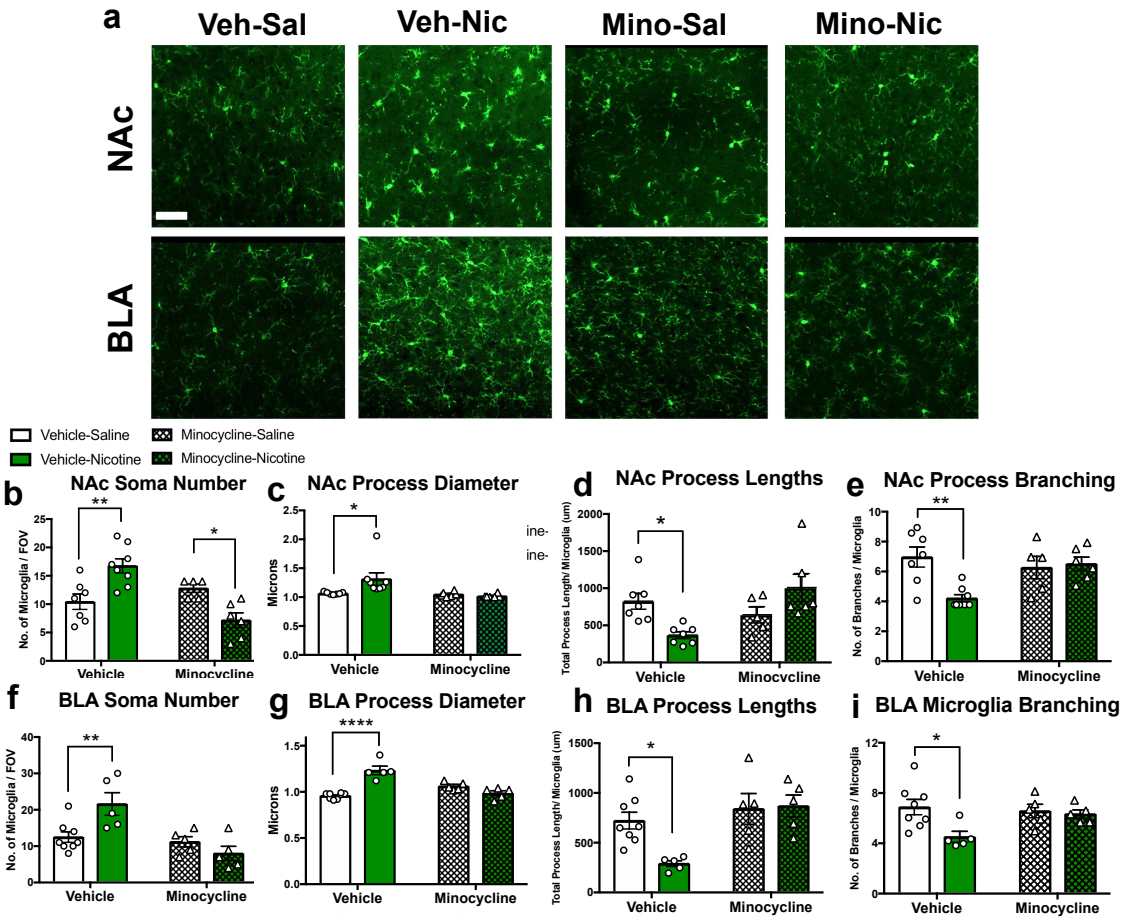


Fig 3.3: Minocycline blocks nicotine-induced increases in microglial activation **a**, Representative images of IBA1 stained microglia in the NAc and BLA of rats pretreated with saline or nicotine and vehicle or minocycline. **b**, Minocycline administration eliminated nicotine-induced increases in NAc soma number ($F(1,22)=7.95$, $p=0.0100$). There was also a significant interaction between minocycline and nicotine administration ($F(1,22)=21.84$, $p=0.0001$) (n= 7 vehicle-saline, 5 minocycline-saline, 8 vehicle-nicotine, 6 minocycline-nicotine). **c**, Nicotine increased NAc diameter ($F(1,23)=4.299$, $p=0.0495$), and this was blocked with minocycline ($F(1,23)=5.447$, $p=0.0287$) (n=6-8)) **d**, Nicotine decreased NAc process lengths, and minocycline blocked this ($F(1,21)=11.49$, $p=0.0028$) (n=5-7). **e**, Nicotine decreased NAc microglial branching ($F(1,22)=5.532$, $p=0.0280$), and minocycline blocked this decrease ($F(1,22)=7.935$, $p=0.0100$) (n=5-8). **f**, Minocycline administration eliminated nicotine-induced increases in BLA soma number ($F(1,19)=13.59$, $p=0.0016$). There was also a significant interaction between minocycline and nicotine administration ($F(1,19)=9.264$, $p=0.0067$) (n=8 vehicle-saline, 5 minocycline-saline, 5 vehicle-nicotine, 5 minocycline-nicotine). **g**, Nicotine increased BLA microglial diameter ($F(1,19)=14.75$, $p=0.0011$ and minocycline blocked this increase ($F(1,19)=7.493$, $p=0.0131$). There was an interaction between nicotine and minocycline ($F(1,19)=45.97$, $p<0.0001$) (n=5-8). **h**, Minocycline blocked nicotine-induced decreases in BLA process lengths ($F(1,19)=11.31$, $p=0.0033$). There was an interaction between nicotine and minocycline ($F(1,19)=4.937$, $p=0.0386$) (n=5-8). **i**, Nicotine decreased BLA process branching ($F(1,19)=5.446$, $p=0.0307$) and this blocked with minocycline administration (n=5-8).

RESULTS

Minocycline blocks increases in cocaine reinforcement after adolescent nicotine exposure.

Minocycline, a general neuroimmune suppressor, blocked nicotine induced increases in microglia number (**Fig. 3.3 b,f**). Minocycline also reversed changes to microglial process diameter (**Fig. 3.3 c,g**), process lengths (**Fig 3.3 d,h**) and process branching (**Fig. 3.3 e,i**). These morphology data confirm that minocycline suppressed microglial activation. Furthermore, minocycline blocked nicotine induced increases in cocaine self-administration (**Fig. 3.4**)

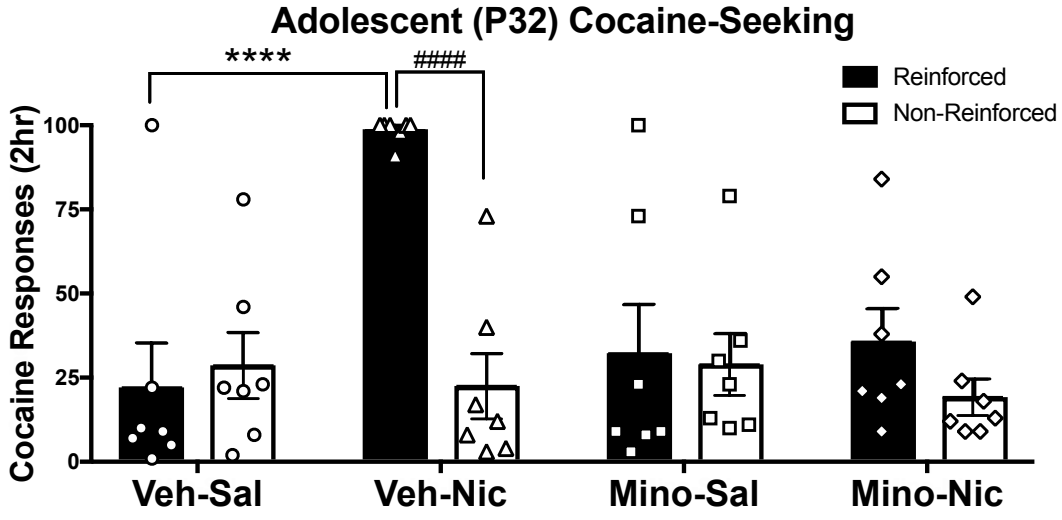


Figure 3.4: Minocycline blocked nicotine-induced increases in cocaine responding. Nicotine selectively increases cocaine responding ($F(1,48)=5.208$, $p=0.0270$) and adolescent rats discriminate between reinforced and non- reinforced responses (####$p<0.0001$). There was a significant effect of response ($F(1,48)=10.1$, $p=0.0026$), interaction between response, nicotine, and minocycline exposure ($F(1,48)=6.128$, $p=0.0169$, n=7 rats per group). Behavioral data was analyzed with Bonferonni post hoc tests (****$p<0.0001$). Bars show mean +/ s.e.m

An antibiotic cocktail blocks increases in cocaine reinforcement after adolescent nicotine exposure, but vancomycin and neomycin do not.

A previously established antibiotic-cocktail treatment (Ampicillin, Vancomycin, and Neomycin) that significantly depletes the gut microbiome (Reikvam et al., 2011, Lee et al., 2018) eliminated nicotine induced increases to cocaine self-administration (**Fig. 3.5**). Treatment with gut-impenetrable antibiotics vancomycin (targets gram-positive bacteria) and neomycin (targets gram-negative bacteria) did not affect increases in cocaine responding after adolescent nicotine exposure (**Fig. 3.6; Fig 3.7)**

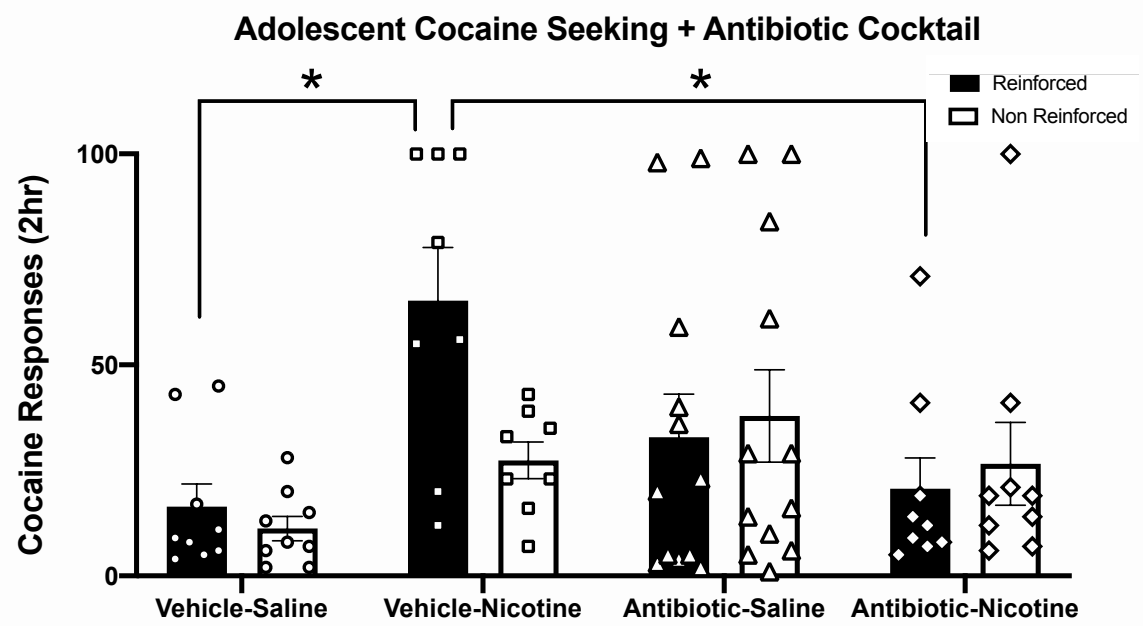


Fig. 3.5: Antibiotic cocktail blocked nicotine-induced increases in cocaine reinforcement. Nicotine significantly increased cocaine responding ($F(1,68)=11.66$, $p=0.0011$), and this was blocked with antibiotic cocktail administration ($F(1,68)=4.355$, $p=0.0406$). (n=8-12 rats). Behavioral data was analyzed with Bonferonni post hoc tests (*$p<0.05$). Bars show mean +/ s.e.m

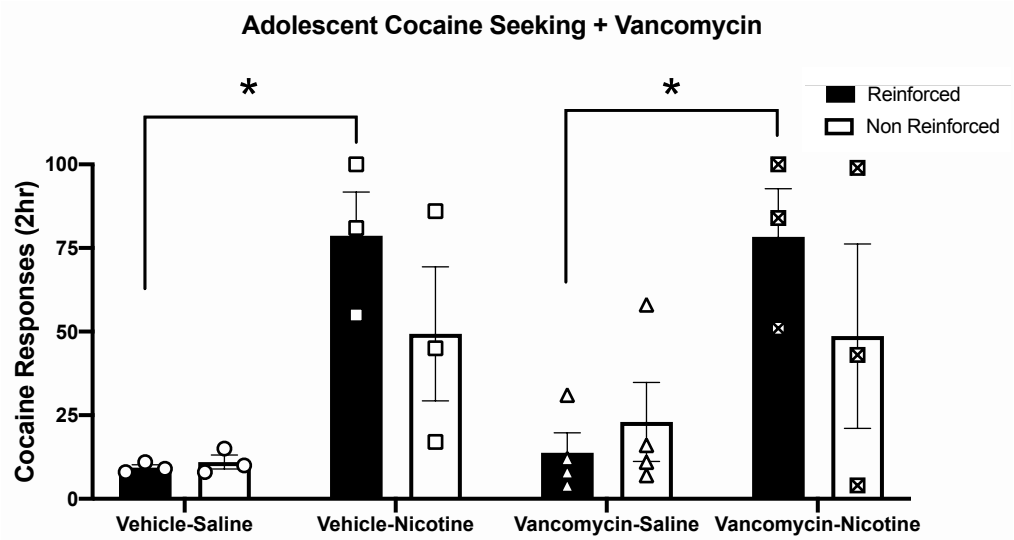


Fig. 3.6: Preliminary results suggest nicotine increased cocaine responding and this was unaffected with vancomycin administration. However, this study is underpowered and requires replication.Three-way ANOVA shows a significant effect of nicotine ($F(1,9)=38.73$, $p=0.0002$) n=3 rats) and no effect of vancomycin ($F(1,9)=0.2350$; $p=0.06394$). Behavioral data was analyzed with Bonferonni post hoc tests (*$p<0.05$). Bars show mean +/ s.e.m

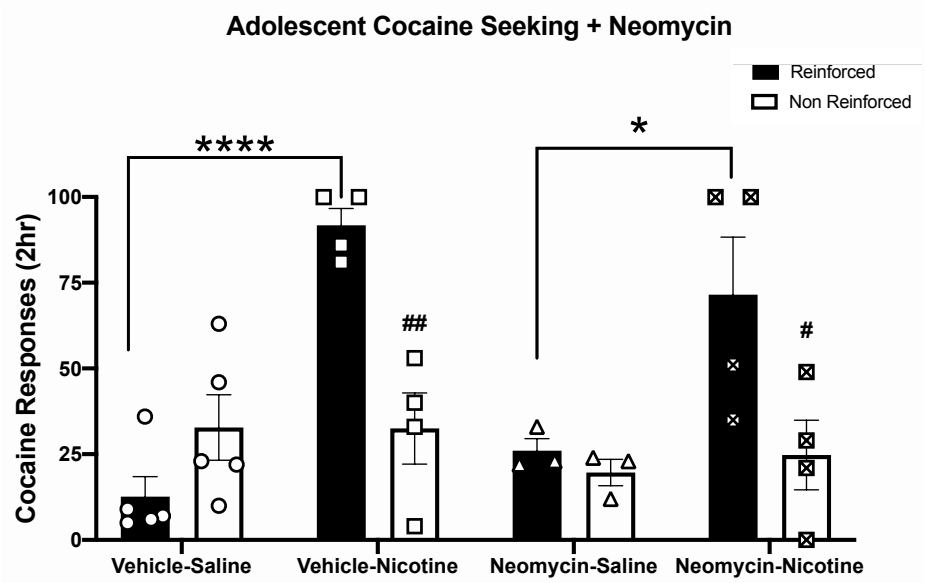


Fig. 3.7: Preliminary results suggest nicotine increased cocaine responding and this was unaffected with vancomycin administration. However, this study is underpowered and requires replication. A three way ANOVA showed a significant effect of nicotine ($F(1, 12)=20.90$; $p=0.0006$) and response ($F,12) = 11.73$; $p=0.0050$) and no significant effect of neomycin ($F(1,12) = 0.9595$, $p=0.3467$). (n= 3-5). Bars show mean +/ s.e.m

Microglia are necessary for increases in cocaine reinforcement after adolescent nicotine exposure

To directly target microglia, we used the more selective CSF1R inhibitor, PLX3397, which eliminates confounds exhibited by minocycline and other antibiotics. PLX3397 eliminated all measurable microglia from regions of interest (**Fig. 3.8 a,c,d**) . PLX also blocked increases in cocaine reinforcement, demonstrating a mechanistic role for microglia in the priming effect of adolescent nicotine on cocaine reinforcement (**Fig. 3.8b**).

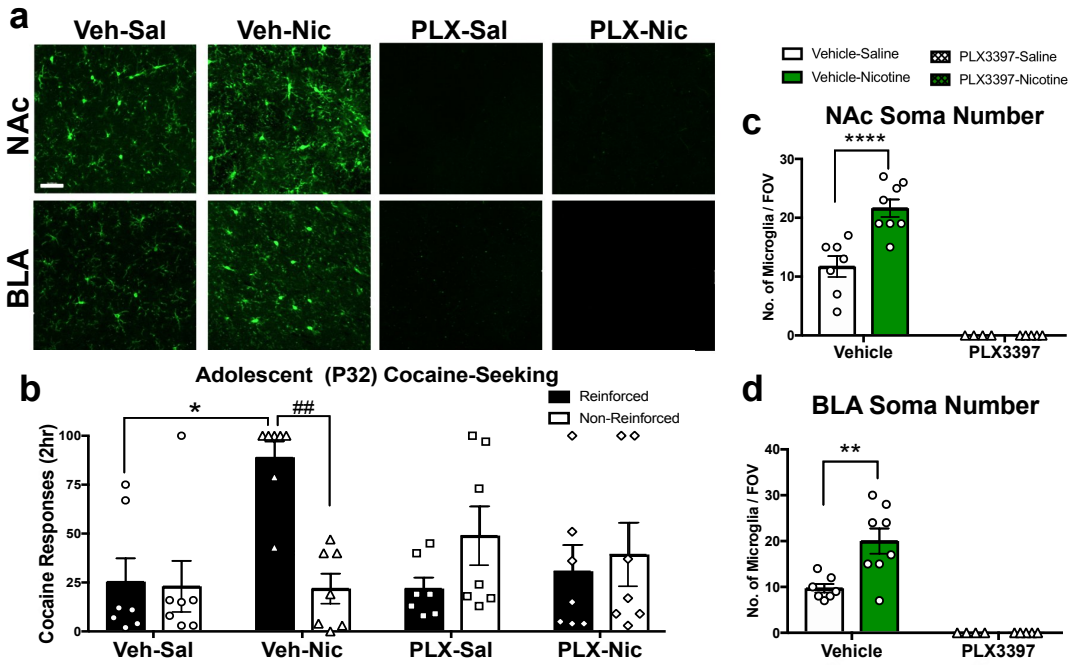


Fig. 3.8: Microglia are necessary for nicotine-induced increases in cocaine seeking, **a**, Representative images of IBA1 stained microglia in the NAc and BLA with vehicle and PLX3397 administration **b**, Nicotine selectively increases cocaine responding ($F(1,48)=6.077$, $p=0.0173$). In addition, a significant interaction between responses and PLX3397 treatment ($F(1,48)=9.725$, $p=0.0031$) was observed. Adolescent rats discriminated between reinforced and non-reinforced responses (##$p=0.0065$; n=7 rats per group).. **c**, Nicotine increased NAc soma number ($F(1,20)=10.55$, $p=0.0040$), and PLX3397 eliminated all observable microglia ($F(1,20)=119.4$, $p<0.0001$) (n=7 vehicle-saline, 8 vehicle-nicotine, 4 PLX3397-saline, 5 PLX3397-nicotine). An interaction between nicotine and PLX3397 treatment was observed ($F(1,20)=10.55$, $p=0.0040$). **d**, Nicotine increase BLA microglia soma number ($F(1,20)=6.388$,$p=0.0200$) and PLX3397 eliminated measurable BLA microglia ($F(1,20)=53.31$,$p<0.0001$). An interaction between nicotine and PLX3397 was observed ($F(1,20)=6.388$,$p=0.0200$) (n=7 vehicle-saline, 8 vehicle-nicotine, 4 PLX3397-saline, 5 PLX3397-nicotine). Behavioral and cellular data was analyzed with Bonferonni post hoc tests (##$p<0.01$; *$p<0.05$; **$p<0.01$). Bars show mean +/ s.e.m.

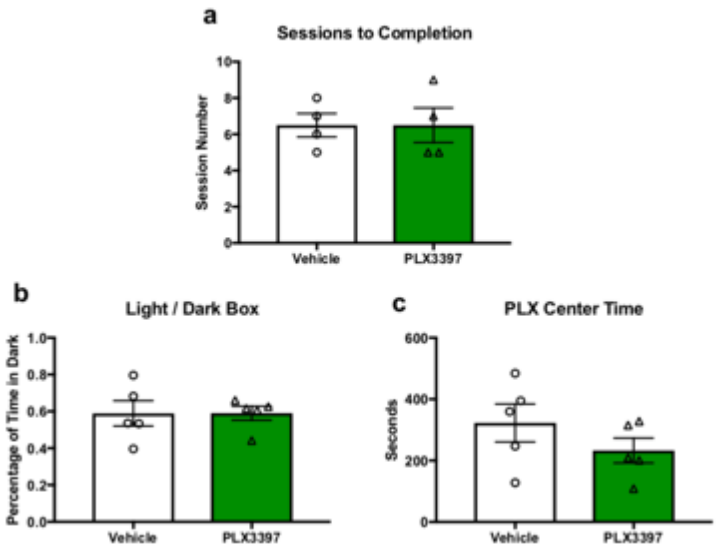


Fig 3.9: PLX3397 did not affect reward or stress behaviors. PLX3397 was administered at least 7 days prior to each behavioral test to ensure clearance of microglia (see Fig. 4). **a,** PLX3397 chow administration did alter food training acquisition. PLX3397 did not alter number of sessions to completion of the food-training paradigm (t_6=0, p>0.9999; n=4). **b**, PLX3397 did not effect light dark box performance (t_8=0.01526, p=0.9882; n=5). **c**, PLX3397 did not significantly change rat center time t_8=1.22, p=0.2573; n=5).

Whereas it has previously been reported that PLX3397 has no negative effects on behavior (Elmore et al., 2014), this has not been studied in adolescents. In a small battery of tests, we found that PLX3397 had no significant effects on adolescent rat reward learning (**Fig. 3.9a**). In addition, PLX and vehicle treated rats did not significantly differ in performance on either the light dark box or center time behavioral tasks (**Fig 3.9b,c**).

DISCUSSION

Adolescent rat nicotine exposure increases cocaine reinforcement, and microglia are necessary for this phenomenon. While decades of research have correlated drug exposure with microglial activation, it was unclear if this activation is instrumental to drug seeking. I have now identified microglia as a novel, yet essential, regulator of

nicotine-induced increases in cocaine-seeking. These data provide a causal link between microglia and drug-seeking and corroborates the correlative evidence from the previous chapter.

Prior literature has demonstrated that minocycline can block rewarding aspects of drugs of abuse, and our data replicates this. In addition, other antibiotic schedules that disturb the gut microbiome can alter psychostimulant reward (Meckel and Kiraly, 2019). An antibiotic cocktail (ampicillin, vancomycin and neomycin) blocked nicotine induced increase to cocaine reinforcement. However, vancomycin and neomycin alone do not. Although this study is underpowered it may highlight the importance of antibiotic choice in studies which investigate the microbiome. Ampicillin crosses the gut and the blood brain barrier and depletes both gram-negative and gram-positive gut bacteria. Vancomycin and neomycin do not cross the gut, and together target both gram-negative (Neomycin) and gram-positive (vancomycin) bacteria. Together these data suggest that antibiotic blockade of cocaine reinforcement is through a direct suppression of the central immune system (ampicillin and minocycline) rather than a disturbance of the gut microbiome. However, future studies must confirm through cellular and transcriptional analysis that ampicillin also blocks nicotine-induced increases to microglial activation. Still, it is important to highlight this behavioral distinction as many studies that investigate the microbiome, use antibiotic which cross the gut and blood brain barrier. My data emphasize that any cellular or behavioral effect should only be attributed to the microbiome if gut-impenetrable antibiotics are used.

Our antibiotic data demonstrate the central immune system is critical for the priming

effect of nicotine on cocaine reinforcement. However, antibiotics are indiscriminate drugs with many effects outside of microglia. It was therefore unclear if microglia are directly involved in reward-associated behaviors. We removed these confounds by eliminating microglia in the CNS with PLX3397. These results unequivocally demonstrate the critical role of microglia in nicotine-induced increases in cocaine reinforcement. Notably PLX- and vehicle-treated rats did not differ in natural reward learning, demonstrating that microglia are primarily involved in drug-associated behaviors. PLX administration also did not alter anxiety-like behavior or overall rat health. These data highlight microglia as a promising therapeutic target for substance use disorders. In future studies the role of microglia should be investigated in association with other addiction-like behaviors, such as dependence, reinstatement and craving.

Evidence for the therapeutic significance of immune suppressant drugs for addiction remains preliminary. In humans, minocycline inhibited reports of rewarding/ “high” feelings of the psychostimulant d-amphetamine. However, minocycline did not alter their choice behavior for this psychostimulant in a progressive ratio task. There were key experimental flaws in this study, (i.e. subjects used were not addicts), but it did identify minocycline as a potential therapeutic for addiction that should be explored further. PLX3397 has not been tested in human addicts. It has, however, recently passed phase 3 clinical trials for Pigmented Villonodular Synovitis, demonstrating safe use for humans. Future studies should examine if clinical doses of PLX3397 in rodents alter drug-associated behaviors.

Since the introduction of e-cigs, adolescent nicotine use is increasing at an exorbitant rate. My data demonstrate suppressing microglia could be a viable therapy for the adverse health consequences that follow adolescent nicotine use.

CHAPTER 4

D2 receptors are a mechanistic interface between nicotine-induced changes to microglia and cocaine reinforcement

INTRODUCTION

Adolescence is the final developmental stage which has distinctive changes to dopaminergic circuitry**.** Dopamine D2 receptors, in particular, are functionally immature during adolescence (Benoit-Marand, M. & O'Donnell, P. 2008), and become sensitized after adolescent nicotine exposure (Dao et al., 2011). In addition, administration of a D2 receptor antagonist during cocaine self-administration, blocked nicotine pretreatment-induced increases in responding (Dao et al., 2011). Still, it remains unclear if blockade of D2 receptors during the nicotine pretreatment, but not cocaine self-administration, will inhibit increases in responding.

Recent literature demonstrates that microglia are especially sensitive to D2 receptor activation. Systemic administration of quinpirole (a D2 receptor agonist) increased inflammatory cytokine release from microglia cells, while D1 and D3 receptor agonists did not (Lewitus et al., 2016). However, this finding does not determine if the inflammatory effect of quinpirole is through microglial D2 receptors, or another cell type. In culture, microglia express D2 receptors. In contrast, sequencing data has failed to identify D2 receptors within isolated microglia (Hammond et al., 2019). Still, the expression of D2 receptors in the adolescent NAc or BLA microglia has yet to be determined.

In this chapter, I used a combination of approaches to determine whether D2 receptors on neurons or glia play a mechanistic role in nicotine-induced increases in cocaine reinforcement in adolescents.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats were group-housed in an AAALAC-accredited vivarium on a 12 hr light/dark cycle with unlimited access to food and water. Pups were weaned on P21. All experiments were performed during the light cycle and carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine.Drugs. Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2 – 7.4; doses were calculated as free base. Cocaine HCl (Sigma, St. Louis, MO) was dissolved in sterile saline.

Catheter Implantation: Rats, aged P24/25 or P82/83, were anesthetized with equithesin (0.0035 ml/g) and surgically implanted with an indwelling catheter in their jugular vein (Belluzzi et al, 2005). Throughout the 2-3 days of recovery, catheters were flushed daily with heparinized saline solution (1000 units heparin per 30 ml bacteriostatic saline) to maintain patency. Catheter patency was verified by testing with propofol (5 mg/kg, i.v.) after drug pretreatment, and only animals showing rapid anesthesia were included in analyses.

Stereotaxic injections: Rats aged P24/25 were anesthetized with equithesin (0.0035 ml/g) and sterotaxically injected in the NAc (+2.1AP, +/-0.5 ML, -6.9DV). One microliter of the control (CCTCTTACCTCAGTTACAATTTATA) or D2 targeted

(TTCCAGCTCCCGCCGCATCCAC) morpholino was injected at a rate of 0.2 µl/min at a concentration of 2µM. After injection, bilateral cannulas remained in the injection site for 10 minutes. Cannulas were then pulled up at a rate of 1.4 mm/min. A morpholino was chosen for RNAi as it induces minimal glial inflammation and induces mRNA suppression within 72 hours. This rapid RNAi allows us to avoid a second surgery which would cause additional inflammatory responses and stress to the animal. .We verified RNAi placement by measure D2 receptor protein density in the targeted area (NAc) with immunohistochemistry. All animals had sufficient knockdown (30% or greater) except for 1 rat, who was removed from the study.

Pretreatment: Adolescent (P28-31) and adult (P86-89) rats received an intravenous pretreatment of nicotine (60 µg/kg), or saline for 4 consecutive days (McQuown et al., 2007). The D2 receptor antagonist raclopride (0.5mg/kg/iv) or vehicle was administered 20 minutes prior to each saline or nicotine infusion (P28-P31). Behavioral testing was conducted following the last day of pretreatment on P32.

Intravenous Self-Administration: Animals were placed into an operant chamber equipped with two nose poke holes and were tested in a single 2 h session on a fixed ratio 1 schedule to deliver a 0.02 ml intravenous infusion of cocaine (0.5 mg/kg). During each 1.1s infusion, a signal light above the reinforced hole illuminated, after which the house light shut off for a 20-s time-out period. To control for nonspecific activating effects of drugs, activity on a non- reinforced hole, whose activation had no programmed consequences, was recorded. A maximum of 100 infusions was allowed.

Immunohistochemistry: All animals were perfused immediately following behavior according to established procedures and brains were post- fixed in 4% paraformaldehyde for 24 h to ensure proper tissue fixation. To investigate changes in protein levels, 40µm coronal sections were run through free-floating immunohistochemistry (IHC). Briefly, tissue was blocked and permeated in 0.2% Triton X-100, 5% Normal Goat Serum (NGS), in PBS. Sectioned tissue is incubated with primary antibody in incubation buffer (5% NGS, 0.2% Triton X-100 in PBS) overnight. Primary antibodies used were IBA1 (1:1000; Wako-Chem; 019-1974), DRD2 (1:500; Merk Millipore AB508P). Tissue was incubated with secondary antibody (goat-anti rabbit IgG Alexa fluor 488, Abcam ab150077) in incubation buffer. Omission of the primary antibody was a negative control.

RNAscope: RNAscope version 2 kit was used (ACD Inc). Animals were perfused post fixed for 72 hours in 4% paraformaldehyde. Manufacturers instructions were followed.

*Microscopy and Analysis***:** Images for absorption analysis were captured on an Olympus Scanner BX61VS and image analysis was performed using ImageJ. A Zeiss LSM 700 was used to capture images for microglial morphological analysis and D2 receptor protein density. Microglia morphology, cell count and soma size were analyzed by IMARIS. Immunohistochemistry and analysis of all experimental groups were performed in parallel. All analysis was done blind.

*Statistics***:** All data are expressed as mean ± SEM. Data distribution was assumed to be normal, with similar variability among groups. Raclopride and D2 knockdown self-

administration data were analyzed by three-way ANOVA for nicotine x raclopride/D2morpholino x response. Immunohistochemistry data was analyzed with a two- way ANOVA for nicotine pretreatment x raclopride exposure. D2 colocalization with immunohistochemistry were analyzed with a one-way ANOVA. RNAscope data were analyzed with a t-test. All significant main or interaction effects were further analyzed with Bonferonni post hoc analysis.

RESULTS

Raclopride blocks nicotine-induced increases in microglial activation and cocaine reinforcement

While previous work indicates D2 receptors are functionally immature during adolescence (Dao et al., 2012), it is unclear if adolescent nicotine exposure alters receptor density. We used immunohistochemistry and Nanostring mRNA analysis to determine that D2 receptor density is increased after adolescent nicotine exposure (Fig.

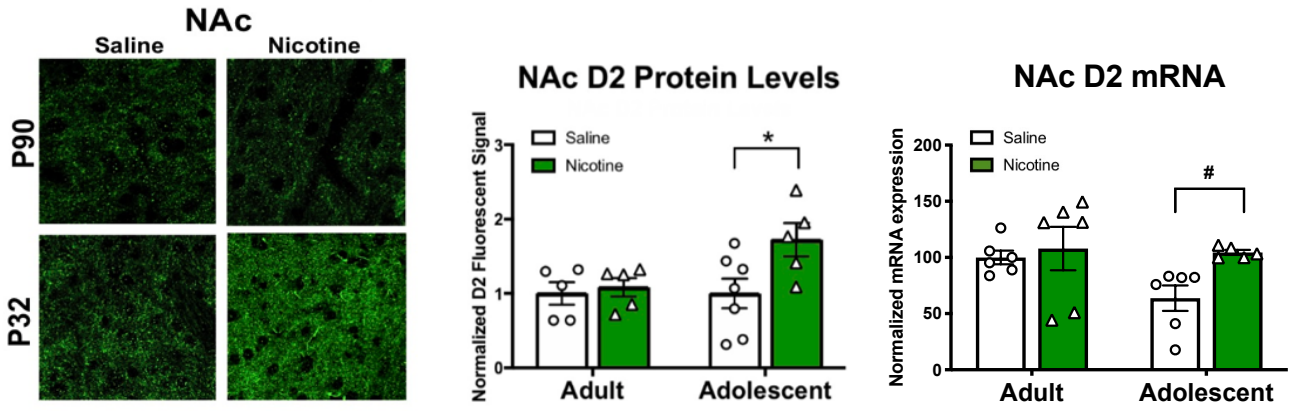


Figure 4.1: D2 Receptor protein and mRNA levels are increased after adolescent nicotine exposure **a**, Representative images of D2 receptors in the adolescent and adult NAc. **b**, Quantification of D2 receptor fluorescent intensity. A significant effect of nicotine ($F(1,18)=4.598$, $p=0.0459$; $\eta 2=16.40\%$; n=5 adult, 7 adol. saline, 5 adol. nicotine) was observed. **c**, Normalized DRD2 mRNA levels from Nanostring panel (($F(1,19)= 3.944$, $p=0.0616$; n=6 adult, n= 6 adolescent saline, 5 adolescent nicotine). Each gene or protein was analyzed with a two- way ANOVA and Bonferonni post hoc tests (#p=.06;;*p<0.05; **p<0.01). Bars show mean +/ s.e.m.

4.1).

This correlative data suggested that D2 receptors might be important for nicotine-induced changes to adolescent behavior and microglia. To investigate the role of D2 receptors in nicotine-induced increase in cocaine reinforcement and microglia activation, the D2 receptor antagonist, raclopride, was administered during nicotine pretreatment, 20 minutes prior to each injection (Fig 4.2a). Importantly, raclopride was not on board during the cocaine self-administration session or brain harvesting.

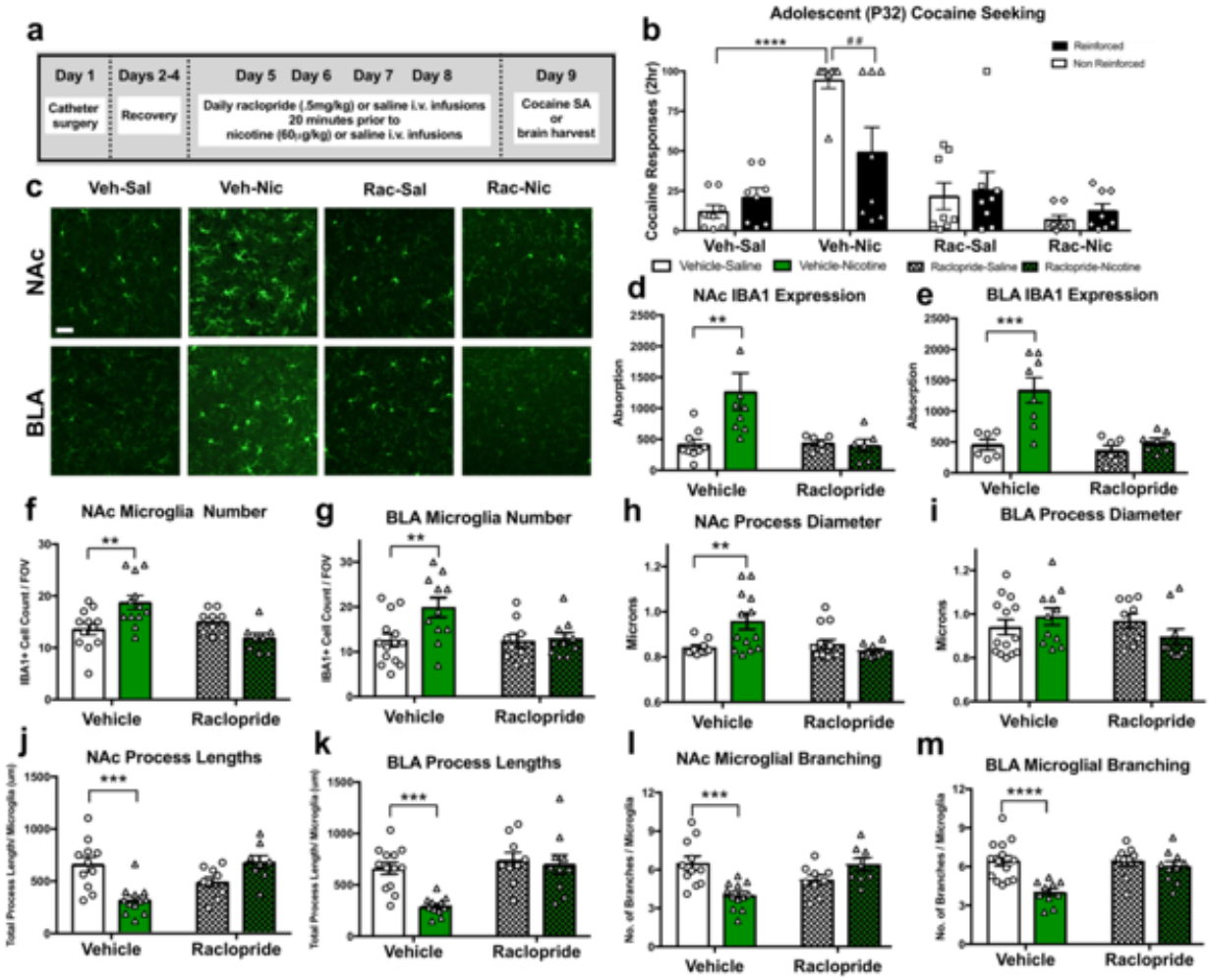


Fig 4.2: Nicotine-induced increases in cocaine seeking and microglial activation are blocked with raclopride administration. **a**, Schematic of paradigm with nicotine pretreatment and raclopride administration. **b**, Adolescent nicotine exposure significantly increased cocaine responding ($F(1,56)=12.84$, $p=0.0007$) with an effect of raclopride treatment ($F(1,56)=22.43$, $p<0.0001$). There was also a significant interaction between, response and nicotine treatment ($F(1,56)=5.146$, $p=0.0272$), nicotine and raclopride treatment ($F(1,56)=35.58$, $p<0.0001$) and response, nicotine and raclopride treatment ($F(1,56)=5.802$, $p=0.0193$). Nicotine-Vehicle treated adolescents distinguished between reinforced and non-reinforced responses (##$p=0.0073$) (n=9-10), and raclopride significantly blocked nicotine-induced increases in cocaine reinforced response **c**, Representative images of IBA1 expression after saline-saline, saline-nicotine, raclopride-saline and raclopride-nicotine exposure. **d**, Nicotine significantly increased NAc IBA1 expression ($F(1,26)=4.154$, $p=0.0518$) and this was blocked with raclopride administration ($F(1,26)=4.479$, $p=0.0440$). We observed a significant interaction between nicotine and raclopride ($F(1,26) =4.991$, $p=0.0343$) (n=6-9). **e**, Nicotine significantly increased BLA IBA1 expression ($F(1,23)=13.79$, $p=0.0011$) and raclopride eliminated this increase ($F(1,23)=7.185$, $p=0.0134$) (n=6-8). **f**, Nicotine increased NAc microglial cell count increased and this was blocked with raclopride administration ($F(1,37)=5.473$, $p=0.0248$). A significant interaction between nicotine and raclopride ($F(1,37)=12.63$, $p=0.0011$) was observed (n=9-12). **g**, Raclopride blocked ($F(1,40)=4.292$, $p=0.0448$) increases in BLA microglial cell count after nicotine exposure ($F(1,40)=5.331$, $p=0.0262$) (n=9-12). **h**, Raclopride blocks nicotine-induced increases adolescent NAc process diameter ($F(1,44)=6.051$, $p=0.0179$). A significant interaction between raclopride and nicotine ($F(1,44)=9.377$, $p=0.0037$) was found (n=10-14). **i**, No significant effects of raclopride or nicotine were found in the BLA process diameter (n=9-14). **j**, Nicotine exposure decreased adolescent NAc microglial process lengths and raclopride blocked this decrease. We found a significant interaction between raclopride and nicotine ($F(1,36)=21.54$, $p<0.0001$; n=9-13). **k**, Raclopride blocks nicotine-induced decreases adolescent BLA microglial process lengths. We observed a significant effect of nicotine ($F(1,39)=9.432$, $p=0.0039$), raclopride ($F(1,39)=13.19$, $p=0.0008$) and interaction between nicotine and raclopride ($F(1,39)=6.24$,$p=0.0168$) (n=9-13). **l,** Raclopride blocked nicotine-induced decreases in microglial branching ($F(1,36)=17.47$, $p=0.0002$, n=9-12) **m**, Raclopride blocked decreases adolescent BLA microglial branching after nicotine exposure. A significant effect of nicotine ($F(1,39)=13.08$, $p=0.0008$), raclopride ($F(1,39)=6,564$, $p=0.0144$), and interaction between nicotine and raclopride ($F(1,39)=6.609$, $p=0.0141$) (n=9-14). All behavioral data analyzed with a three-way ANOVA and all morphology data analyzed with a two-way ANOVA, both Bonferonni post hoc analysis (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Scale bar is 50μm. Bars show mean +/ s.e.m.

D2 receptor blockade with raclopride eliminated nicotine-induced increases in cocaine reinforcement (**Fig. 4.2b**). D2 receptor activity is also required for nicotine-induced increases in microglial activation after adolescent nicotine exposure. In both the NAc and BLA, raclopride suppressed nicotine induced increases in IBA1 expression (**Fig 4.2c,d,e**) and microglia count (**Fig 4.2f,g**). D2 receptor blockade also significantly blocked nicotine-induced increases in microglial process diameter (**Fig. 4.2h**), decreases in process lengths (**Fig. 4.2j**), and decreases in microglial branching in the adolescent NAc (**Fig. 4.2l**). In the adolescent BLA, raclopride also significantly blocked nicotine-induced decreases in microglial process lengths (**Fig. 4.2k**), and microglial branching (**Fig. 4.2m**). These morphological differences demonstrate that D2 receptors mediate microglial activation after nicotine exposure. Furthermore, the behavioral and

cellular data together reveal D2 receptors as a critical interface for nicotine-induced changes to adolescent cocaine use and microglia. However, it is unclear if these D2 receptors are on microglia or neurons.

Adolescent NAc and BLA microglia do not express D2 receptors

While our data and others demonstrate microglia are particularly sensitive to systemic D2 receptor activation (Lewitus et al., 2016), it is unclear if these cells express D2 receptors in vivo in the NAc or BLA. Cultured microglia express functional D2 receptors (Pocock and Kettenmann 2007). However, recent single cell sequence studies indicate microglia isolated from the brain do not (Hammond et al., 2019). To our knowledge, no study has investigated if D2 receptors are expressed in adolescent NAc or BLA microglia. To determine this, we used both immunohistochemistry (**Fig. 4.3a**) and RNAscope (**Fig. 4.3b**) to visualize D2 receptor colocalization. Our immunohistochemical analysis reveals the microglia marker Cd11b and D2 receptors have minimal colocalization compared to Neurotrace and D2 receptors, and the astrocyte marker GFAP and D2 (**Fig. 4.3a**). Our mRNA analysis corroborates this, showing little to no colocalization of D2 receptors on microglia (**Fig. 4.3**).

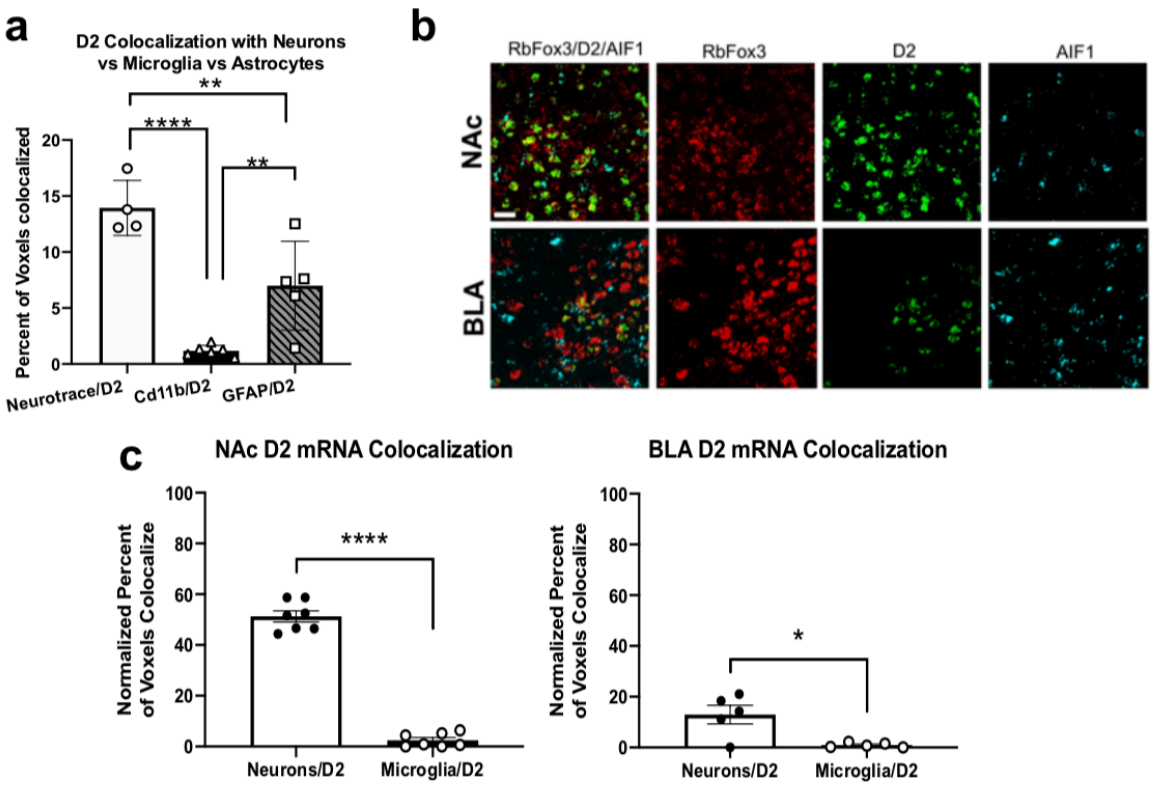


Fig. 4.3 Neurons express D2 receptors while microglia do not**. a,** Within the NAc, microglia have little to no D2 receptors compared to neurons and astrocytes. Percent of voxels colocalized with D2 receptors is highest in neurons, with minimal colocalization with cd11b ($F(2,12)=28.57$, $p<0.0001$) (n=4-6). **b**, Representative image of NAc / BLA RNAscope for visualization of neurons (RbFox3), D2, and microglia (AIF1). For analysis of RNAscope data, voxels colocalized were normalized to total D2 voxels per field of view. **c**, Percent of voxels colocalized between D2 receptors, microglia (AIF1) or neurons (Rbfox3) was normalized to total D2+ voxels. Within the NAc little to no D2 receptors were expressed in microglia, with ~50% of neurons expressing D2 receptors ($t12=20.06$, $p<0.0001$) (n=7 neurons/D2, n=7 microglia/D2) **d**, Within the BLA little to no D2 receptors were expressed on microglia and ~20% of neurons expressed D2 receptors ($t8=3.269$, $p=0.0114$) (n=5 neurons/D2, n=5 microglia/D2). Immunohistochemistry data (panel a) analyzed with bonferroni post hoc tests ((*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Scale bar is 20 µm. Bars show mean +/- s.e.m.

Neuronal NAc D2 receptors are required for nicotine-induced increases to cocaine self-administration and microglia

To determine if neuronal D2 receptors are required for nicotine-induced increases in cocaine reinforcement and microglial activation, we used a vivo-morpholino

for rapid D2 RNA interference (RNAi). I confirmed knockdown with immunohistochemistry (**Fig. 4.5a**). Although microglia do not express D2 receptors, astrocytes do (**Fig. 4.3a**). Under our injection parameters, vivo-morpholinos are unable to interfere with astrocyte mRNA in any measurable way (Reissner et al., 2012) and we confirmed this (**Fig. 4.5b,c**).

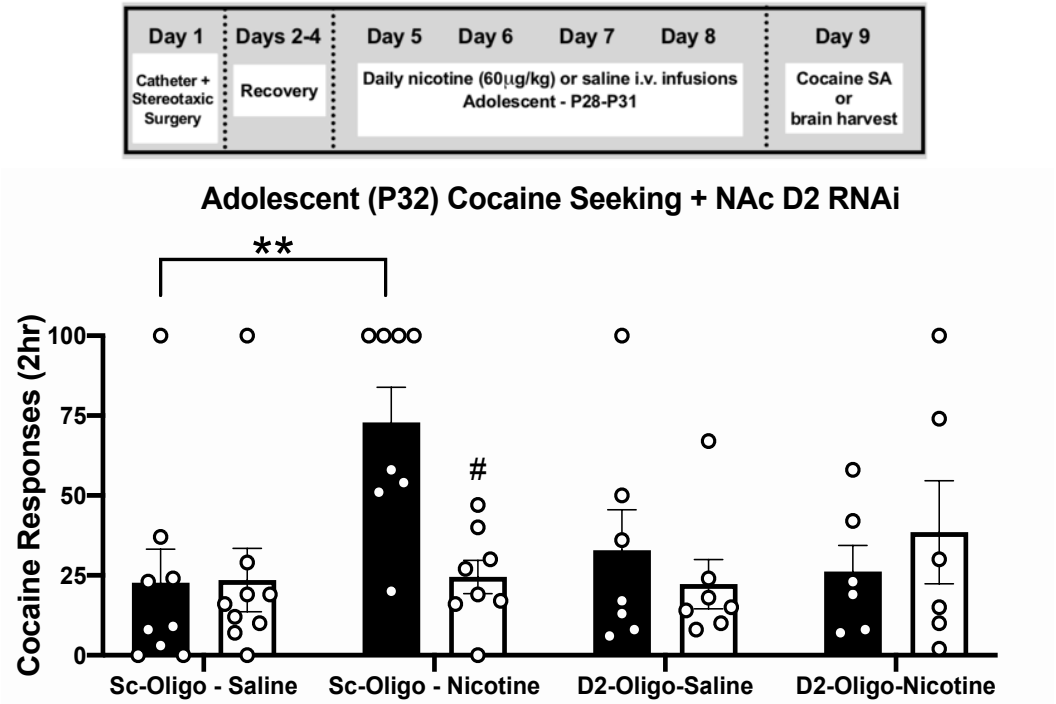


Fig. 4.4:.RNAi of D2 receptors blocks nicotine-induced increases in cocaine-seeking **a**, Schematic of paradigm with nicotine pretreatment and D2 targeted morpholino stereotaxically injected into the NAc. **b**, Adolescent nicotine exposure significantly increased cocaine responding and with significant interaction between nicotine, D2-RNAi and response ($F(1,26)=6.195$, $p=0.0195$; $\eta 2=8.101\%$) Nicotine-Vehicle treated adolescents distinguished between reinforced and non-reinforced responses (#$p=0.0075$) (n=9 scramble-saline, 8 scrambled-nicotine, 7 D2-targeted-saline, 7 D2-targed-nicotine). Data analyzed with Bonferroni post hoc tests (**$p<0.01$). Bars show mean +/- s.e.m.

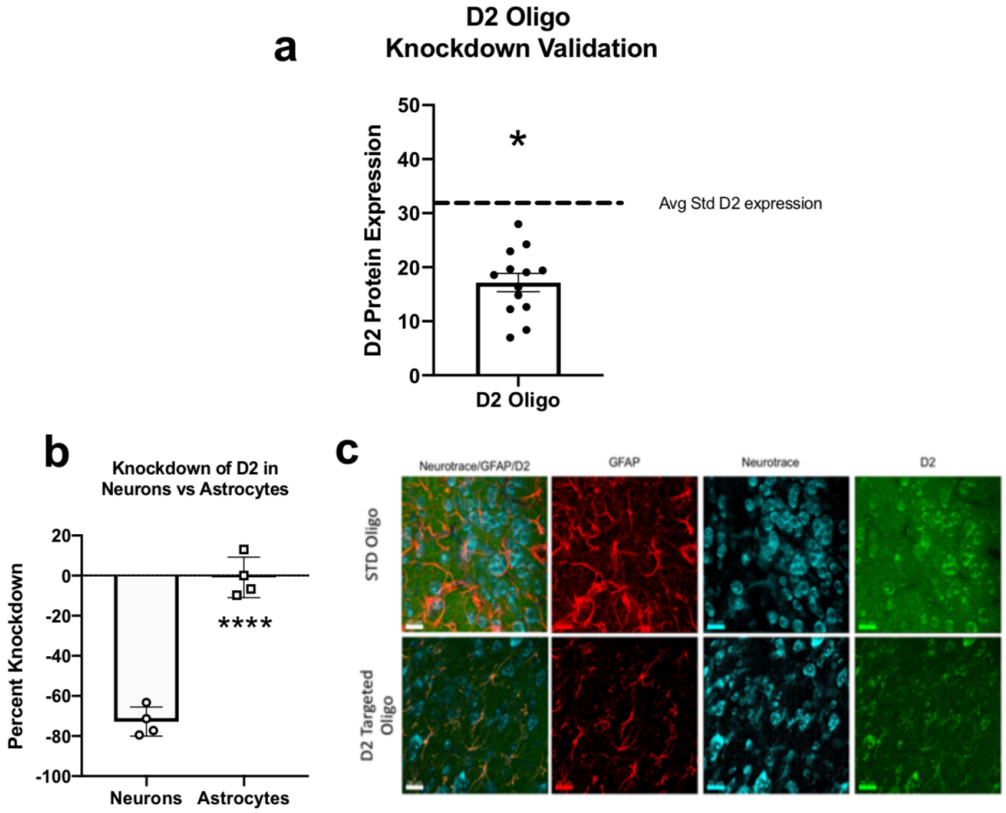


Fig. 4.5 Validation of D2 knockdown and demonstration that knockdown of D2 receptors is predominantly in neurons. **a**, Validation of knockdown with D2 receptor protein knockdown. **b**, Percent knockdown in astrocytes compared to neurons. Percent knockdown determined by (1-Percent decrease in voxels colocalized) compared to sc-oligo treatment. Decrease in voxels colocalized was significantly higher in neurons compared to astrocytes (t_6=11.59, $p<0.0001$, n=4) **c**, Representative immunohistochemistry images of colocalization between Neurotrace, GFAP, and D2. Bonferroni post hoc tests (****$p<0.0001$). Scale bar is 20 µm. Bars show mean +/- s.e.m.

RNAi of predominantly neuronal NAc D2-receptors morpholino inhibited nicotine-induced increases to cocaine self-administration, as compared to scrambled oligo controls (**Fig. 4.4**). In addition, NAc neuronal D2-RNAi blocked nicotine-induced increases in microglial activation. Knockdown of D2 receptors blocked increases in NAc microglia number (**Fig. 4.6b**), process thickness (**Fig. 4.6c**), and decreases in process lengths (**Fig. 4.6d**) and branching (**Fig. 4.6e**). In conclusion, neuronal NAc D2 receptors

mediate both nicotine-induced increases in cocaine reinforcement and microglial activation.

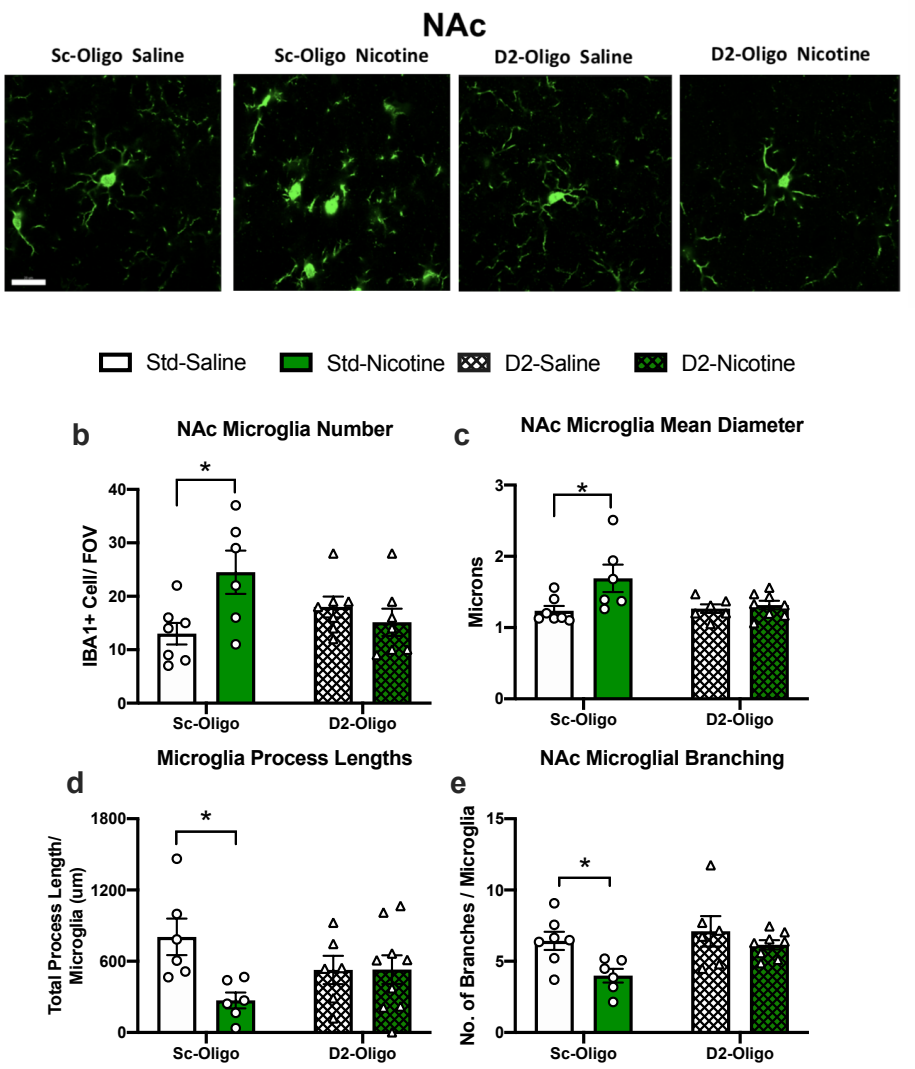


Fig 4.6: Neuronal D2 knockdown blocks nicotine-induced increases in microglial activated morphology **a**, Representative images of IBA1 stained NAc with standard and D2-targeted RNAi. **b**, D2-targeted morpholino eliminates nicotine-induced increases NAc microglial count ($F=1,23)=7.2$, $p=0.0129$, $\eta2=22.02\%$) (n=7 scramble-saline, 6 scrambled-nicotine, 7 D2-targeted-saline, 7 D2-targed-nicotine).**c,** Nicotine thickens NAc microglial process diameter, and this is blocked with a knockdown of D2 receptors ($F(1,23)=6.085$, $p=0.0215$, $\eta2=17.42\%$) (n=7 scramble-saline, 6 scrambled-nicotine, 6 D2-targeted-saline, 8 D2-targed-nicotine) **d**, D2 knockdown blocks ($F(1,23)=4.678$, $p=0.0412$, $\eta2=14.85\%$) nicotine-induced retraction of microglial processes ($F(1,23)=4.587$, $p=0.0430$, $\eta2=14.56\%$). (n=6 scramble-saline, 6 scrambled-nicotine, 6 D2-targeted-saline, 9 D2-targed-nicotine) **e**, D2 knockdown blocks ($F(1,23)=4.809$, $p=0.0387$, $\eta2=13.79\%$) nicotine-induced decreases to microglial branching ($F(1,23)=6.864$, $p=0.0153$, $\eta2=19.69\%$) (n=7 scramble-saline, 5 scrambled-nicotine, 6 D2-targeted-saline, 8 D2-targed-nicotine). All morphology data analyzed with a two-way ANOVA, both Bonferonni post hoc analysis (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Scale bar is 50μm. Bars show mean +/- s.e.m.

DISCUSSION

D2 receptor activation is a shared mechanism underlying nicotine-induced changes to behavior and microglia morphology. Neuronal D2 receptors are functionally immature during adolescence and are therefore poised to have unique contributions to adolescent behavior. Neurons and astrocytes express D2 receptors, but microglia do not. Systemically blocking D2 receptors with raclopride and knocking down predominantly neuronal NAc D2 receptors with a morpholino eliminates nicotine-induced increases in cocaine reinforcement. This two-pronged approach allowed us to definitively determine that D2 receptors are necessary for this behavior. Interestingly, raclopride and the RNAi of predominantly neuronal D2 receptors also blocked nicotine-induced changes to microglia morphology.

Although neuronal D2 receptors underlie increases in microglia activation after nicotine exposure, it remains unclear how a neuronal receptor has such profound effects on microglia. One hypothesis is that D2 receptor activation may cause neurons to release an unidentified dissociable signal that activates microglia. A second hypothesis is that nicotine-induced dopamine release elevates firing of D2-expressing neurons within the NAc, which attracts local microglia and promotes reactive morphology. Unlike adult MSNs, adolescent D2 receptor activation increases the probability of MSN firing. Increased synaptic activity can reduce extracellular calcium levels, attract microglia to neuronal dendrites, and alter their morphology (Eyo et al., 2015, Dissing-Olesen et al., 2014). Moreover, neuronal activation can increase extracellular ATP which increases microglial activation through purinergic receptors (Xu et al., 2015). Importantly, my data highlight the impact of neuron-microglia

communication on drug-seeking behavior. As such, identifying which signals mediate this communication is essential for understanding how this contributes to maladaptive behaviors and may create promising new therapeutic targets.

CHAPTER 5

Nicotine-induced changes to synaptic plasticity and architecture are microglial and D2-dependent

INTRODUCTION

Synaptic maturation is an essential part of adolescent development, and nicotine disrupts this progress (Yuan et al., 2015). Glutamatergic transmission is developmentally regulated in the NAc and is important for behavioral adaptations in response to drugs of abuse. Long-term potentiation (LTP) is one candidate mechanism underlying drug-associated learning, and adolescents exhibit immature LTP characteristics. Adolescent NAc LTP is more readily induced compared to adults (Schramm et al., 2002), and nicotine pretreatment in adolescent mice increases LTP amplitude (Kandel et al., 2015). LTP induction increases microglial synaptic contact (Pfieffer et al., 2016), and suppressing neuroinflammation with minocycline alters LTP induction (Riazi et al., 2015). Yet, it is unclear if microglia contribute to nicotine-induced changes to adolescent LTP.

Synaptic pruning is a hallmark of adolescent development, and while much of this pruning is cell autonomous, increasing evidence indicates that microglia can contribute to developmental synaptic pruning. Foundational studies investigating development of the visual system have revealed the critical role microglia play in developmental pruning and the behavioral significance of this pruning. However, little is known about the role microglia play in adolescent synaptic pruning or how drugs of abuse alter microglial synaptic elimination.

Several signaling pathways contribute to microglia-dependent synaptic pruning, one of which is the fractalkine signaling pathway. This pathway utilizes the chemokine fractalkine, or CX3CL1, which binds to it's receptor, CX3CR1, which is exclusively expressed on microglia. An extensive literature largely based on transgenic mouse models has revealed that fractalkine signaling plays a critical role in regulating a spectrum of microglial properties, in particular their migration and surveillance of the brain parenchyma, survival of developing neurons, as well as the maturation, activity and plasticity of synapses (Paolicelli et al., 2014). Importantly CX3CL1-CX3CR1 signaling is a critical component of developmental microglial synaptic pruning (Paolicelli et al., 2011; 2014).

In this chapter I characterize nicotine-induced changes to synaptic architecture and plasticity. I investigate the role of microglia and D2 receptors in nicotine-induced synaptic depletion and LTP enhancement.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats were group-housed in an AAALAC-accredited vivarium on a 12 hr light/dark cycle with unlimited access to food and water. Pups were weaned on P21. All experiments were performed during the light cycle and carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine.Drugs. Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2 – 7.4; doses were calculated as free base. Cocaine HCl (Sigma, St. Louis, MO) was dissolved in sterile saline.

Catheter Implantation: Rats, aged P24/25 or P82/83, were anesthetized with equithesin (0.0035 ml/g) and surgically implanted with an indwelling catheter in their jugular vein (Belluzzi et al, 2005). Throughout the 2-3 days of recovery, catheters were flushed daily with heparinized saline solution (1000 units heparin per 30 ml bacteriostatic saline) to maintain patency. Catheter patency was verified by testing with propofol (5 mg/kg, i.v.) after drug pretreatment, and only animals showing rapid anesthesia were included in analyses.

Pretreatment: Adolescent (P28-31) and adult (P86-89) rats received a intravenous drug pretreatment of nicotine (60 µg/kg), or saline for 4 consecutive days (McQuown et al., 2007). The D2 receptor antagonist raclopride (0.5mg/kg) was administered 20 minutes prior to each saline or nicotine infusion (P28-P31).

Behavioral testing was conducted following the last day of pretreatment on P32.
Intravenous Self-Administration: Animals were placed into an operant chamber equipped with two nose poke holes, and were tested in a single 2 h session on a fixed

ratio 1 schedule to deliver a 0.02 ml intravenous infusion of cocaine (0.5 mg/kg). During each 1.1s infusion, a signal light above the reinforced hole illuminated, after which the house light shut off for a 20-s time-out period. To control for nonspecific activating effects of drugs, activity on a non- reinforced hole, whose activation had no programmed consequences, was recorded. A maximum of 100 infusions was allowed.

Immunohistochemistry: All animals were perfused according to established procedures and brains were post- fixed in 4% paraformaldehyde for 24 h to ensure proper tissue fixation. To investigate changes in protein levels, 40µm coronal sections were run through free-floating immunohistochemistry (IHC). Briefly, tissue was blocked and permeated in 0.2% Triton X-100, 5% Normal Goat Serum (NGS), in PBS. Sectioned tissue is incubated with primary antibody in incubation buffer (5% NGS, 0.2% Triton X-100 in PBS) overnight. Primary antibody used was Synaptophysin (1:250; Abcam, 32127). Tissue was incubated with secondary antibody (goat-anti rabbit IgG Alexa fluor 488, Abcam ab150077) in incubation buffer. Omission of the primary antibody was a negative control.

RT-qPCR

Complementary DNA was made from 150 ng of total RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). Primers used were: GAPDH: Forward (CTGCACCACCAACTGCTTAG), Reverse (TGATGGCATGGACTGTGG);); Casp3: Forward (CCGACTTCCTGTATGCTTACTCTA), Reverse (CATGACCCGTCCCTTGAA); Arc: Forward (GCTGAAGCAGCAGACCTGA), Reverse

(TCTGCTTTTCTTCACTGGTATGA); CHRNA7: Forward (GGCAAAATGCCTAAGTGGAC) Reverse (CTTCATGCGCAGAAACCAT). Primers were derived from the Roche Universal Probe Library. Gapdh probe is conjugated to Lightcycler Yellow555 and all other probes were conjugated to a reporter fluorophore. The non-overlapping dyes and quencher on the reference gene allow for multiplexing in the Roche LightCycle 480 II machine (Roche Applied Science). All values were normalized to Gapdh expression levels. Analysis and statistics were performed using the Roche proprietary algorithms based on the Pfaffl method.

Slice preparation and recording

Parasagittal slices containing the NAc core were prepared from adolescent rats. Following isoflurane anaesthesia, rats were decapitated and the brain was quickly removed and submerged in ice-cold, oxygenated dissection medium containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 5 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose. Parasagittal slices (320 µm) were cut from the blocked brain using a FHC vibrating tissue slicer (Model:OTS-5000) before being transferred to an interface recording chamber containing preheated artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 glucose and 10 µM picrotoxin to reduce feedforward inhibition (refs 56, 57, 58). Slices were continuously perfused with this solution at a rate of 1.0–1.5 ml min^{-1}, while the surface of the slices were exposed to warm, humidified 95% O_2/5% CO_2 at 31±1 °C. Recordings began following at least 1.5 h of incubation.

Stimulation of afferent fibers within the NAc was achieved by placing a bipolar stainless steel stimulation electrode (25 µm diameter, FHC) just below the anterior commissure. Activation of fEPSPs were recorded using a glass pipette (2–3 MΩ) positioned caudal or caudal–ventral to the stimulation electrode (Fig. 4a). Thus, correct placement of electrodes within the NAc was confirmed by visual inspection of the slice and comparison with mouse brain atlas (Paxinos and Watson; 0.84–1.08 lateral to midline). Two parasagittal slices/hemisphere containing a large portion of the NAc core were obtained for each animal. Pulses were administered at 0.05 Hz using a current that elicited a 30–40% maximal response. Measurements of fEPSP amplitude were recorded during a minimum 20 min stable baseline period at which time LTP was induced by delivering three to five trains (intertrain interval of 1 min), each train containing five 'theta' bursts, with each burst consisting of four pulses at 100 Hz and the bursts themselves separated by 200 ms (that is, TBS). The stimulation intensity was not increased during the delivery of TBS. Data were collected and digitized by NAC 2.0 Neurodata Acquisition System (Theta Burst Corp., Irvine, CA) and stored on a disk.

*Microscopy and Analysis***:** A Zeiss LSM 700 was used to capture images of synaptophysin. Microglia morphology, cell count and soma size were analyzed by IMARIS. IHC and analysis of all experimental groups was performed in parallel. All analysis was done blind.

*Statistics***:** All data are expressed as mean ± SEM. Data distribution was assumed to be normal, with similar variability among groups, but this was not formally tested. Immunohistochemistry was analyzed with a two- way ANOVA for nicotine pretreatment

x raclopride exposure, nicotine pretreatment x minocycline exposure, and nicotine pretreatment x PLX3397 exposure. All significant main or interaction effects were further analyzed with Bonferonni post hoc analysis.

RESULTS

Nicotine modifies synaptic plasticity genes in an age-dependent manner

Using a Nanostring multiplex gene panel for analysis of transcripts associated with synaptic and neurotransmitter function in the adolescent and adult male NAc (**Fig 5.1a**), we found that nicotine suppressed expression of the activity related, immediate early genes *Arc* and *Fos* in adults, while increasing Arc expression in adolescents. Nicotine selectively decreased expression of the α7 nicotinic acetylcholine receptor (*Chrna7*) in adults but not adolescents, a receptor that is known to underlie the suppressive effects of nicotine on adult microglia[25]. Other synaptic associated genes that were also altered in adolescents after nicotine exposure included *Fus* and *Emp2* (**Fig. 5.1a**). Nicotine also significantly altered transcripts associated with dopamine transmission in an age-dependent manner (**Fig 5.1a**). *PAH*, which encodes for the enzyme phenylalanine hydroxylase that catalyzes the hydroxylation of phenylalanine to tyrosine, was selectively decreased by nicotine pretreatment in adolescence. In addition, the Parkinson's associated gene *Park2*, which regulates dopaminergic neuronal health and synaptic function, is decreased following adult nicotine exposure, but increased after adolescent nicotine exposure. Arc and CHRNA7 transcripts to were confirmed with RT-qPCR (**Fig. 5.1b,c**). This extensive gene expression investigation

demonstrates that nicotine has age-dependent effects on synaptic plasticity, and indicates that nicotine may alter synaptic quantity and structure.

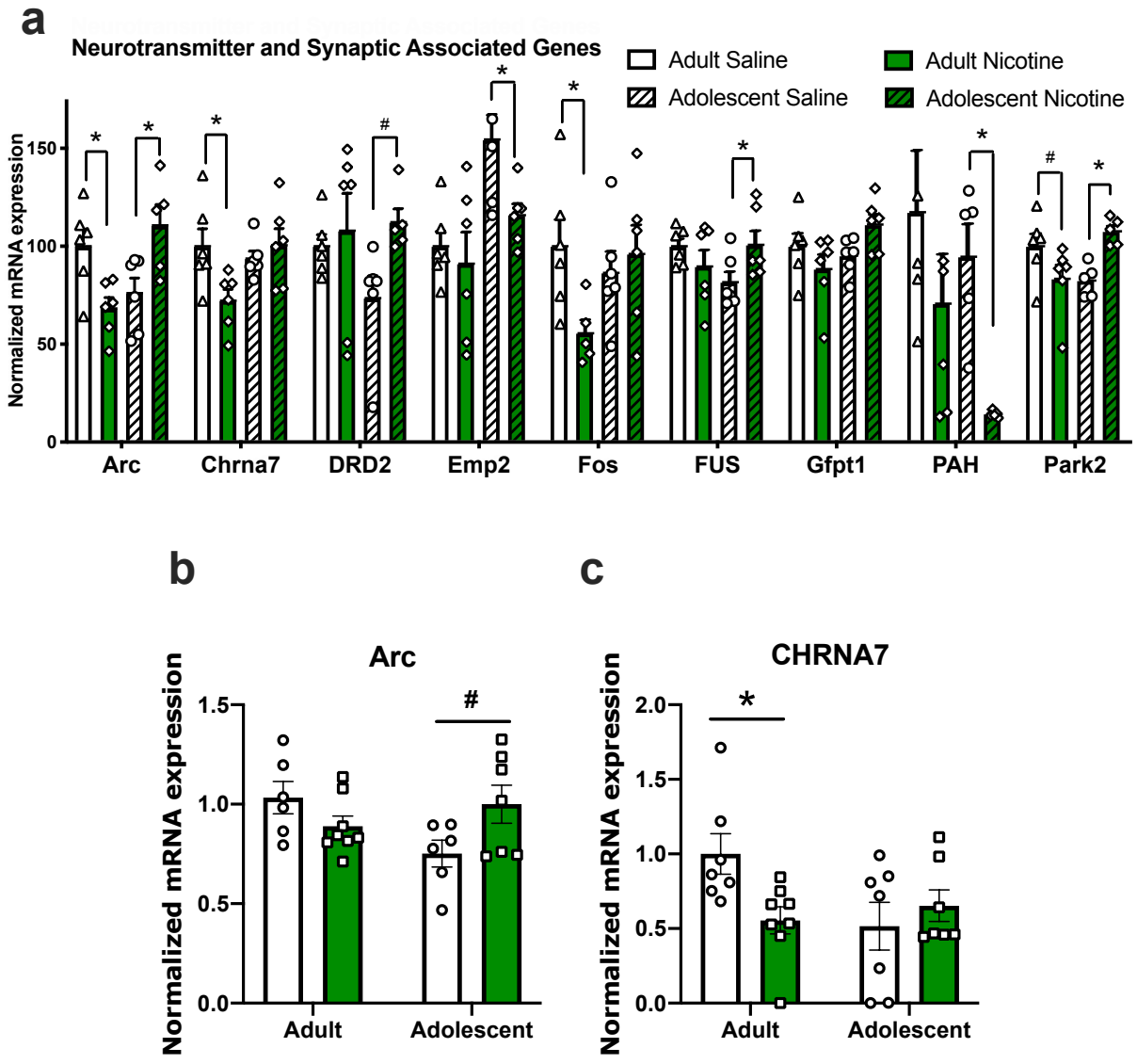


Fig. 5.1: Nicotine alters synaptic genes in an age-dependent manner. **a**, Nanostring panel analysis of specific synaptic and neurotransmitter associated genes, expressed as a percentage of adult saline (n=6). Each gene or protein was analyzed with a two-way ANOVA and Bonferonni post hoc tests **b**, Nicotine increased adolescent Arc mRNA expression with a significant interaction between age and nicotine pretreatment ($F(1, 23)=6.774$, $p=0.0159$) (n=6 adult saline, 8 adult nicotine, 6 adolescent saline, 7 adolescent nicotine). **c**, Nicotine significantly decreased adult CHRNA7 mRNA expression ($F(1, 25)=5.507$, $p=0.0272$) (n=7 adult saline, 8 adult nicotine, 7 adolescent saline, 7 adolescent nicotine). (#p<.1;*p<0.05; **p<0.01). Bars show mean +/ s.e.m.

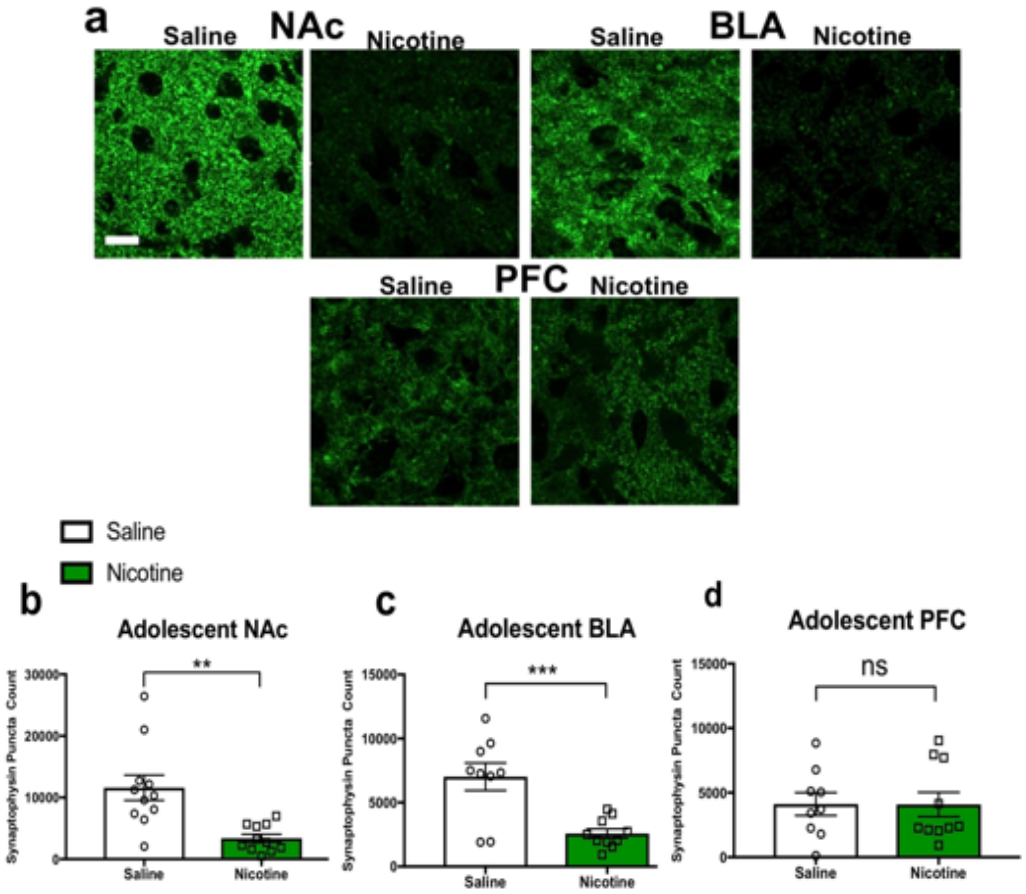


Fig 5.2: Nicotine suppresses synaptophysin levels in the NAc and BLA **a**, Representative images of NAc, BLA and PFC synaptophysin, with and without prior nicotine exposure. **b**, Nicotine decreases NAc synaptophysin puncta count (t20=3.804, **p=0.0011; r2=0.4198; n=11 rats). **c**, Nicotine decreases BLA synaptophysin puncta count (t17=4.075,***p=0.0008, r2=0.4941; n=9 saline, nicotine). **d**, Nicotine did not alter PFC synaptophysin levels (t17=0.02202, p=0.9827, r2=2.853e-005; n=9 saline, 10 nicotine).

Nicotine suppresses presynaptic proteins levels in the NAc and BLA but not the PFC

Nicotine induces microglial inflammation, and increased microglial activation is correlated with increased synaptic phagocytosis (Wu et al., 2015). To address whether nicotine induces changes in synapse number, slices were stained with the presynaptic marker, synaptophysin, after nicotine pretreatment. IMARIS software was used to analyze synaptophysin puncta count in the NAc, BLA and PFC (**Fig. 5.2a**). Adolescent nicotine exposure significantly decreased synaptophysin count in both the NAc (**Fig. 5.2b**) and BLA (**Fig. 5.2c**). In contrast, there was no change in synaptophysin levels in the PFC, a region which is actively maturing during adolescence but did not show

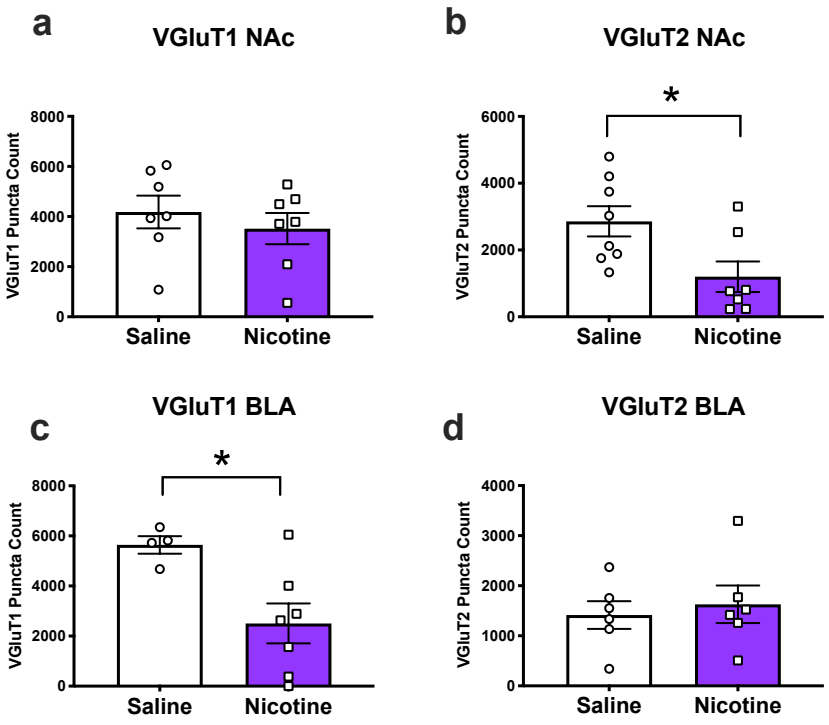


Fig. 5.3: Adolescent nicotine exposure has region-specific effects on VGlut1 and VGlut1 expression. **a**, Nicotine did not affect VGlut1 levels, but did suppress ($t_{13}(2.565)=0.0235$), NAc VGlut2 levels (**b**). Nicotine did decrease ($t_9(2.832)=9$) BLA VGlut1 levels (**c**), but not BLA VGlut2 levels (**d**).

nicotine- induced increases in microglia (**Fig. 5.2b**; **see Chapter 2 Fig.2.4**).

Nicotine causes region-specific depression in the presynaptic glutamate vesicle release proteins, VGluT1 and VGluT2. Adolescent nicotine exposure suppresses VGluT2 levels in the NAc, but not VGluT1 (**Fig. 5.3a,b**). In contrast, nicotine exposure suppressed adolescent VGluT1 levels in the BLA, but not VGluT2 (**Fig. 5.3c,d**).

Decreased synaptophysin levels in the NAc are microglia- and D2 receptor-dependent.

Both microglia and D2 receptor activation are required for decreases in NAc synaptophysin (**Fig. 5.4**). Minocycline (**Fig. 5.4a,b**), PLX3397 (**Fig. 5.4c,d**), and raclopride (**Fig. 5.4e,f**) blocked nicotine-induced decreases in synaptophysin. In contrast, only PLX3397 was able to eliminate nicotine-induced decreases in synaptophysin in the BLA (**Fig. 5.5**).

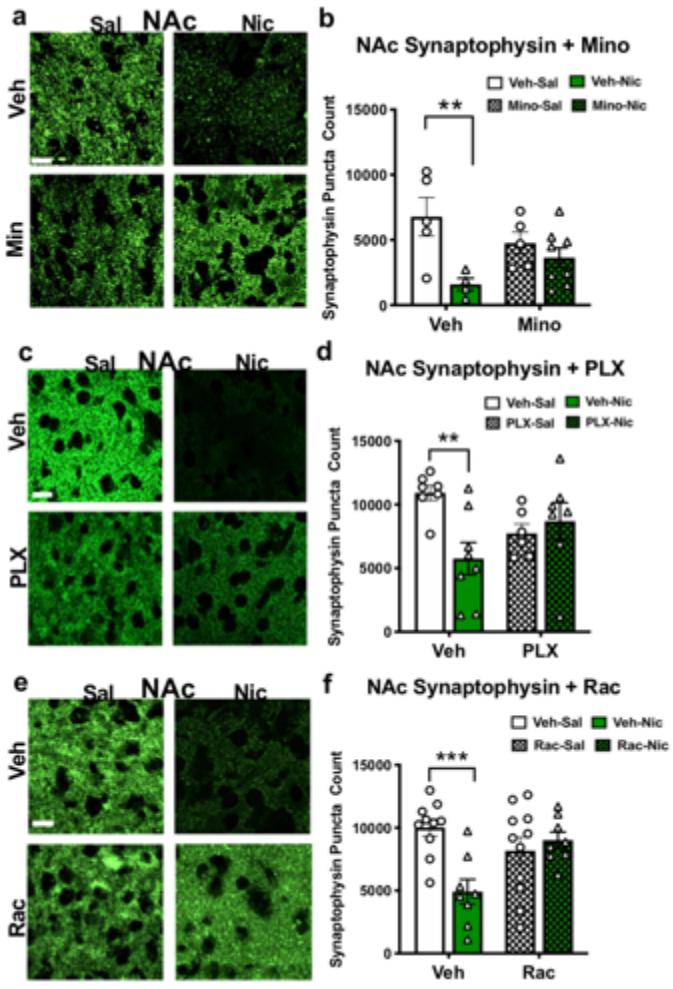


Fig. 5.4: Nicotine induces a decrease in synaptic density that is microglia and D2 dependent. **a**, Nicotine decreases NAc synaptophysin puncta count (t_{20}=3.804, **p=0.0011; n=11 rats). **b**, Nicotine decreases BLA synaptophysin puncta count (t_{17}=4.075,***p=0.0008, n=9 saline, nicotine). **c**, Nicotine did not alter PFC synaptophysin levels (t_{17}=0.02202, p=0.9827; n=9 saline, 10 nicotine). **e**, Representative images of synaptophysin levels in the NAc following saline/nicotine and vehicle/minocycline exposure. **f,** Minocycline administration released nicotine-induced suppression of NAc synaptophysin count (F(1,18)=9.692), p=0.0055) (n= 5 vehicle-saline, 4 vehicle-nicotine, 5 minocycline saline, 8 minocycline nicotine).**h**, Representative images of synaptophysin levels in the NAc with nicotine/saline and/or PLX3397/vehicle chow administration. **i**, PLX3397 blocks nicotine induced decreases in NAc synaptophysin levels (F(1,24)=7.412, p=0.0119, n=7 vehicle-saline, 8 vehicle-nicotine, 6 PLX3397-saline, 7 PLX3397-nicotine). **k**, Representative images of synaptophysin in the NAc with nicotine and/or raclopride administration. **l**, Nicotine-induced decreases NAc synaptophysin counts (F(1,33)=5.58, p=0.0242) were blocked with raclopride (F(1,33)=10.88, p=0.0023) (n=10 vehicle-saline, 8 vehicle-nicotine, 11 raclopride-saline, 8 raclopride nicotine). Data was analyzed with a two way ANOVA with Bonferroni post hoc tests (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Scale bar 15 μm Bars show mean +/ s.e.m.

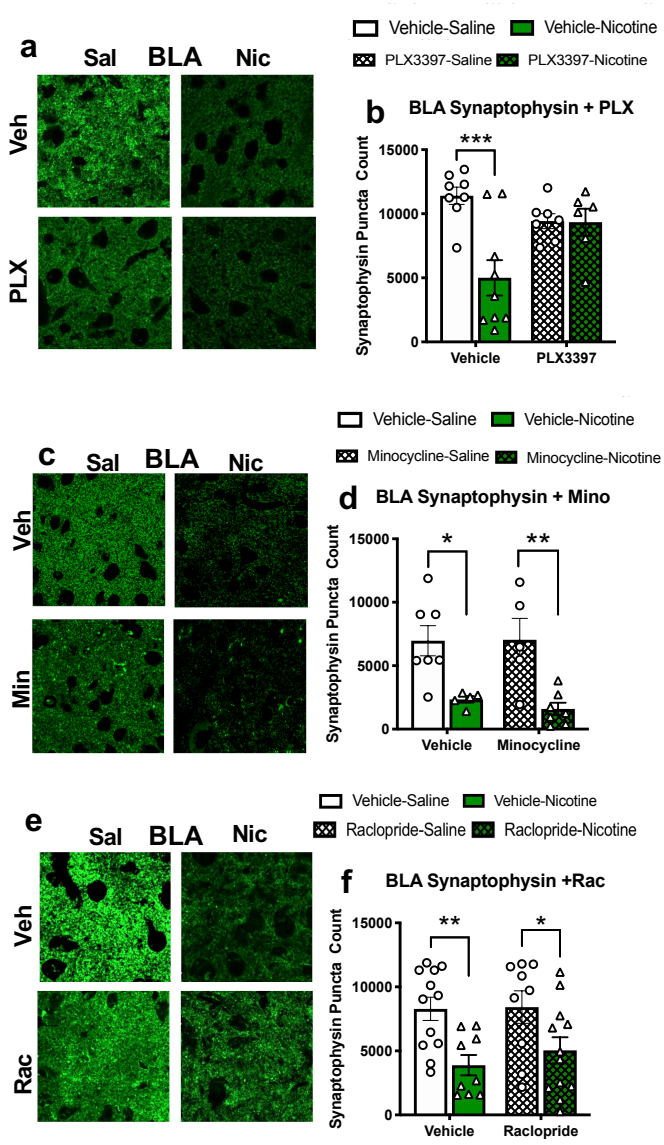


Fig. 5.5: Nicotine suppresses BLA synaptophysin levels, which is blocked with PLX3397, but not raclopride or minocycline. **a**, Representative images of synaptophysin levels in the BLA with nicotine/saline and/or PLX3397/vehicle chow administration. **b**, PLX3397 blocks nicotine induced decreases in BLA synaptophysin levels with a significant of nicotine ($F(1.26)=9.517$, $p=0.0048$;) and interaction between nicotine and PLX3397 ($F(1,26)=9.039$, $p=0.0058$) (n=8 vehicle-saline, 9 vehicle- nicotine, 7 PLX3397-saline, 6 PLX3397-nicotine). **c**, Representative images of synaptophysin levels in the BLA following saline/nicotine and vehicle/minocycline exposure. **d**, Nicotine significantly suppressed BLA synaptophysin count ($F(1,20)=23.25$), $p=0.0001$), and minocycline did affect this depletion (n= 7 vehicle-saline, 5 vehicle-nicotine, 5 minocycline saline, 7 minocycline nicotine). **e**, Representative images of synaptophysin in the BLA with nicotine and/or raclopride administration. **f**, Raclopride did not elevate nicotine induced suppression of synaptophysin in the BLA ($F(1,38)=14.49$, $p=0.0005$, (n=12 vehicle-saline, 9 vehicle-nicotine, 9 raclopride-saline, 12 raclopride nicotine. Data was analyzed with a two way ANOVA with Bonferroni post hoc tests (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Bars show mean +/ s.e.m.

Nicotine increases fractalkine signaling pathways in the adolescent NAc

The fractalkine signaling pathway (CX3CL1-CX3CR1) regulates microglial synaptic pruning. To investigate if this pathway is altered following adolescent nicotine exposure, we used RT-qPCR to measure both the neuronal (CX3CL1) and microglial (CX3CR1) components. Nicotine increases adolescent NAc CX3CR1 and CX3CL1 mRNA expression (**Fig. 5.6a,b**) while decreasing adult CX3CR1 mRNA expression (**Fig. 5.6c**).

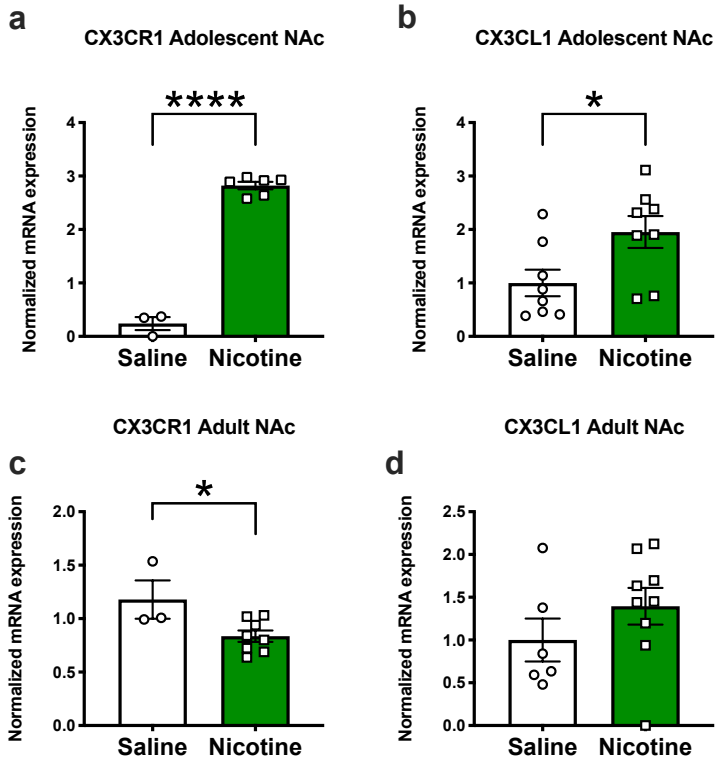


Fig. 5.6: Nicotine increases fractalkine signaling in the adolescent NAc. **a,** Nicotine increased (t_{20}=7, p<0.0001) adolescent CX3CR1, and CX3CL1 (t_{14}=2,455, p=0.0278) (**b**). **c,** In adults, nicotine decreased CX3CR1 (t_9=2.576, p=0.0299) with no significant effects on CX3CL1 expression (**d**)

Arc is increased following adolescent NAc nicotine exposure

We previously found that nicotine increases Arc mRNA levels only in adolescent NAc (**Fig. 5.1**). To determine if this increase in Arc was dependent on microglia, we measure immuno-labeled Arc+ cells in the presence or absence of nicotine or PLX3397 (**Fig. 5.7**). Nicotine pretreatment increased Arc+ cells and this was blocked with the elimination of microglia (**Fig. 5.7b**). We found no significant differences in BLA Arc levels (**Fig. 5.7c**)

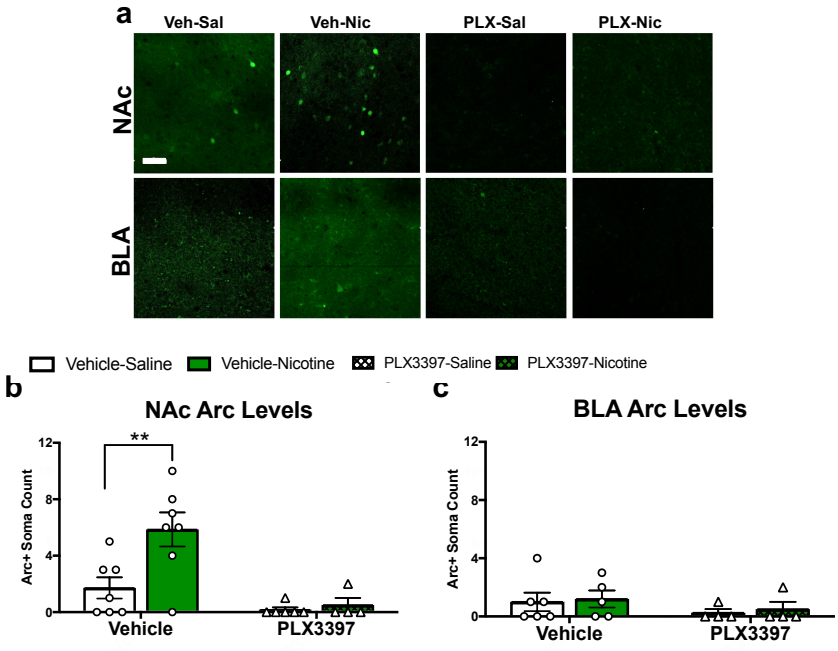


Fig. 5.7: Nicotine causes a microglia-dependent increase in NAc Arc+ cells **a**, Representative images of Arc staining in the NAc and BLA. **b**, Nicotine increased Arc expression ($F(1,20)=6.474$, $p=0.0193$) and PLX3397 blocked this increase ($F(1,20)=15.41$, $p=0.0008$;There was an interaction between PLX3397 administration and nicotine ($F(1,20)=4.69$, $p=0.0426$) (n=7 vehicle-saline/nicotine, 5 PLX3397-saline, 4 PLX3397-nicotine). **c**, There was no significant effect of PLX3397 or nicotine administration on BLA Arc expression (n=6 vehicle-saline, 5 vehicle-nicotine, 4 PLX3397-saline/nicotine). Data were analyzed with a two-way ANOVA with Bonferroni post hoc tests (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Scale bar 50 µm. Bars show mean +/- s.e.m.

Adolescent nicotine exposure enhances long-term synaptic plasticity in the NAc

Nicotine has been shown to enhance long-term potentiation (LTP) in the NAc in adult rats, and increases in NAc LTP are associated with heightened drug-associated behaviors. We have also shown in adolescent rats that nicotine caused a microglia-dependent increase in Arc positive cells in the NAc, but not the BLA (**Fig. 5.7**). We therefore hypothesized that nicotine would cause microglia-dependent changes in long-term synaptic plasticity. LTP in the NAc was induced by applying high frequency stimulation to glutamate projections onto medium spiny neurons in the NAc. Nicotine significantly increased NAc LTP without affecting baseline synaptic transmission **(Fig. 5.8a-d)**. The nicotine-induced enhancement in LTP was blocked by inhibiting microglial activation with minocycline and blocking D2 receptors with raclopride (**Fig. 5.8**). However, clearing microglia from the CNS severely suppressed LTP induction, and nicotine pretreatment restored this depression **(Fig. 5.8e-p)**. Taken together these data demonstrate that suppressing microglial activation and blocking D2 receptors eliminates nicotine induced increases in LTP, while eliminating microglia does not.

Nicotine did not affect baseline synaptic transmission, as measured by paired pulse facilitation (PPF) (**Fig. 5.8c**) and input/output (I/O) curve **(Fig. 5.8d)**. Minocycline, PLX3397 and raclopride did not influence nicotine-induced changes to PPF (**Fig. 5.8g,k,o**). Input/output (i/o) curves were unchanged by nicotine with co-administration of minocycline and PLX3397, but nicotine lowered the I/O curve in raclopride treated

animals (**Fig. 5.8h,l,p**). However, I/O curves were lower in both minocycline and PLX treated rats (**Fig. 5.8h,l**).

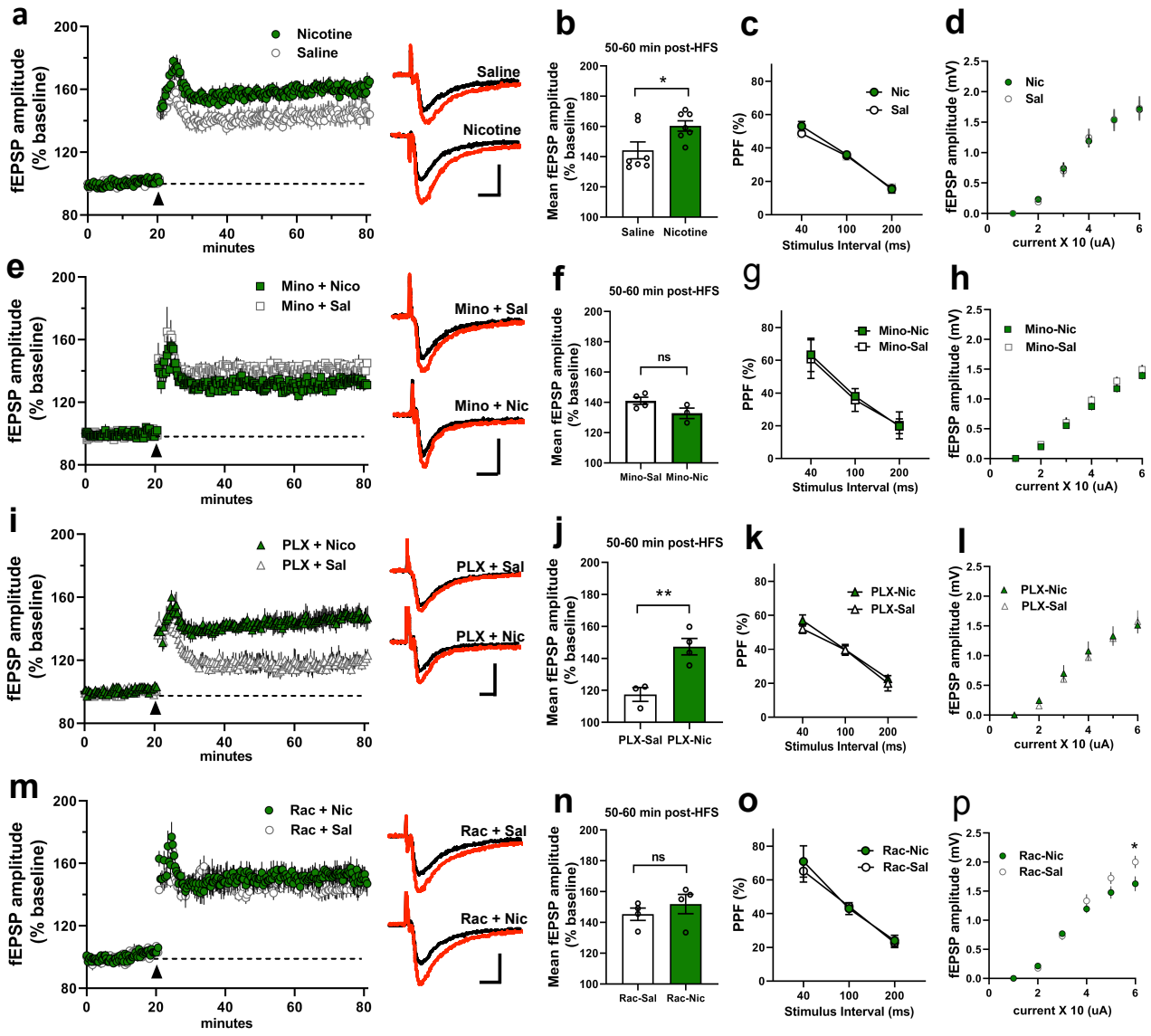

a
fEPSP amplitude (% baseline)
Nicotine
Saline
minutes
Saline
Nicotine
b
50-60 min post-HFS
Mean fEPSP amplitude (% baseline)
*
Saline
Nicotine
c
PPF (%)
Nic
Sal
Stimulus Interval (ms)
d
fEPSP amplitude (mV)
Nic
Sal
current X 10 (uA)
e
Mino + Nico
Mino + Sal
Mino + Sal
Mino + Nic
f
ns
Mino-Sal
Mino-Nic
g
Mino-Nic
Mino-Sal
h
i
PLX + Nico
PLX + Sal
PLX + Sal
PLX + Nic
j
**
PLX-Sal
PLX-Nic
k
PLX-Nic
PLX-Sal
l
m
Rac + Nic
Rac + Sal
Rac + Sal
Rac + Nic
n
Rac-Sal
Rac-Nic
o
Rac-Nic
Rac-Sal
p
*

Fig 5.8. Nicotine-induced enhancement in LTP in the NAc core is microglia and D2 dependent. **a**, Nicotine pretreatment significantly enhanced high frequency-induced LTP (black arrow head) in the NAc. Representative traces on the right were collected during baseline (black line) and 60 min post-HFS (red line) in saline and nicotine treated rat slices. **b**, Mean fEPSP amplitude as a percentage of baseline during the last 10 minutes of recording is significantly greater in nicotine than saline treated rats ($t_{12}=2.46$, $p=0.0298$; n=7 saline, 7 nicotine). **c,d** No significant differences were found between nicotine and saline treated rats following paired pulse facilitation (PPF) ($F(1,12)=0.3946$, $p=0.5417$; n=7 saline, 7 nicotine) or input/output curve ($F(1,11)=0.0002$, $p=0.9881$; n=7 saline, 6 nicotine) respectively. **e**, The microglial inhibitor, Minocycline (Mino), effectively blocked nicotine enhancement in LTP. Representative traces on the right were collected during baseline (black line) and 60 min post-HFS (red line) in Mino+saline and Mino+nicotine treated rat slices. **f**, No difference were found in mean fEPSP amplitude during the last 10 minutes of recording in Mino-nicotine and Mino-saline treated rats ($t_5=2.043$, $p=0.0965$; n=4 mino+saline, 3 mino+nicotine). **g,h**, No significant differences were found between Mino-nicotine and Mino-saline treated rats following PPF ($F(1,5)=0.0583$; n=4 mino+saline, 3 mino+nicotine) or input/output curve ($F(1,5)=1.251$, $p=0.3142$; n=4 mino+saline, 3 mino+nicotine), respectively. **i**, Nicotine pretreatment notably increased NAc LTP in the presence of a selective CSF1R inhibitor, PLX3397 (PLX). Representative traces on the right were collected during baseline (black line) and 60 min post-HFS (red line) in PLX-saline and PLX-nicotine treated rat slices. **j**, Mean fEPSP amplitude during the last 10 minutes of recording was significantly elevated in PLX-nicotine than PLX-saline treated rats ($t_5=4.238$, $p=0.0082$; n=3 PLX+saline, 4 PLX+nicotine). **k,l**, No significant differences were found between PLX-nicotine and PLX- saline treated rats following paired PPF ($F(1,5)=2.158$, $p=0.2017$; n=3 PLX+saline, 4 PLX+nicotine) or input/output curve ($F(1,5)=0.1119$, $p=0.7515$; n=3 PLX+saline, 4 PLX+nicotine), respectively. **m**, Raclopride (Rac) pretreatment effectively blocked nicotine induced increases in LTP. Representative traces on the right were collected during baseline (black line) and 60 min post-HFS (red line) in Rac+saline and Rac+nicotine treated rat slices. **n**, No difference were found in mean fEPSP amplitude during the last 10 minutes of recording in Rac-nicotine and Rac-saline treated rats ($t_6=0.8906$, $p=0.4704$; n=4 Rac+saline, 4 Rac+nicotine). **o,** No significant differences were found between Rac-nicotine and Rac-saline treated rats following PPF ($F(1,6)=0.1201$, $p=0.7407$;n=4 Rac+saline, 4 Rac+nicotine). **p**, An interaction, however, between groups was observed in input/output curve. Specifically, post-hoc analysis showed a significant difference between Rac-nicotine and Rac-saline in fEPSP amplitude at the highest current setting ($F(5,30)=3.199$, $p=0.0197$; n=4 Rac+saline, 4 Rac+nicotine). LTP data were analyzed with a Students unpaired t test. Paired pulse facilitation and input/output curve data were analyzed using a two-way ANOVA with Bonferroni post hoc tests (*$p<0.05$, **$p<0.01$).. Scale bars = 0.5 mV/5 ms.

DISCUSSION

Nicotine causes a decrease in synaptophysin puncta count and elevated LTP amplitude within the adolescent NAc. Co-administration of minocycline, PLX3397 and raclopride blocked nicotine-induced suppression of NAc synaptophysin levels. Minocycline and raclopride also block nicotine-induced increases in LTP, but PLX administration does not. Together these data demonstrate that nicotine significantly alters adolescent synaptic plasticity and architecture, and these changes require D2 receptors and microglial activation.

Nicotine caused a suppression of synaptophysin within the NAc and BLA, but different mechanisms underlie this decrease. In the NAc, VGlut2 levels decrease, while in the BLA VGlut1 levels are depressed by nicotine. VGlut1 and VGlut2 have regionally dimorphic expression (Mestikawy et al., 2011), and these data may indicate that nicotine alters different afferents to the NAc and BLA. In addition, within the NAc, administration of minocycline, PLX or raclopride eliminated nicotine-induced changes in synaptophysin. In contrast, only PLX reverses nicotine-induced synaptic depression within the BLA. The incapability of raclopride to restore BLA synapse count may be due to circuit dynamics within this region, or because neurons in the BLA expresses far fewer D2 receptors than the NAc (see Chapter 3). PLX, and not minocycline, -induced synaptic restoration within the BLA could be because the more severe effects of PLX (microglial elimination) may enable recovery.

To address potential mechanisms of microglia-dependent synaptic pruning we

evaluated levels of the microglial CX3CR1 receptor, and the neuronal CX3CL1 ligand. Both CX3CR1 and CX3CL1 were increased after adolescent nicotine exposure, while CX3CR1 was decreased after adult nicotine exposure. The increase in CX3CR1 is predicted given the observed nicotine-induced increase in microglia (Chapter 1). However, the concomitant increase in CX3CL1 may indicate this receptor signaling cascade is involved in the microglia-dependent decreases in synaptophysin.

Alongside decreases in synaptophysin are increases in Arc positive cells within the NAc that are microglia dependent. This decrease in synaptic markers with a concurrent increase in IEG expression may indicate a selective synaptic pruning that allows for heightened drug-induced neuronal activation. A similar phenomenon is observed in adolescent development, where select synapses are pruned, while there is a simultaneous strengthening of the remaining connections (Yuan et al., 2015).

Baseline synaptic plasticity remained largely unchanged by nicotine, as input/output (I/O) curves and paired-pulse facilitation (PPF) did not significantly differ. The I/O curve generally refers to the relationship between the excitatory input to a neuron and the probability it will generate an action potential. Interestingly, rats treated with microglial inhibitors (minocycline, PLX), had suppressed I/O curves as compared to saline/nicotine treated rats. This potentially indicates microglia increase the excitability of neurons and suppressing or eliminating these cells decreases the probability of neuronal firing. In contrast, raclopride elevated I/O curves, indicating D2 receptor blockade increased the probability of excitatory neuron transmission.

LTP is a long-term synaptic plasticity measure that can be correlated with heightened drug reward, and nicotine pretreatment elevated adolescent NAc LTP. While minocycline and raclopride eliminate nicotine-induced increases in LTP, PLX administration does not. However, raclopride, minocycline, and PLX all block nicotine-induced increases in cocaine self-administration (See Chapter 2,3), indicating that LTP is an imperfect analogue for nicotine-induced increases cocaine reinforcement. PLX severely depresses HFS-induced NAc LTP, and nicotine restores this depression. Although the mean fEPSP amplitude of PLX-Nic treated animals is lower than that of nicotine alone treated animals, it is difficult to interpret these results because the PLX-Sal treated animals have such blunted LTP. The mechanism by which microglia contribute to LTP maintenance is unclear but is an intriguing phenomenon that should be assessed in future studies. The restoration of LTP by nicotine may indicate cholinergic or dopaminergic transmission is involved in the PLX-induced suppression of LTP. How microglia contribute to the maintenance of LTP is a fundamental question that should be assessed in future studies. Together these LTP data demonstrate microglial activation and D2 receptor activity are critical for long-term changes in nicotine-induced neural transmission.

In conclusion, using both functional and imaging techniques, I determined that neuroinflammation and D2 receptors are required for nicotine-induced enhancement of synaptic plasticity.

CHAPTER 6

Discussion

I. Significance

Adolescence is a sensitive developmental period with enhanced vulnerability to developing substance use disorders (Kessler et al., 2005; Crews et al., 2007). Although social influences may be a factor, clinical and preclinical evidence highlight that this adolescent sensitivity originates from underlying neurobiological mechanisms (Yuan et al., 2015). Teen smokers have disproportionately high rates of future substance abuse compared to their nonsmoking peers, suggesting that tobacco acts as a “gateway” to future illicit drug use (Kandel et al., 1992; Degenhardt et al., 2010, Lai et al., 2000; Hanna et al., 2001; Biederman et al., 2006, Yuan et al., 2015). While tobacco use has recently declined, e-cigaratte use is increasing at an exponential rate (CDC, 2019). E-cigarettes are now the most commonly used tobacco product among teenagers and have the lowest perceived health risk (Dutra and Glantz, 2014; Wills et al., 2015). Teens are heavily exposed to e-cigarette advertising and e-cigarettes are readily accessible (Marynak et al., 2018; Paradise, 2014). Teens also believe e-cigarettes are safer, and many consume nicotine unintentionally (Boykan et al., 2019). This dramatic rise in teen e-cigarette use is alarming, especially since adolescence is a vulnerable developmental period where nicotine use is associated with future drug use.

During adolescence, there is a dynamic reorganization of neurocircuitry regulating the maturation of cognitive, executive, and emotional function. In particular,

DA and limbic systems are still under development, exhibiting both physiological and functional changes (Yuan et al., 2015). The neuroimmune system is also maturing during adolescence (Brenhouse et al., 2016). Fundamental adolescent transformations including synaptic pruning, neurite outgrowth, and neurotransmitter release all regulate—and are regulated by—immune signals (Linker et al., 2018).

Microglia are the resident immune cells of the brain, and the first defenders against biological threats – including drugs of abuse. Adolescent microglia are more sensitive to immune insults than adult (Brenhouse et al., 2016), and nearly all drugs of abuse activate microglia (Coller and Hutchinson, 2012). Inhibiting neuroinflammation can block rewarding effects of drugs. In contrast, nicotine suppresses microglia in the adult brain, and this suppression occurs via the α7 nicotinic receptor (Shytle et al., 2004; De Simmone et al., 2005; Mishra et al., 2015; Noda et al., 2017). However, it has been unclear how nicotine interacts with adolescent microglia. I therefore tested the hypothesis that exposure to nicotine produces unique alterations to adolescent microglia, which contribute to adolescent vulnerability for increased cocaine use. Specifically, my dissertation identifies microglia as a novel, yet essential, regulator of nicotine-induced increases in synaptic plasticity and cocaine-seeking, with D2 receptors as a critical interface for these phenomena.

II. Microglia are Activated By Adolescent Nicotine Exposure and Increase Cocaine-Seeking

Characterizing Nicotine-Induced Changes to Adolescent and Adult Microglia

Our lab has consistently demonstrated that adolescent, but not adult, nicotine exposure increases cocaine reinforcement (McQuown et al. 2007; McQuown et al. 2009; Dao et al., 2011). I have now shown that, correlated with this behavioral change, is an increase in microglial activation that is specific to the adolescent brain. In addition, I found that nicotine-induced microglial activation is confined to regions that encode reward: the NAc and BLA. Through extensive protein, mRNA and cellular analysis, I have demonstrated that nicotine has fundamentally dichotomous effects on adolescent and adult microglia.

Increasing evidence demonstrates that adult nicotine exposure suppresses microglia through the α7 receptor (Mishra et al., 2015; Noda et al., 2017), but mechanisms underlying nicotine effects on adolescent microglia have not been elucidated. However, both systemically inhibiting D2 receptors, and knocking down neuronal NAc D2, blocks nicotine-induced activation of adolescent microglia. The mechanism by which neuronal D2 receptors cause significant microglial morphological changes is currently unknown. Still, these data highlight the substantial communication between neurons and microglia, and the importance of understanding this crosstalk.

Microglia Increase Adolescent Cocaine Reinforcement

While decades of research have correlated drug exposure with microglial activation, it was ambiguous if this activation is required for drug-seeking. Using a multivariate approach, with different microglial inhibitors, I have identified microglia as a

remarkably important regulator of nicotine-induced increases in cocaine-seeking. Minocycline, an antibiotic cocktail (ampicillin, vancomycin, neomycin), and PLX3397 all block nicotine-induced increases in cocaine-seeking. Importantly, antibiotics that cannot directly interact with the CNS (vancomycin or neomycin) do not block this behavior, suggesting that inhibition of the central, but not the peripheral, immune system is mechanistically vital. These data highlight the need for more clinical and preclinical studies to investigate microglia as a therapeutic target for adverse health consequences following adolescent nicotine exposure.

III. The Role of D2 Receptors

Neuronal D2 receptors are a critical regulator for nicotine-induced changes to cocaine reinforcement and microglial activation. D2 receptors are functionally immature during adolescence and have distinct contributions to adolescent behavior. Prior work from our lab has demonstrated that adolescent nicotine pretreatment enhances adolescent D2-locomotor behaviors and D2 functional receptor activity (Mojica et al., 2014; Dao et al., 2011). My work showing that concurrent blockade of D2 receptors eliminates nicotine-induced increases in subsequent cocaine-seeking, supplements these findings. These data highlight the dissimilarity between adults and adolescents, as D2 receptor antagonism in adults has the opposite effect and enhances cocaine reinforcement (Self et al., 1996).

Pharmacological and transcriptional inhibition of D2 receptors blocked increases in microglial activation. Interestingly, I have found that NAc and BLA microglia do not

express D2 receptors, which contradicts cell culture studies (Pocock and Kettenmann 2007; Lewitus et al., 2016). These data emphasize the differences between in vivo and in vitro microglia, and highlights regional differences in microglia. The downstream signaling cascade that causes neuronal D2 receptors to prevent microglial activation is presently unknown, but an important question. Potential dissociable signals that underlie this phenomenon are calcium and ATP, as both extracellular concentrations are modified upon neuronal activation and alter microglial morphology (Eyo et al., 2015, Dissing-Olesen et al., 2014; Xu et al., 2015). Although measuring extracellular ATP is difficult, pharmacologically inhibiting ATP receptors (P2X) would determine if extracellular ATP signaling was necessary for nicotine-induced increases in cocaine seeking.

IV. Neuroinflammation and D2 Receptors are Required for Nicotine-Induced Enhancement of Synaptic Plasticity

Administration of minocycline, PLX3397 and raclopride blocked nicotine-induced suppression of NAc synaptophysin levels. This suggests presynaptic plasticity mechanisms may be altered, but input/output (I/O) curves and paired-pulse facilitation (PPF) did not significantly differ. A more thorough investigation would look at mini EPSCs and IPSCs using patch clamp to better asses a presynaptic mechanism. However, our data may indicate that a decrease in a presynaptic marker is not necessarily tied to a decrease in presynapses or changes in vesicular release.

While minocycline and raclopride eliminate nicotine-induced increases in LTP in

the NAc , PLX administration does not. In contrast, raclopride, minocycline, and PLX all block nicotine-induced increases in cocaine self-administration, indicating that LTP is an imperfect representation for drug-associated learning. PLX severely depresses HFS-induced NAc LTP, and nicotine restores this depression. The mechanism by which microglia contribute to LTP maintenance is unclear, but is an intriguing phenomenon that should be assessed in future studies. The restoration of LTP by nicotine may indicate that cholinergic or dopaminergic transmission is involved in the PLX-induced suppression of LTP. Importantly, microglial activation and D2 receptor activity are critical for long-term changes in nicotine-induced synaptic architecture and plasticity.

V. Limitations and future directions

Sex differences

I observed no sex differences in cocaine seeking or IBA1 expression at either age. However, upon closer examination of microglial morphology I did observe some baseline sex differences in adult, but not adolescent, microglial morphology. Adult male microglia soma are bigger than female, and have altered process morphology. This difference in microglia size reflects recent literature (Guneykaya et al., 2018). The developmental delay in microglial sex differences may indicate that pubertal hormonal brain changes influence microglia morphology and function. Interestingly, microglia isolated from adult brains maintain the sex-specific features when put in culture or transplanted in the brain of the opposite sex (Villa et al., 2018), suggesting that

developmental hormonal or genetic (X-linked) factors influence microglial sex differences.

Modeling the Clinical Condition

The paradigm used throughout this thesis was carefully developed by the Leslie laboratory to mimic adolescent smoking initiation. Early adolescent rats are given a very low-dose (60μg/kg/day) of nicotine, and this dose was specifically chosen as it yields initial blood nicotine levels similar to that produced by 1-2 cigarettes per day (McQuown et al. 2007; McQuown et al. 2009; Dao et al., 2011). This level of nicotine is equivalent to initial smoking experiences in teens (Klein et al., 2013). However, all of our pharmacological manipulations were administered *during* the nicotine pretreatment paradigm. This was in order to tease out the mechanism by which nicotine increased cocaine reinforcement, but is not necessarily clinically relevant. Follow up studies should administer microglial inhibitors, *after* nicotine pretreatment, as cocaine reinforcement stays elevated for 10 days (Dao et al., 2011). This would better model an intervention or rehab condition where treatment is typically given after drug exposure.

Microglia are Intractable Cells

Here we have demonstrated that nicotine has age-dependent effects on genes predominantly expressed by microglia. However, to perform this transcriptional analysis, we did not separate microglia from other cell types. This isolation could be accomplished with fluorescent activated cell sorting (FACS). However, FACS is often done with genetic, not antibody, -based fluorescence and requires larger brain regions

for sufficient RNA (De Biasi et al., 2017). We would require antibody-based separation and both the NAc and BLA are quite small regions. As such, FACS sorting microglia in our paradigm would have been extremely difficult. Still, FACS sorting of microglia to investigate cell-type specific changes in gene expression is vital to understanding drug-induced changes to adolescent microglia.

Microglia are immune competent cells that do not readily express virus' or RNAi molecules. As such, it is extraordinarily difficult to specifically manipulate these cell types. We used PLX3397 to eliminate microglia, which circumvents nonspecific effects of neuroimmune suppressors like minocycline. However, PLX is a robust manipulation and causes significant changes to astrocyte gene expression and morphology (Elmore et al., 2014). Ideally, one would knockdown a specific inflammatory gene (TLR4; NR4a2) within microglia and examine the effect on behavioral and molecular phenomenon. However, the lack of tools to genetically manipulate microglia prevents these critical experiments (Lacagnina et al., 2017, Linker et al., 2018).

Astrocytes

Astrocytes are abundant, bushy glial cells that provide metabolic support for neurons, control synaptic microenvironments, and influence circuit development (Kim et al., 2017). I demonstrated that nicotine robustly increased the astrocyte marker GFAP in the adolescent NAc. I did not pursue this phenomenon further, as this increase was confined to the NAc and not observed in the BLA. However, the mechanism of this

increase and the role of astrocytes in nicotine-associated behaviors should be addressed in future studies.

Conclusion

Overall, I have shown that adolescent nicotine exposure promotes a maladaptive reactive microglial state, which causes an increase in cocaine reinforcement and synaptic plasticity in reward encoding regions. Moreover, I have demonstrated neuronal D2 receptors as a critical interface for nicotine-induced changes to cocaine reinforcement, and microglial activation. Importantly, my data emphasize the exceptionally deleterious effects of nicotine on the adolescent brain. Since the introduction of e-cigarettes, teen nicotine use is increasing at an astounding rate, and over 40% of high school students have used e-cigarettes (MTF, 2018; CDC 2019). Given this, understanding how nicotine affects the adolescent brain, and identifying novel therapeutics is essential to addressing this growing teen nicotine crisis (MTF, 2018; CDC 2019). I propose microglia are a promising therapeutic target that remains understudied.

A. Lárraga, J.D. Belluzzi, F.M. Leslie. Nicotine increases alcohol intake in adolescent male rats. Front. Behav. Neurosci., 11 (2017), p. 25

Adriani, W., Macri, S., Pacifici, R. & Laviola, G. (2002) Peculiar vulnerability to nicotine oral self- administration in mice during early adolescence. Neuropsychopharmacology, 27, 212-224.

Adriani, W., Spijker, S., Deroche-Gamonet, V., Laviola, G., Le Moal, M., Smit, A.B. & Piazza, P.V. (2003) Evidence for enhanced neurobehavioral vulnerability to nicotine during periadolescence in rats. J Neurosci, 23, 4712-4716.

Agrawal, R.G., Hewetson, A., George, C.M., Syapin, P.J. & Bergeson, S.E. (2011) Minocycline reduces ethanol drinking. Brain Behav. Immun., 25 (Suppl. 1), S165–S169.

Alajaji M, Lazenka MF, Kota D, Wise LE, Younis RM, Carroll FI, Levine A, Selley DE, Sim-Selley LJ, Damaj MI (2016). Early adolescent nicotine exposure affects later-life cocaine reward in mice. Neuropharmacology 105, 308-317.

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A.M., Sanchez-Vera, I. & Guerri, C. (2010) Pivotal role of TLR4 receptors in alcohol-induced neu- roinflammation and brain damage. J. Neurosci., 30, 8285–8295.

Alvarez VM, Rama Rao KV, Brahmbhatt M, Norenberg MD. Interaction between cytokines and ammonia in the mitochondrial permeability transition in cultured astrocytes. J Neurosci. Res. 2011;89(12):2028–2040. doi:10.1002/jnr.22708 [doi]. Retrieved from PM:21748779.

Andersen, S.L., Rutstein, M., Benzo, J.M., Hostetter, J.C., & Teicher, M.H. (1997) Sex differences in dopamine receptor overproduction and elimination. Neuroreport, 8, 1495–1498.

Anderson, D. J., Malysz, J., Grønlien, J. H., El Kouhen, R., Håkerud, M., Wetterstrand, C., Briggs, C. A. and Gopalakrishnan, M. (2009). Stimulation of dopamine release by nicotinic acetylcholine receptor ligands in rat brain slices correlates with the profile of high, but not low, sensitivity alpha4beta2 subunit combination. Biochemical Pharmacology 78(7), 844–851.

Aurelian, L., Warnock, K.T., Balan, I., Puche, A. & June, H. (2016) TLR4 signaling in VTA dopaminergic neurons regulates impulsivity through tyr- osine hydroxylase modulation. Transl. Psychiatry, 6, e815.

Beardsley PM, Hauser KF (2014). Glial modulators as potential treatments of psychostimulant abuse. Adv Pharmacol.;69:1–69.

Beattie, E.C., Stellwagen, D., Morishita, W., Bresnahan, J.C., Ha, B.K., Von Zastrow, M., Beattie, M.S. & Malenka, R.C. (2002) Control of synaptic strength by glial TNFalpha. Science, 295, 2282–2285.

Becker JB (2009) Sexual differentiation of motivation: a novel mechanism? Horm Behav 55:646–654. doi:10.1016/j.yhbeh.2009.03.014 pmid:19446081

Becker JB, Perry AN, Westenbroek C (2012) Sex differences in the neural mechanisms mediating addiction: a new synthesis and hypothesis. Biol Sex Differ 3:14. doi:10.1186/2042-6410-3-14 pmid:22676718

Bell, R.L., Lopez, M.F., Cui, C., Egli, M., Johnson, K.W., Franklin, K.M. & Becker, H.C. (2015) Ibudilast reduces alcohol drinking in multiple animal models of alcohol dependence. Addict. Biol., 20, 38–42.

Belluzzi, J.D., Lee, A.G., Oliff, H.S. & Leslie, F.M. (2004) Age-dependent effects of nicotine on locomotor activity and conditioned place preference in rats. Psychopharmacology (Berl), 174, 389-395.

Benes FM, Taylor JB, Cunningham MC. (2000) Convergence and plasticity of monoaminergic systems in the medial prefrontal cortex during the postnatal period: implications for the development of psychopathology. Cereb Cortex 10:1014–1027. 10.1093/cercor/10.10.1014

Bernheim, A., Halfon, O., & Boutrel, B. (2013) Controversies about the enhanced vulnerability of the adolescent brain to develop addiction. Front Pharmacol, 4, 118.

Bessis, A., Bechade C, Bernard D, and Roumier A. (2007). Microglial control of neuronal death and synaptic properties. Glia, 55, 233–238

Bialas AR, Stevens B. (2013). TGF-β signaling regulates neuronal C1q expression and developmental synaptic refinement. Nature Neuroscience 12, 1173-82.

Biederman, J., Monuteaux, M.C., Mick, E., Wilens, T.E., Fontanella, J.A., Poetzl, K.M., Kirk, T., Masse, J., & Faraone, S.V. (2006) Is cigarette smoking a gateway to alcohol and illicit drug use disorders? A study of youths with and without attention deficit hyperactivity disorder. Biol Psychiatry, 59, 258-264.

Blanco, A.M., Perez-Arago, A., Fernandez-Lizarbe, S. & Guerri, C. (2008) Ethanol mimics ligand-mediated activation and endocytosis of IL-1RI/ TLR4 receptors via lipid rafts caveolae in astroglial cells. J. Neurochem., 106, 625–639.

Boykan, R., Messina, C. R., Chateau, G., Eliscu, A., Tolentino, J. and Goniewicz, M. L. (2019). Self-Reported Use of Tobacco, E-cigarettes, and Marijuana Versus Urinary Biomarkers. Pediatrics 143(5).

Boykan, R., Messina, C. R., Chateau, G., Eliscu, A., Tolentino, J. and Goniewicz, M. L. (2019). Self-Reported Use of Tobacco, E-cigarettes, and Marijuana Versus Urinary Biomarkers. Pediatrics 143(5).

Brenhouse, H. C. and Schwarz, J. M. (2016). Immunoadolescence: Neuroimmune development and adolescent behavior. Neuroscience and Biobehavioral Reviews 70288–299.

Brenhouse, H.C., Sonntag, K.C., & Andersen, S.L. (2008) Transient D1 dopamine receptor expression on prefrontal cortex projection neurons: relationship to enhanced motivational salience of drug cues in adolescence. J Neurosci, 28, 2375–2382.

Bunnell, R.E., Agaku, I.T., Arrazola, R.A., Apelberg, B.J., Caraballo, R.S., Corey, C.G., Coleman, B.N., Dube, S.R. & King, B.A. (2015) Intentions to Smoke Cigarettes Among Never-Smoking US Middle and High School Electronic Cigarette Users: National Youth Tobacco Survey, 2011-2013. Nicotine Tob Res, 17, 228-235.

Camenga, D.R., Delmerico, J., Kong, G., Cavallo, D., Hyland, A., Cummings, K.M., & Krishnan- sarin, S. (2014) Addictive Behaviors Trends in use of electronic nicotine delivery systems by adolescents. Addict Behav, 39, 338–340.

Casey, B. J., Getz, S., & Galvan, A. (2008). The adolescent brain. Developmental Review, 28, 62–77.

CDC Centers for Disease Control and Prevention. Notes from the Field: Electronic Cigarette Use Among Middle and High School Students — United States, 2011–2012. Morbidity and Mortality Weekly Report 2013; 62(35):729-730.

Cengelli, S., O'Loughlin, J., Lauzon, B., & Cornuz, J. (2012) A systematic review of longitudinal population-based studies on the predictors of smoking cessation in adolescent and young adult smokers. Tob Control, 21, 355-362.

Chen, H., Matta, S.G. & Sharp, B.M. (2007) Acquisition of nicotine self-administration in adolescent rats given prolonged access to the drug. Neuropsychopharmacology, 32, 700-709.

Chen, J. & Millar, W.J. (1998) Age of smoking initiation: implications for quitting. Health Rep, 9, 39-46(Eng); 39-48 (Fre).

Colby, S.M., Tiffany, S.T., Shiffman, S. & Niaura, R.S. (2000) Are adolescent smokers dependent on nicotine? A review of the evidence. Drug Alcohol Depend, 59 Suppl 1, S83-95.

Coller, J.K. & Hutchinson, M.R. (2012) Implications of central immune signaling caused by drugs of abuse: mechanisms, mediators and new thera- peutic approaches for prediction and treatment of drug dependence. Pharmacol. Ther., 134, 219–245.

Corongiu, S., Dessì, C. and Cadoni, C. (2019). Adolescence versus adulthood: Differences in basal mesolimbic and nigrostriatal dopamine transmission and response to drugs of abuse. Addiction Biology.

Coyle JT, Molliver ME. (1977) Major innervation of newborn rat cortex by monoaminergic neurons. Science 196:444–447. 10.1126/science.850788

Cui, C., Shurtleff, D. & Harris, R.A. (2014) Neuroimmune mechanisms of alcohol and drug addiction. Int. Rev. Neurobiol., 118, 1–12.

Dao, J.M., McQuown, S.C., Loughlin, S.E., Belluzzi, J.D. & Leslie, F.M. (2011) Nicotine alters limbic function in adolescent rat by a 5-HT1A receptor mechanism. Neuropsychopharmacology, 36, 1319-1331.

De Biase, L.M., Schuebel, K.E., Fusfeld, Z.H., Jair, K., Hawes, I.A., Cimbro, R., Zhang, H.Y., Liu, Q.R. et al. (2017) Local cues establish and maintain region-specific phenotypes of basal ganglia microglia. Neuron, 95, 341– 356. e6.

De Simone R, Ajmone-Cat MA, Carnevale D, Minghetti L (2005). Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. J Neuroinflammation. 25, 4.

Defoe, I. N., Dubas, J. S., Figner, B., & van Aken, M. A. (2015). A meta-analysis on age differences in risky decision making: Adolescents versus children and adults. Psychological Bulletin, 141, 48–84.

Degenhardt, L., Dierker, L., Chiu, W.T., Medina-Mora, M.E., Neumark, Y., Sampson, N., Alonso, J., Angermeyer, M., Anthony, J.C., Bruffaerts, R., de Girolamo, G., de Graaf, R., Gureje, O., Karam, A.N., Kostyuchenko, S., Lee, S., Lepine, J.P., Levinson, D., Nakamura, Y., Posada-Villa, J., Stein, D., Wells, J.E. & Kessler, R.C. (2010) Evaluating the drug use "gateway" theory using cross- national data: consistency and associations of the order of initiation of drug use among participants in the WHO World Mental Health Surveys. Drug Alcohol Depend, 108, 84-97.

Diamond A. (2002) Normal development of prefrontal cortex from birth to young adulthood: cognitive functions, anatomy, and biochemistry. In: Principles of frontal lobe function, Ed 2 (Stuss D KR, editor. , ed), pp 466–503. Oxford: Oxford UP.

Dierker L, Swendsen J, Rose J, He J, Merikangas K. Transitions to regular smoking and nicotine dependence in the adolescent national comorbidity survey (NCS-A) Ann. Beh. Med. 2012;43:394–401. doi: 10.1007/s12160-011-9330-9.

DiFranza JR, Rigotti NA, McNeill AD, Ockene JK, Savageau JA, St Cyr D, Coleman M. Initial symptoms of nicotine dependence in adolescents. Tob Control. 2000;9:313–319.

DiFranza, J.R., Savageau, J.A., Fletcher, K., Pbert, L., O'Loughlin, J., McNeill, A.D., Ockene, J.K., Friedman, K., Hazelton, J., Wood, C., Dussault, G. & Wellman, R.J. (2007) Susceptibility to nicotine dependence: the Development and Assessment of Nicotine Dependence in Youth 2 study. Pediatrics, 120, e974-983.

Dissing-Olesen, L., LeDue, J. M., Rungta, R. L., Hefendehl, J. K., Choi, H. B. and MacVicar, B. A. (2014). Activation of neuronal NMDA receptors triggers transient ATP-mediated microglial process outgrowth. The Journal of Neuroscience 34(32), 10511–10527.

Dudley, M. N. et al. Oral bacitracin vs vancomycin therapy for Clostridium difficile-induced diarrhea. A randomized double-blind trial. Arch Intern Med 146, 1101–1104 (1986).

Dutra, L.M. & Glantz, S.A. (2014) Electronic cigarettes and conventional cigarette use among U.S. adolescents: a cross-sectional study. JAMA Pediatr, 168, 610-617.

Dwyer, J.B., Broide, R.S., & Leslie, F.M. (2008) Nicotine and brain development. Birth Defects Res Part C - Embryo Today Rev, 84, 30-44.

Dwyer, J.B., McQuown, S.C. & Leslie, F.M. (2009) The dynamic effects of nicotine on the developing brain. Pharmacol Ther, 122, 125-139.

El Mestikawy, S., Wallén-Mackenzie, A., Fortin, G. M., Descarries, L. and Trudeau, L.-E. (2011). From glutamate co-release to vesicular synergy: vesicular glutamate transporters. Nature Reviews. Neuroscience 12(4), 204–216.

Eldreth, D., Hardin, M. G., Pavletic, N., & Ernst, M. (2013). Adolescent transformations of behavioral and neural processes as potential targets for prevention. Prevention Science, 14, 257–266.

Elmore MR, Najafi AR, Koike MA, Dagher NN, Spangenber EE, Rice RA, Kitazawa M, Matusow B, Nguyen H, West BL, Green KN. (2014) Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron:* 82(2) 380-97

Elmore, M. R. P., Elischer, M. F., Claeys, M. C. and Pajor, E. A. (2015). The effects of different flooring types on the behavior, health, and welfare of finishing beef steers. Journal of Animal Science 93(3), 1258–1266.

Erblich B, Zhu L, Etgen AM, Dobrenis K, Pollard JW (2011). Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. PLoS ONE, 6, p. e26317

Ernst, M. (2014). The triadic model perspective for the study of adolescent motivated behavior. Brain and Cognition, 89, 104–111.

Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in expression (2011). PLoS ONE.;6:e17996.

Eyo, U. B., Gu, N., De, S., Dong, H., Richardson, J. R., & Wu, L.-J. (2015). Modulation of microglial process convergence toward neuronal dendrites by extracellular calcium. The Journal of Neuroscience, 35(6), 2417–2422.

Felger, J.C. & Miller, A.H. (2012) Cytokine effects on the basal ganglia and dopamine function: the subcortical source of inflammatory malaise. Front. Neuroendocrinol., 33, 315–327.

Fombonne E (2009) Epidemiology of pervasive developmental disorders. Pediatr Res 65:591–598. doi:10.1203/PDR.0b013e31819e7203 pmid:19218885

Foster, J. A., Rinaman, L. and Cryan, J. F. (2017). Stress & the gut-brain axis: Regulation by the microbiome. Neurobiology of stress 7124–136.

Fröhlich, E. E. et al. Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication (2016). Brain Behav Immun 10.1016/j.bbi.2016.02.020

Frost JL, Schager DP (2016). Microglia: Architects of the Developing Nervous System. Trends in Cellular Biology 8, 587 -97.

Fujita Y, Kunitachi S, Iyo M, Hashimoto K (2012). The antibiotic minocycline prevents methamphetamine-induced rewarding effects in mice. Pharmacol Biochem Behav. 101(2):303–306.

Garwood CJ, Pooler AM, Atherton J, Hanger DP, Noble W (2011). Astrocytes are important mediators of Abeta-induced neurotoxicity and tau phosphorylation in primary culture. Cell Death Dis.

Ginhoux F, Greter M,Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, L.G. Ng, Stanley ER. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science, 330 (2010), pp. 841-845

Ginzel, K.H., Maritz, G.S., Marks, D.F., Neuberger, M., Pauly, J.R., Polito, J.R., Schulte-Hermann, R., & Slotkin, T.A. (2007) Critical review: nicotine for the fetus, the infant and the adolescent? J Health Psychol, 12, 215–224.

Gogtay, N. & Thompson, P.M. (2010) Mapping gray matter development: Implications for typical development and vulnerability to psychopathology. Brain and Cognition 72, 6–15.

Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greenstein, D., Vaituzis, A. C. et al. (2004). Dynamic mapping of human cortical development during childhood through early adulthood. Proceedings of the National Academy of Sciences United States of America, 101, 8174–8179.

Graber JA (2013) Pubertal timing and the development of psychopathology in adolescence and beyond. Horm Behav 64:262–269. doi:10.1016/j.yhbeh.2013.04.003 pmid:23998670

Grana, R., Benowitz, N. & Glantz, S.A. (2014) E-cigarettes: a scientific review. Circulation, 129,

Guan, Y.-Z., Jin, X.-D., Guan, L.-X., Yan, H.-C., Wang, P., Gong, Z., Li, S.-J., Cao, X., Xing, Y.-L. and Gao, T.-M. (2015). Nicotine inhibits microglial proliferation and is neuroprotective in global ischemia rats. Molecular Neurobiology 51(3), 1480–1488.

Guneykaya, D., Ivanov, A., Hernandez, D. P., Haage, V., Wojtas, B., Meyer, N., Maricos, M., Jordan, P., Buonfiglioli, A., Gielniewski, B., Ochocka, N., Cömert, C., Friedrich, C., Artiles, L. S., Kaminska, B., Mertins, P., Beule, D., Kettenmann, H. and Wolf, S. A. (2018). Transcriptional and Translational Differences of Microglia from Male and Female Brains. Cell reports 24(10), 2773–2783.e6.

Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Hammond, T. R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., Walker, A. J., Gergits, F., Segel, M., Nemesh, J., Marsh, S. E., Saunders, A., Macosko, E., Ginhoux, F., Chen, J., Franklin, R. J. M., Piao, X., McCarroll, S. A. and Stevens, B. (2019). Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. Immunity 50(1), 253–271.e6.

Hanna, E.Z., Yi, H., Dufour, M.C., & Whitmore, C.C. (2001) The relationship of early-onset regular smoking to alcohol use, depression, illicit drug use, and other risky behaviors during early adolescence: Results from the youth supplement to the Third National Health and Nutrition Examination Survey. J Subst Abuse, 13, 265-282.

Hashimoto, K. (2006) Protective effects of minocycline on behavioral changes and neurotoxicity in mice after administration of methamphetamine. Prog. Neuropsychopharmacol. Biol. Psychiatry, 30, 1381–1393.

Heyes W.F., . NeomycinK. Florey, N.W. Atwater, L. Chafetz, R. Bishara, B.T. Kho, G.A. Brewer, H.G. Leemann, B.C.R.Z. Rudy (Eds.), Host microbiota constantly control maturation and function of microglia. Analytical Profiles of Drug Substances, vol. 8, Academic Press, Inc., London (1979), pp. 399-488

Jenssen, B. P. and Boykan, R. (2019). Electronic cigarettes and youth in the united states: A call to action (at the local, national and global levels). Children (Basel, Switzerland) 6(2).

Kalsbeek A, Voorn P, Buijs RM, Pool CW, Uylings HB. (1988) Development of the dopaminergic innervation in the prefrontal cortex of the rat. J Comp Neurol 269:58–72. 10.1002/cne.902690105

Kandel DB, Chen K. Extent of nicotine dependence and smoking in the United States: 1991-1993. Nicotine Tob Res. 2000;2:263–274

Kandel, D.B., Yamaguchi, K., & Chen, K. (1992) Stages of progression in drug involvement from adolescence to adulthood: further evidence for the gateway theory. J Stud Alcohol, 53, 447– 457.

Kendler, K. S., Myers, J., Damaj, M. I., & Chen, X. (2014) Early smoking onset and risk for subsequent nicotine dependence: a monozygotic co-twin control study. Am J Psychiatry, 170, 408–413.

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O. and Vaiserman, A. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC Microbiology 17(1), 120.

Konstantoudaki, X., Chalkiadaki, K., Vasileiou, E., Kalemaki, K., Karagogeos, D. and Sidiropoulou, K. (2018). Prefrontal cortical-specific differences in behavior and synaptic plasticity between adolescent and adult mice. Journal of Neurophysiology 119(3), 822–833.

Kremer, Marcel & Henn, Anja & Kolb, Cornelia & Basler, Michael & Moebius, Jacqueline & Guillaume, Benoît & Leist, Marcel & Van den Eynde, Benoît & Groettrup, Marcus. (2010). Reduced Immunoproteasome Formation and Accumulation of Immunoproteasomal Precursors in the Brains of Lymphocytic Choriomeningitis Virus Infected Mice. Journal of immunology.

Kucukdereli, H., Allen, N.J., Lee, A.T., Feng, A., Ozlu, M.I., Conatser, L.M., Chakraborty, C., Workman, G. et al. (2011) Control of excitatory CNS synaptogenesis

by astrocyte-secreted proteins Hevin and SPARC. Proc. Natl Acad. Sci. USA, 108, E440–E449.

Kuhn C, Johnson M, Thomae A, Luo B, Simon SA, Zhou G, Walker QD (2010) The emergence of gonadal hormone influences on dopaminergic function during puberty. Horm Behav 58:122–137. doi:10.1016/j.yhbeh.2009.10.015 pmid:19900453

Lacagnina, M.J., Rivera, P.D. & Bilbo, S.D. (2017) Glial and neuroimmune mechanisms as critical modulators of drug use and abuse. Neuropsy- chopharmacology, 42, 156–177.

Lai, S., Lai, H., Page, J.B. & McCoy, C.B. (2000) The association between cigarette smoking and drug abuse in the United States. J Addict Dis, 19, 11-24.

Lambe EK, Krimer LS, Goldman-Rakic PS. (2000) Differential postnatal development of catecholamine and serotonin inputs to identified neurons in prefrontal cortex of rhesus monkey. J Neurosci 20:8780–8787.

Lee, M.S. & Kim, Y.-J. (2007) Signaling pathways downstream of pattern- recognition receptors and their cross talk. Annu. Rev. Biochem., 76, 447– 480.

Levin, E.D., Lawrence, S.S., Petro, A., Horton, K., Rezvani, A.H., Seidler, F.J. & Slotkin, T.A. (2007) Adolescent vs. adult-onset nicotine self-administration in male rats: duration of effect and differential nicotinic receptor correlates. Neurotoxicol Teratol, 29, 458-465.

Levitt P, Moore RY. (1979) Development of the noradrenergic innervation of neocortex. Brain Res 162:243–259. 10.1016/0006-8993(79)90287-7

Lewitus, G.M., Konefal, S.C., Greenhalgh, A.D., Pribiag, H., Augereau, K. & Stellwagen, D. (2016) Microglial TNF-a suppresses cocaine-induced plasticity and behavioral sensitization. Neuron, 90, 483–491.

Lidov HG, Grzanna R, Molliver ME. (1980) The serotonin innervation of the cerebral cortex in the rat: an immunohistochemical analysis. Neuroscience 5:207–227. 10.1016/0306-4522(80)90099-8

Linker, K. E., Cross, S. J. and Leslie, F. M. (2018). Glial mechanisms underlying substance use disorders. The European Journal of Neuroscience.

Maex, R., Grinevich, V. P., Grinevich, V., Budygin, E., Bencherif, M. and Gutkin, B. (2014). Understanding the role α7 nicotinic receptors play in dopamine efflux in nucleus accumbens. ACS Chemical Neuroscience 5(10), 1032–1040.

Manitt C, Mimee A, Eng C, Pokinko M, Stroh T, Cooper HM, Kolb B, Flores C. (2011) The netrin receptor DCC is required in the pubertal organization of mesocortical dopamine circuitry. J Neurosci 31:8381–8394. 10.1523/JNEUROSCI.0606-11.2011

Marynak, K.; Gentzke, A.; Wang, T.W.; Neff, L.; King, B.A. Exposure to Electronic Cigarette Advertising Among Middle and High School Students—United States, 2014–2016. MMWR Morb. Mortal. Wkly. Rep. 2018, 67, 294–299.

McCutcheon, J.E., Conrad, K.L., Carr, S.B., Ford, K. a, McGehee, D.S., & Marinelli, M. (2012) Dopamine neurons in the ventral tegmental area fire faster in adolescent rats than in adults. J Neurophysiol, 108, 1620–1630.

McQuown, S.C., Belluzzi, J.D. & Leslie, F.M. (2007) Low dose nicotine treatment during early adolescence increases subsequent cocaine reward. Neurotoxicol Teratol, 29, 66-73.

McQuown, S.C., Dao, J.M., Belluzzi, J.D. & Leslie, F.M. (2009) Age-dependent effects of low-dose nicotine treatment on cocaine-induced behavioral plasticity in rats. Psychopharmacology (Berl), 207, 143-152.

Meckel, K. R. and Kiraly, D. D. (2019). A potential role for the gut microbiome in substance use disorders. Psychopharmacology 236(5), 1513–1530.

Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A (2015). Harmful effects of nicotine. Indian Journal of Medicine Paediatrics Oncology 36, 24-31.

Miyamoto A, Wake H, Moorhouse AJ, Nabekura J (2013). Microglia and synapse interactions: fine tuning neural circuits and candidate molecules 15, 7-70.

Miyamoto, N., Maki, T., Shindo, A., Liang, A.C., Maeda, M., Egawa, N., Itoh, K., Lo, E.K. et al. (2015) Astrocytes promote oligodendrogenesis after white matter damage via brain-derived neurotrophic factor. J. Neurosci., 35, 14002–14008.

Mychasiuk, R. and Metz, G. A. S. (2016). Epigenetic and gene expression changes in the adolescent brain: What have we learned from animal models? Neuroscience and Biobehavioral Reviews 70189–197.

Nandi S, Gokhan S, Dai XM, Wei S, Enikolopov G, Lin H, Mehler MF, Stanley ER. The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation (2012). Dev. Biol., 367, pp. 100-113

Naneix F, Marchand AR, Di Scala G, Pape JR, Coutureau E. (2012) Parallel maturation of goal-directed behavior and dopaminergic systems during adolescence. J Neurosci 32:16223–16232. 10.1523/JNEUROSCI.3080-12.2012

Natividad, L.A., Torres, O.V., Friedman, T.C. & O'Dell, L.E. (2013) Adolescence is a period of development characterized by short- and long-term vulnerability to the rewarding effects of nicotine and reduced sensitivity to the anorectic effects of this drug. Behav Brain Res, 257, 275- 285.

Neurauter, G., Schr€ocksnadel, K., Scholl-B€urgi, S., Sperner-Unterweger, B., Schubert, C., Ledochowski, M. & Fuchs, D. (2008) Chronic immune stim- ulation correlates with reduced phenylalanine turnover. Curr. Drug Metab., 9, 622–627.

Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science, 308, 1314–1318.

Noda M, Kobayashi AI (2017). Nicotine inhibits activation of microglial proton currents via interactions with α7 acetylcholine resceptors. Journal of Physiological Science 67, 235-245.

Northcutt, A.L., Hutchinson, M.R., Wang, X., Baratta, M.V., Hiranita, T., Cochran, T.A., Pomrenze, M.B., Galer, E.L. et al. (2015) DAT isn't all that: cocaine reward and reinforcement require Toll-like receptor 4 signal- ing. Mol. Psychiatry, 20, 1525–1537.

O'Dell, L.E., Torres, O.V., Natividad, L.A. & Tejeda, H.A. (2007) Adolescent nicotine exposure produces less affective measures of withdrawal relative to adult nicotine exposure in male rats. Neurotoxicol Teratol, 29, 17-22.

O'Loughlin J, DiFranza J, Tyndale RF, Meshefedjian G, McMillan-Davey E, Clarke PB, Hanley J, Paradis G (2003). Nicotine-dependence symptoms are associated with smoking frequency in adolescents. Am J Prev Med. 25:219–225.

O'Dell, L.E., Bruijnzeel, A.W., Smith, R.T., Parsons, L.H., Merves, M.L., Goldberger, B.A., Richardson, H.N., Koob, G.F. & Markou, A. (2006) Diminished nicotine withdrawal in adolescent rats: implications for vulnerability to addiction. Psychopharmacology (Berl), 186, 612-619.

O'Donnell, P. (2010) Adolescent maturation of cortical dopamine. Neurotox Res, 18, 306–312.

Ostby Y, Tamnes CK, Fjell AM, Westlye LT, Due-Tonnessen P, Walhovd KB (2009) Heterogeneity in Subcortical Brain Development: A Structural Magnetic Resonance Imaging Study of Brain Maturation from 8 to 30 Years. J Neurosci, 29, 11772-11782.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Pan- zanelli, P., Giustetto, M., Ferreira, T.A. et al. (2011) Synaptic pruning by microglia is necessary for normal brain development. Science, 333, 1456–1458.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Pan- zanelli, P., Giustetto, M., Ferreira, T.A. et al. (2011) Synaptic pruning by microglia is necessary for normal brain development. Science, 333, 1456–1458.

Paradise, J. (2014) Electronic cigarettes: smoke-free laws, sale restrictions, and the public health. Am J Public Health, 104, e17–8.

Pascual-Lucas, M., Fernandez-Lizarbe, S., Montesinos, J. & Guerri, C. (2014) LPS or ethanol triggers clathrin- and rafts/caveolae-dependent endocytosis of TLR4 in cortical astrocytes. J. Neurochem., 129, 448–462.

Paus, T. (2010) Growth of white matter in the adolescent brain: Myelin or axon? Brain and Cognition 72, 26–35.

Pfeiffer, T., Avignone, E. and Nägerl, U. V. (2016). Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines. Scientific reports 632422.

Pocock, J. M. and Kettenmann, H. (2007). Neurotransmitter receptors on microglia. Trends in Neurosciences 30(10), 527–535.

Pribiag, H. & Stellwagen, D. (2013) TNF-a downregulates inhibitory neuro- transmission through protein phosphatase 1-dependent trafficking of GABA(A) receptors. J. Neurosci., 33, 15879–15893.

Primus RJ, Kellogg CK (1989) Pubertal-related changes influence the development of environment-related social interaction in the male rat. Dev Psychobiol 22:633–643. doi:10.1002/dev.

Primus RJ, Kellogg CK (1990) Developmental influence of gonadal function on the anxiolytic effect of diazepam on environment-related social interaction in the male rat. Behav Pharmacol 1:437–446

Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene the CNS(2015). Nat Neurosci;18:965–77

Riazi, K., Galic, M. A., Kentner, A. C., Reid, A. Y., Sharkey, K. A. and Pittman, Q. J. (2015). Microglia-dependent alteration of glutamatergic synaptic transmission and plasticity in the hippocampus during peripheral inflammation. The Journal of Neuroscience 35(12), 4942–4952.

Rosenberg DR, Lewis DA. (1994) Changes in the dopaminergic innervation of monkey prefrontal cortex during late postnatal development: a tyrosine hydroxylase immunohistochemical study. Biol Psychiatry 36:272–277. 10.1016/0006-3223(94)90610-6.

SAMHSA Substance Abuse and Mental Health Services Administration. Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings. N Series H-41. Rockville (MD): U.S. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, 2011. HHS Publication No. (SMA) 11-4658.

Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Santello, M. & Volterra, A. (2012) TNFa in synaptic function: switching gears. Trends Neurosci., 35, 638–647.

Schramm, N. L., Egli, R. E. and Winder, D. G. (2002). LTP in the mouse nucleus accumbens is developmentally regulated. Synapse 45(4), 213–219.

Schulz KM, Zehr JL, Salas-Ramirez KY, Sisk CL (2009) Testosterone programs adult social behavior before and during, but not after, adolescence. Endocrinology 150:3690–3698. doi:10.1210/en.2008-1708 pmid:19423759

Schwarz JM, Bilbo SD (2013). Adolescent morphine exposure affects long-term microglial function and later-life relapse liability in a model of addiction. J Neurosci;33:961–971.

Schwarz JM, Hutchinson MR, Bilbo SD. Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. J Neurosci. 2011;31:17835–17847.

Self, D.W., Belluzzi, J.D., Kossuth, S. & Stein, L. (1996) Self-administration of the D1 agonist SKF 82958 is mediated by D1, not D2, receptors. Psychopharmacology (Berl), 123, 303-306.

Shram, M.J., Funk, D., Li, Z. & Le, A.D. (2007) Acute nicotine enhances c-fos mRNA expression differentially in reward-related substrates of adolescent and adult rat brain. Neurosci Lett, 418, 286-291.

Shram, M.J., Funk, D., Li, Z., & Lê, A.D. (2006) Periadolescent and adult rats respond differently in tests measuring the rewarding and aversive effects of nicotine. Psychopharmacology (Berl), 186, 201-208.

Shytle, R.D., Mori, T., Townsend, K., Vendrame, M., Sun, N., Zeng, J., Ehrhart, J., Silver, A.A. et al. (2004) Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J. Neurochem., 89, 337–343.

Slotkin, T.A. (2004) Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol, 198, 132– 151.

Smith LK, Field EF, Forgie ML, Pellis SM (1996) Dominance and age-related changes in the play fighting of intact and post-weaning castrated male rats (Rattus norvegicus). Aggressive Behav 22:215–226

Snider, S.E., Vunck, S.A., van den Oord, E.J.C.G., Adkins, D.E., McClay, J.L. & Beardsley, P.M. (2012) The glial cell modulators, ibudilast and its amino analog, AV1013, attenuate methamphetamine locomotor activity and its sensitization in mice. Eur. J. Pharmacol., 679, 75–80.

Soczynska JK, Mansur RB, Brietzke E, Swardfager W, Kennedy SH, Woldeyohannes HO, McIntyre RS (2012). Novel therapeutic targets in depression: Minocycline as a candidate treatment. Behavioural Brain Research. 235(2):302–317.

Spear, L.P. (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev, 24, 417–463.

Steinberg, L. (2008) A Social Neuroscience Perspective on Adolescent Risk-Taking. Dev Rev, 28.

Stevens B, Allen NJ, Vazsques LE, Howell, GR, Chritopherson KS, Navid N (2007). The classical complement cascade mediates CNS synapse elimination. *Cell* 131, 1164–1178.

Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., Micheva, K.D., Mehalow, A.K. et al. (2007) The classical com- plement cascade mediates CNS synapse elimination. Cell, 131, 1164– 1178.

Syapin, P.J., Martinez, J.M., Curtis, D.C., Marquardt, P.C., Allison, C.L., Groot, J.A., Baby, C., Al-Hasan, Y.M. et al. (2016) Effective reduction in high ethanol drinking by semisynthetic tetracycline derivatives. Alcohol. Clin. Exp. Res., 40, 2482–2490.

Teicher, M.H., Andersen, S.L., & Hostetter, J.C. (1995) Evidence for dopamine receptor pruning between adolescence and adulthood in striatum but not nucleus accumbens. Dev Brain Res, 89, 167–172.

Thomas, AM *et al.* Adolescent nicotine exposure alters GABAA receptor signaling in the ventral tegmental area and increases adult ethanol self administration (2018). Cell Rep., 23, pp. 68-77

Torres, O.V., Tejeda, H.A., Natividad, L.A. & O'Dell, L.E. (2008) Enhanced vulnerability to the rewarding effects of nicotine during the adolescent period of development. Pharmacol Biochem Behav, 90, 658-663.

Tremblay, M.-E., Lowery, R.L. & Majewska, A.K. (2010) Microglial interac- tions with synapses are modulated by visual experience. PLoS Biol., 8, e1000527.

Trias, E., Beilby, P. R., Kovacs, M., Ibarburu, S., Varela, V., Barreto-Núñez, R., Bradford, S. C., Beckman, J. S. and Barbeito, L. (2019). Emergence of microglia bearing senescence markers during paralysis progression in a rat model of inherited ALS. Frontiers in aging neuroscience 1142.

Trotman HD, Holtzman CW, Ryan AT, Shapiro DI, MacDonald AN, Goulding SM, Brasfield JL, Walker EF (2013) The development of psychotic disorders in adolescence: a potential role for hormones. Horm Behav 64:411–419. doi:10.1016/j.yhbeh.2013.02.018 pmid:23998682

Trotta, T., Porro, C., Calvello, R. & Panaro, M.A. (2014) Biological role of Toll-like receptor-4 in the brain. J. Neuroimmunol., 268, 1–12.

Tseng, K.Y. & O'Donnell, P. (2005) Post-pubertal emergence of prefrontal cortical up states induced by D1-NMDA co-activation. Cereb Cortex, 15, 49-57.

Tseng, K.Y. & O'Donnell, P. (2007) D2 dopamine receptors recruit a GABA component for their attenuation of excitatory synaptic transmission in the adult rat prefrontal cortex. Synapse, 61, 843-850.

USDHHS U.S. Department of Health and Human Services. Preventing Tobacco Use Among Youth and Young Adults: A Report of the Surgeon General. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2012.

Van Rensburg, K. J., Taylor, A., & Hodgson, T. (2009). The effects of acute exercise on attentional bias towards smoking-related stimuli during temporary abstinence from smoking. Addiction (Abingdon, England), 104, 1910–1917.

Vetter-O'Hagen CS, Spear LP (2011) The effects of gonadectomy on age- and sex-typical patterns of ethanol consumption in Sprague-Dawley rats. Alcohol Clin Exp Res 35:2039–2049. doi:10.1111/j.1530-0277.2011.01555.x pmid:21651581

Volkow ND, Fowler JS, Wang G-J (2004). The addicted human brain viewed in the light of imaging studies: brain circuits and treatment strategies. Neuropharmacology.;47:3–13. doi: 10.1016/j.neuropharm.2004.07.019.

Wahlstrom, D., Collins, P., White, T., & Luciana, M. (2010) Developmental changes in dopamine neurotransmission in adolescence: behavioral implications and issues in assessment. Brain Cogn, 72, 146–159.

Wake, H., Moorhouse, A.J., Jinno, S., Kohsaka, S. & Nabekura, J. (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J. Neurosci., 29, 3974–3980.

Watkins, L.R., Hutchinson, M.R., Rice, K.C. & Maier, S.F. (2009) The “toll” of opioid-induced glial activation: improving the clinical efficacy of opi- oids by targeting glia. Trends Pharmacol. Sci., 30, 581–591.

Walker, D. M., Bell, M. R., Flores, C., Gulley, J. M., Willing, J. and Paul, M. J. (2017). Adolescence and reward: making sense of neural and behavioral changes amid the chaos. The Journal of Neuroscience 37(45), 10855–10866.

WHO Report on the Global Tobacco Epidemic, 2011: Warning About the Dangers of Tobacco. Geneva: World Health Organization.

Willing J, Cortes LR, Brodsky JM, Kim T, Juraska JM. (2017) Innervation of the medial prefrontal cortex by tyrosine hydroxylase immunoreactive fibers during adolescence in male and female rats. Dev Psychobiol 59:583–589. 10.1002/dev.21525

Wills, T.A., Knight, R., Williams, R.J., Pagano, I. & Sargent, J.D. (2015) Risk factors for exclusive e- cigarette use and dual e-cigarette use and tobacco use in adolescents. Pediatrics, 135, e43-51.

Wommack JC, Delville Y (2007) Stress, aggression, and puberty: neuroendocrine correlates of the development of agonistic behavior in golden hamsters. Brain Behav Evol 70:267–273. doi:10.1159/000105490 pmid:17914258

Wu Y, Dissing-Olesen L, MacVicar BA, and Stevens B (2015). Microglia: Dynamic Mediators of Synapse Development and Plasticity. Trends in Cellular Biology 36, 605-613 .

Wu, Y., Dissing-Olesen, L., MacVicar, B.A. & Stevens, B. (2015) Microglia: dynamic mediators of synapse development and plasticity. Trends Immu- nol., 36, 605–613.

Xu, P., Xu, Y., Hu, B., Wang, J., Pan, R., Murugan, M., Wu, L.-J. and Tang, Y. (2015). Extracellular ATP enhances radiation-induced brain injury through microglial activation and paracrine signaling via P2X7 receptor. Brain, Behavior, and Immunity 5087–100.

Yuan M, Cross SJ, Loughlin SE, Leslie FM (2015). Nicotine and the adolescent brain. Journal of Physiology 593, 3397-3412

Zhang L, Kitaichi K, Fujimoto Y, Nakayama H, Shimizu E, Iyo M, Hashimoto K. Protective effects of minocycline on behavioral changes and neurotoxicity in mice after administration of methamphetamine (2006). Prog Neuropsychopharmacol Biol Psychiatry. 30(8):1381–1393.