12-2013

Full-Gestational Exposure to Nicotine and Ethanol Results in Long-Term Dysregulation in the Ventral Tegmental Area

Emily Elizabeth Roguski
University of Tennessee Health Science Center

Full-Gestational Exposure to Nicotine and Ethanol Results in Long-Term Dysregulation in the Ventral Tegmental Area

Abstract

Nicotine is considered a gateway drug for other drugs of abuse. The majority of smokers that begin smoking in adolescence, continue into adulthood. Multiple factors have been linked with smoking initiation, including maternal smoking. Drug exposure during pregnancy has long been suspected to exert deleterious effects on the fetal brain. Concurrent use of smoking and drinking alcohol throughout pregnancy is an all too frequent occurrence in the human population especially in disadvantaged and younger women.

The present study investigated the potential contributing factors for the enhanced nicotine selfadministration as seen in offspring with gestational exposure to nicotine and ethanol (Nic+EtOH).* We focused on the mesocorticolimbic pathway, specifically the λ-aminobutyric acid (GABA) and glutamate modulators of ventral tegmental area (VTA) dopamine (DA) neurons that project to the nucleus accumbens (NAcc).

Our animal model of full gestational exposure to Nic+EtOH exposes the developing brain to both drugs throughout the entire 3 trimester human equivalent [i.e. gestational days (GD) 1-22 and postnatal days (PN) 2-12]. Using self-administration (SA), in vivo microdialysis, quantitative PCR, and western blot analysis, we investigated alterations in the VTA of offspring.

We found that the enhancement of nicotine SA acquisition of offspring with gestational Nic+EtOH exposure is not due to changes in maternal-pup interactions. The offspring with gestational Nic+EtOH exposure have a dramatic change in neurotransmitter release in response to 30μg i.v. nicotine; that displays a 75% increase of NAcc DA and a 50% reduction of VTA GABA release. In comparison, pair-fed (PF) offspring show a 20% increase in NAcc DA and a 20% decrease in VTA GABA in response to nicotine. Nic+EtOH offspring also show sensitivity to n-methyl-d-aspartate (NMDA) in the VTA of offspring that results in a greater increase in NAcc DA and VTA glutamate release compared to the PF offspring. The enhanced acquisition of nicotine SA seen in Nic+EtOH offspring can be reduced to PF levels with disruption to the mesocorticolimbic pathway by infusion of either a GABA agonist or a NMDA receptor antagonist into the VTA.

These results correlate with the epidemiologic data that suggests that children with gestational drug exposure are more likely to smoke. The alteration of the mesocorticolimbic pathway present in the offspring with gestational Nic+EtOH exposure is present in both adult and adolescent offspring, suggesting that the neurochemical changes are long-lasting.

* Matta SG, Elberger AJ (2007) Combined exposure to nicotine and ethanol throughout full gestation results in enhanced acquisition of nicotine self-administration in young adult rat offspring. Psychopharmacology (Berl) 193: 199-213.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biomedical Sciences

Full-Gestational Exposure to Nicotine and Ethanol Results in Long-Term Dysregulation in the Ventral Tegmental Area

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Emily Elizabeth Roguski
December 2013

DEDICATION

I dedicate this work in memory of my father George F. Roguski for instilling in me the importance of hard work to achieve your goals and all the years of unconditional love and support.

I also dedicate my dissertation work in memory my mentor Dr. Shannon G. Matta for all her hours of reflecting, reading, encouraging, and patience. Most of all for taking a chance on me and helping me further my education.

ACKNOWLEDGEMENTS

I wish to thank my committee members who were more than generous with their expertise and precious time. A special thanks to Dr. Jeff Steketee, my committee chairman for always being there for help when need and most importantly for stepping in to help me finish. I would also like to thank Dr. Burt M Sharp for always having extra time throughout all my years at UT. Thank you Dr. Andrea J Elberger, Dr. Edwards A Park, and Dr. Steve Tavalin for agreeing to serve on my committee.

I would like to thank the University of Tennessee Health Science Center Neuroscience Institute for their support over the years.

Thank you to Dr. Hao Chen for teaching me every technique used in my experiments. I truly would not have been able to accomplish these experiments without your help.

Finally, to my Mum, Alex, and Bobbi Jo thank you for being there at any time and the love and support over the years.

ABSTRACT

Nicotine is considered a gateway drug for other drugs of abuse. The majority of smokers that begin smoking in adolescence, continue into adulthood. Multiple factors have been linked with smoking initiation, including maternal smoking. Drug exposure during pregnancy has long been suspected to exert deleterious effects on the fetal brain. Concurrent use of smoking and drinking alcohol throughout pregnancy is an all too frequent occurrence in the human population especially in disadvantaged and younger women.

The present study investigated the potential contributing factors for the enhanced nicotine self-administration as seen in offspring with gestational exposure to nicotine and ethanol (Nic+EtOH).* We focused on the mesocorticolimbic pathway, specifically the λ-aminobutyric acid (GABA) and glutamate modulators of ventral tegmental area (VTA) dopamine (DA) neurons that project to the nucleus accumbens (NAcc).

Our animal model of full gestational exposure to Nic+EtOH exposes the developing brain to both drugs throughout the entire 3 trimester human equivalent [i.e. gestational days (GD) 1-22 and postnatal days (PN) 2-12]. Using self-administration (SA), *in vivo* microdialysis, quantitative PCR, and western blot analysis, we investigated alterations in the VTA of offspring.

We found that the enhancement of nicotine SA acquisition of offspring with gestational Nic+EtOH exposure is not due to changes in maternal-pup interactions. The offspring with gestational Nic+EtOH exposure have a dramatic change in neurotransmitter release in response to 30μg i.v. nicotine; that displays a 75% increase of NAcc DA and a 50% reduction of VTA GABA release. In comparison, pair-fed (PF) offspring show a 20% increase in NAcc DA and a 20% decrease in VTA GABA in response to nicotine. Nic+EtOH offspring also show sensitivity to n-methyl-d-aspartate (NMDA) in the VTA of offspring that results in a greater increase in NAcc DA and VTA glutamate release compared to the PF offspring. The enhanced acquisition of nicotine SA seen in Nic+EtOH offspring can be reduced to PF levels with disruption to the mesocorticolimbic pathway by infusion of either a GABA agonist or a NMDA receptor antagonist into the VTA.

These results correlate with the epidemiologic data that suggests that children with gestational drug exposure are more likely to smoke. The alteration of the mesocorticolimbic pathway present in the offspring with gestational Nic+EtOH exposure is present in both adult and adolescent offspring, suggesting that the neurochemical changes are long-lasting.

---

*Matta SG, Elberger AJ (2007) Combined exposure to nicotine and ethanol throughout full gestation results in enhanced acquisition of nicotine self-administration in young adult rat offspring. Psychopharmacology (Berl) 193: 199-213

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-CSF	Artificial Cerebrospinal Fluid
ACh	Acetylcholine
AP5	DL-2-Amino-5-phosphonopentanoic acid
BEC	Blood Ethanol Concentration
Bwt	Body Weight
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DA	Dopamine
EtOH	EtOH Alone
GABA	λ-Aminobutyric Acid
GD	Gestational Day
HPA	Hypothalamic-pituitary-adrenal
LDTg	Laterodorsal Tegmental Nucleus
MLA	Methyllycaconitine
mPFC	Medial Prefrontal Cortex
MW	Molecular Weight
NAcc	Nucleus accumbens
nACh	Nicotinic Acetylcholine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
Nic	Nicotine Alone
Nic+EtOH	Nicotine and EtOH
NMDA	N-methyl-D-aspartate
NRT	Nicotine Replacement Therapy
OPA	Phthaldialdehyde
PF	Pair Fed Offspring
PN	Postnatal Day
PPTg	Pedunculopontine Tegmental Nucleus
qtPCR	Quantitative Polymerase Chain Reaction
SA	Self-Administration
SDS	Sodium Dodecyl Sulfate
SHR	Spontaneous Hypertensive Rats
VP	Ventral Pallidum
VTA	Ventral tegmental area
ZK20775	1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(fluoro-methyl) quinoxalin-1-yl] methylphosphonate
5-HT	Serotonin

CHAPTER 1. INTRODUCTION

Nicotine is considered a gateway drug that may lead to other drugs of abuse, however unlike other drugs, nicotine use is less likely to decline later in life (Breslau and Peterson 1996). The age of smoking initiation is an important factor in determining dependence and methods of smoking cessation. It has been reported that 90 percent of smokers tried their first cigarette before the age of 18 (Kandel et al. 2007) and those smokers are more probable to continue to smoke into adulthood (Colby *et al.* 2000). Symptoms of nicotine dependence in adolescent smokers do not appear to have a minimum requirement of nicotine intake (DiFranza *et al.* 2002). Adolescent smokers are less inclined to be daily smokers but when they are, they are likely to smoke less cigarettes compared to adult smokers (Colby et al. 2000). The adolescents who experience symptoms of nicotine dependence do not represent a small portion of the population and for many those symptoms began before the onset of daily smoking (DiFranza *et al.* 2006). A study demonstrated that the rapid onset of symptoms for nicotine dependence, as seen in adolescent smokers, appears to represent a large portion of the population (DiFranza *et al.* 2000). Nearly 21 percent of smokers display their first symptom of nicotine dependence within 3 months (Kandel et al. 2007). Research has indicated two factors associated with continued smoking: the number of cigarettes smoked and the sensation associated with the first cigarette (positive or negative experience) (Kandel et al. 2007).

The use of tobacco products annually is reported by 26.5 percent of the population over the age of 12 according to the most recent data, with the highest reported use by young adults aged 18-25 years and 66.5 percent of these smokers reporting concurrent use of alcohol (Substance Abuse and Mental Health Services Administration 2012). The availability of tobacco and alcohol because of legal sales allows both drugs to be seen as gateway drugs to other drugs of abuse (Grucza and Bierut 2006a; Myers and Kelly 2006). Sociocultural (i.e. family, peer, and economic) influences have been reported to influence smokers to drink alcohol, and those that drink alcohol to smoke (Bobo and Husten 2000). Smoking and drinking alcohol is an all too frequent occurrence especially in disadvantaged and younger women (Kandel et al. 1997; Substance Abuse and Mental Health Services Administration 2012). The prevalence of nicotine and alcohol dependence together in an individual suggests that the occurrence of one may not be independent of the other (Anthony and Echeagaray-Wagner 2000; Grucza and Bierut 2006b; Kahler et al. 2008).

Drug exposure during pregnancy has long been suspected to exert deleterious effects on the fetal brain. Compared with other likely drugs of abuse, smoking during pregnancy is less likely to decline throughout and continue after birth, thus exposing the child to second hand smoke (Cornelius and Day 2000). Maternal smoking has been correlated with low birth weight, behavioral problems, and initiation of smoking (Ernst et al. 2001; Kandel et al. 2007). In addition, the occurrence of sudden infant death has been correlated with maternal smoking (Lavezzi *et al.* 2013). Adolescent male children of mothers that smoke demonstrated a four-fold increase in the incidence of conduct disorder compared to male children of nonsmokers (Ernst et al. 2001). The female

adolescent children had a five-fold increase in frequency of drug abuse compared to children of nonsmokers (Ernst et al. 2001). It has also been shown that respiratory illnesses such as asthma are more common in children born to mothers who smoke (Maritz and Mutemwa 2012).

Evidence suggests that alcohol exposure during pregnancy at any stage in development can cause harm to the child (Kuczkowski 2007). The harmful properties of alcohol are well known because of its ability to easily cross the placental barrier (Kuczkowski 2007). Children exposed to larger amounts of alcohol prenatally can have changes in brain function as well as brain structure (Mattson *et al.* 2001). There is no safe level of alcohol intake during pregnancy, moreover, since alcohol can be transmitted through breast milk it is recommended that mothers refrain from drinking after giving birth (Substance Abuse and Mental Health Services Administration). Consequences of alcohol exposure during pregnancy are characterized as fetal alcohol syndrome and fetal alcohol spectrum disorder, which includes behavioral problems and poor social skills (Greenbaum *et al.* 2009).

An investigation into the occurrence of smoking during pregnancy by teenage mothers found that 58 percent of those who smoked reported concurrent use of alcohol during pregnancy (Cornelius *et al.* 2007). Given the frequency of concurrent nicotine and alcohol use, the disorders seen in children with gestational drug exposure may be partially due to the effects of both drugs (Buka et al. 2003; Cornelius and Day 2000; Greenbaum et al. 2009; Kandel et al. 2007). The low birth weight seen in children with prenatal nicotine exposure often normalizes over time, however not when combined with prenatal alcohol exposure (Ernst et al. 2001). Both nicotine and alcohol attack the developing brain. Despite the frequency of concurrent use of both drugs, the gestational exposure to these drugs is typically studied separately, rather than together.

Our lab has developed a 3-trimester gestational model to study the combined effects of nicotine and ethanol exposure (Matta and Elberger 2007). Epidemiologically, women who do not stop smoking and/or drinking upon learning they are pregnant (first trimester), usually continue drug use throughout the entire pregnancy (Substance Abuse and Mental Health Services Administration), thereby exposing the fetal brain throughout the entire three trimesters. Most animal models of human drug use during pregnancy expose only the pregnant dam to the drug, and therefore, once the pups are born they no longer receive drug exposure. However, the first 12-14 postnatal days in a rodent are critical because they are the rodent developmental equivalent of the human third trimester (Dwyer *et al.* 2009). This is a period of rapid brain growth and synaptogenesis. Therefore, full gestational drug exposure, equivalent to the entire 3-human trimesters, requires postnatal drug treatment as well. We have previously shown the enhanced acquisition of nicotine self-administration in nicotine and ethanol (Nic+EtOH) offspring as adults (Matta and Elberger 2007) demonstrating that neuroplasticity induced by gestational drug exposure is a long lasting brain alteration. However, the mechanisms underlying this alteration are unknown.

The mesocorticolimbic pathway, which is essential for motivated drug-taking behavior, is comprised of the midbrain ventral tegmental area (*meso*; VTA), the forebrain nucleus accumbens (*limbic*; NAcc), and the medial prefrontal cortex (*cortico;* mPFC) (Koob and Nestler 1997). We are focused on the critical VTA dopaminergic (DA) neurons projecting to NAcc and their regulation, in part, by inhibitory λ-aminobutyric acid (GABA) (**Figure 1.1**) (Laviolette and van der Kooy 2004), as well as excitatory glutamatergic inputs (Mansvelder *et al.* 2003). The excitatory glutamatergic inputs arrive from cortex, laterodorsal tegmental nucleus (LDTg, and from local VTA glutamate neurons (Tolu et al. 2013). The inhibitory transmitter GABA modulates the DA neurons by inputs from NAcc, ventral pallidum (VP), pedunculopontine tegmental nucleus (PPTg), and from local interneurons (Adell and Artigas 2004; Tolu et al. 2013).

We have previously shown that i.v. nicotine resulted in an increase in accumbal DA release at doses as low as 60μg/kg in adult rats (Fu *et al.* 2000). However, only higher doses of nicotine (90μg/kg and higher) were able to produce an increase of VTA glutamate release (Fu et al. 2000). In addition, we have shown that the increase in NAcc DA and VTA glutamate in response to nicotine can be disrupted with intra-VTA infusion of an NMDA receptor antagonist (Fu et al. 2000). Disruption of reward-related learning can also be disrupted with the use of a NMDA receptor antagonist (Zellner *et al.* 2009). Additional studies have examined the modulation by inhibitory GABA on VTA DA neurons. The infusion of intra-VTA baclofen (GABA B receptor agonist) was able to reduce extracellular accumbal DA (Westerink *et al.* 1996). In addition it has been shown that the intra-VTA baclofen is able to disrupt nicotine SA behavior (Paterson *et al.* 2004).

The mesocorticolimbic pathway, specifically in the VTA, has been implicated in the use of EtOH. Studies using anesthetized rats demonstrated that low dose ethanol (0.01-0.03 g/kg) increases VTA GABA firing (Steffensen et al. 2009), whereas higher doses of ethanol (0.2-2.0 g/kg) demonstrate an inhibition of GABAergic activity (Gallegos et al. 1999). An electrophysiological study examined modulation of VTA DA neurons after chronic exposure to ethanol in C57BL/6J mice. VTA DA neurons following chronic ethanol displayed less inhibition by GABA compared to saline controls (Brodie 2002). However, the neurons with chronic ethanol displayed no difference in the presence of NMDA when compared to those with saline exposure (Brodie 2002). Voluntary intake of ethanol by C57BL/6J mice using a model called "drinking in the dark" showed that the mice would drink until intoxicated. This model of ethanol intake can be disrupted by mecamylamine (nACh antagonist) (Hendrickson et al. 2009).

In addition, a study has shown that low doses of Nic+EtOH administered systemically produce an additive effect on NAcc shell DA release (Tizabi et al. 2007). This release can be blocked using systemic mecamylamine (Tizabi et al. 2007). This study provides some evidence for the prevalence of concurrent use of nicotine and alcohol.

In summary, nicotine and ethanol each exert profound effects on brain development, resulting in long-lasting behavioral alterations. The clinical outcomes of such developmental aberrations in mesocorticolimbic mechanisms that underlie addictive behavior are just now being investigated. Yet, the consequences of exposure to both

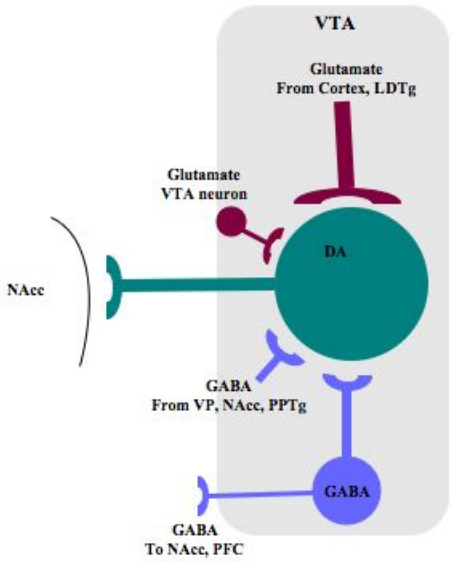


Figure 1.1: Schematic representation of the VTA and afferents. The DA (Green) output neuron is regulated by glutamate (Purple) and GABA (Blue) neurotransmitters in the VTA (grey shaded area). DA neurons are modulated by glutamate projections from cortex, LDTg, PPTg and from local interneurons. GABA local interneuons inhibit DA neurons as well as projections from NAcc and VP.

Source: Adapted by permission from Macmillan Publishers Ltd: Tolu S, Eddine R, Marti F, David V, Graupner M, Pons S, Baudonnat M, Husson M, Besson M, Reperant C, Zemdegs J, Pages C, Hay YA, Lambolez B, Caboche J, Gutkin B, Gardier AM, Changeux JP, Faure P, Maskos U (2013) Co-activation of VTA DA and GABA neurons mediates nicotine reinforcement. Mol Psychiatry 18: 382-93.

agents has not been studied experimentally, despite strong epidemiological evident for co-morbid effects due to the high prevalence of cigarette smoking by young women who consume alcohol during their child-bearing years.

For these reasons, we used our model of gestational Nic+EtOH exposure to examine the long-term alterations in the offspring. We hypothesize, that the gestational drug exposure to the combination of nicotine and ethanol results in a long-term alteration in glutamatergic and GABAergic function in the VTA of adult and adolescent Nic+EtOH offspring, which contributes to their enhanced acquisition of nicotine self-administration behavior. The objectives of the studies were to: (A) Determine whether the enhanced nicotine SA of Nic+EtOH offspring is the result of maternal-offspring interactions or the gestational drug treatment. (B) Investigate the role of the mesocorticolimbic pathway in nicotine SA, specifically changes in glutamate and GABA modulation of VTA DA neurons of Nic+EtOH offspring compared to PF controls.

CHAPTER 2. FOSTERING *ITSELF* INCREASES NICOTINE SELF-ADMINISTRATION IN YOUNG ADULT MALE RATS*

Introduction

Drug exposure during pregnancy has long been reported to negatively affect the development of the fetal brain (Stanwood and Levitt 2004). Maternal behavior in the postnatal environment is also known to play an important role in determining offspring behavior in adolescence and adulthood (Barbazanges et al. 1996). Fostering offspring to drug naïve dams has long been considered necessary to control for lingering drug effects on the dam that may further alter offspring growth and development (Abel and Dintcheff 1978; Golub and Kornetsky 1975). It has become almost dogma in the literature that maternal prenatal drug exposure is a negative contributor to subsequent maternal-offspring interaction. This has resulted in the implementation of fostering as a standard practice without actual empirical testing of the effect that fostering *itself* may exert.

Our laboratory has previously shown that young adult male rats exposed to nicotine and ethanol (Nic+EtOH) throughout full gestation [i.e., gestational days (GD) GD2-20 and during postnatal days 2-12 (PN2-12), the rodent third trimester-equivalent of human gestation during which rapid brain growth and synaptogenesis occur (Dwyer et al. 2009; Puglia and Valenzuela 2010), exhibit increased acquisition of nicotine self-administration (SA) behavior (Matta and Elberger 2007). In addition, there were no significant differences in standard birth parameters between Nic+EtOH offspring and controls (Matta and Elberger 2007). Regardless, a frequent concern of grant and manuscript reviewers is adherence to the literature standard insists on fostering/cross-fostering all offspring to surrogate dams. With careful examination of the historical literature on gestational drug treatment protocols, it appears that the requirement of fostering has been based, at least in part, on studies of fetal alcohol syndrome (FAS). In such studies, the pregnant/nursing dam received gavaged EtOH at doses that raised her blood ethanol concentration (BEC) to approximately 340mg/dl or greater (Abel and Dintcheff 1978), doses rarely seen in the human population. In these reports, the dams that did manage to survive the EtOH dosing showed significant intoxication, including staggering gait, slowed respiration, and in many cases comatose behavior that lasted for > 2h. Offspring that survived this dosing protocol were born late, had low birth weights, and showed delayed maturation parameters (Eppolito and Smith 2006). Obviously, maternal-offspring interactions were compromised. In contrast, our dosing protocol produced BECs' of approximately 150mg/dl and the dams showed no differences in retrieval or covering behavior (data not shown). The aim of the current study was to determine whether fostering would modify the increased acquisition of nicotine SA behavior, previously seen in offspring with full gestational (GD2-PN12) exposure to Nic+EtOH compared to pair-fed (PF) controls.

---

*Reprinted with permission from Springer and Psychopharmacology. Roguski EE, Chen H, Sharp BM, Matta SG. Fostering *itself* increases nicotine self-administration in young adult male rats. Psychopharmacology 2013 Sept;229(2)227-34.

Based on the complete lack of differences in litter parameters reported by Matta and Elberger (2007), we felt it was critical to determine if the increased SA behavior of the Nic+EtOH offspring compared to PF controls was the due to the effect of the drugs on the developing pups or the indirect effect of changes in maternal-offspring interaction. We found that fostering itself has a significant effect on locomotor activity regardless of gestational treatment. Surprisingly, fostering increased nicotine SA only in the fostered PF controls, so that the behavior resembled that seen in the drug treated Nic+EtOH offspring. Therefore, our results show that fostering *itself* can contribute to altered behavior in the offspring, demonstrating that fostering is not an neutral control. Rather, it should be considered a unique experimental condition that specifically interacts with other treatments.

Materials and Methods

Breeding and Gestational Drug Exposure: The model of gestational exposure was conducted as described in Matta and Elberger (2007). Briefly, Sprague-Dawley rats (Harlan, IN, USA) were group housed with a reverse light cycle (11 AM off and 11 PM on). Female rats were paired with male breeders overnight and then randomly assigned to the experimental Nic+EtOH group, or the controls (Nicotine-alone or PF groups) on sperm positive day 1 (GD1). Pair feeding was accomplished by providing the control dams with the same amount of food consumed by the Nic+EtOH dam on the same gestational day. For prenatal drug exposure of the experimental Nic+EtOH group, EtOH was administered by daily gastric gavage of 4g/kg from GD2 until GD20 and nicotine 2mg/kg/day (pH = 7.2; calculated as free base; (−)-Nicotine hydrogen tartrate salt; Sigma Aldrich Co., St. Louis, MO, USA) was delivered by mini-osmopump (Alzet 2ML4; Durect, Cuppertino, CA, USA) implanted on GD3 (**Figure 2.1A**). For prenatal exposure in PF dams, isocaloric maltose dextran (MD) was gavaged, instead of EtOH, and dams received a water-filled mini-osmopump (**Figure 2.1B**). Prenatal Nicotine-alone dams were gavaged with MD and fitted with a 2mg/kg/day nicotine mini-osmopump, to serve as the surrogate for Nic+EtOH offspring (**Figure 2.2A**). After birth (PN0), litters were randomly culled to 10 pups (5 female + 5 male when possible) on PN1 and drug treatments continued from postnatal PN 2-12, the rodent equivalent of the human third trimester. For both fostered and non-fostered Nic+EtOH offspring, the nicotine mini-osmopump in their dam (Nic+EtOH and Nicotine-alone) was replaced on PN2 with one containing 8mg/kg/day, in order to deliver sufficient nicotine to the pups through the dams' milk, as reported previously (Matta and Elberger 2007); EtOH was delivered by gavaging the offspring twice daily, 30 minutes apart, for a total of 4g/kg (**Figure 2.1A**). The non-fostered PF pups received 2 isocaloric MD gavages daily and nursed from their own dam, fitted with a new water mini-osmopump replaced on PN2 to control for surgical effects (**Figure 2.1B**). All offspring were weaned at PN21, group housed by litter, and allowed to mature with unrestricted access to food and water until they were single housed starting on PN60 for nicotine SA experiments. Daily handling and pseudo-gavaging of dams prior to pregnancy minimized stress. Pups were never separated from their littermates, except when an individual pup was being gavaged (<1.5 min); each litter

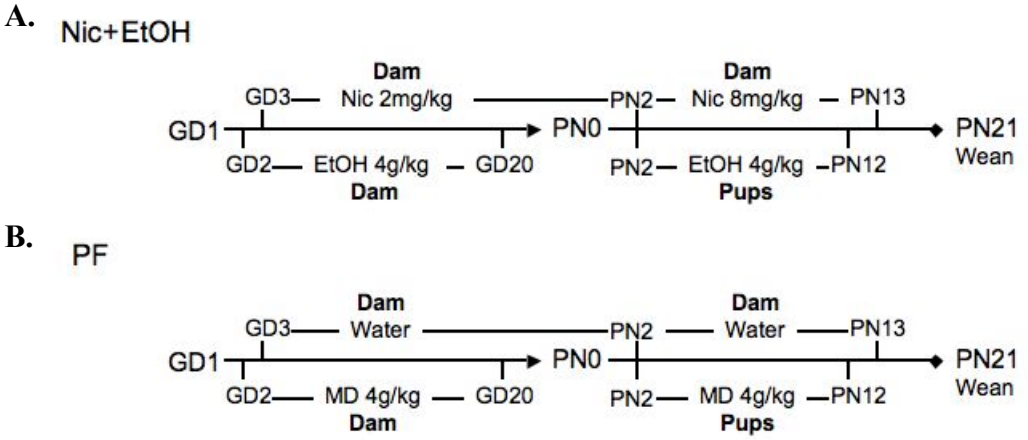


Figure 2.1: Protocol for drug treatments in non-fostered offspring. (A) Dams designated as Nic+EtOH on GD1, received EtOH gavages (4g/kg) beginning on GD2 and a nicotine mini-osmopump (2mg/kg/day) implantation on GD3. The non-fostered pups remained with their dam postnatally. Postnatal exposure to EtOH began on PN2 with twice daily gavages of EtOH (4g/kg total) to each pup. Nicotine exposure was administered by replacing the nicotine pump (8g/kg) in the dam, so that the pups received nicotine through the dams' milk. **(B)** PF dams received daily gavages beginning GD2 of MD (4g/kg) and a control mini-osmopump of water on GD2. These non-fostered pups also remained with their dam postnatally. Postnatal pup exposure with twice daily gavages of MD (4g/kg total) began on PN2 and a replacement control mini-osmopump was inserted in their dam on PN2.

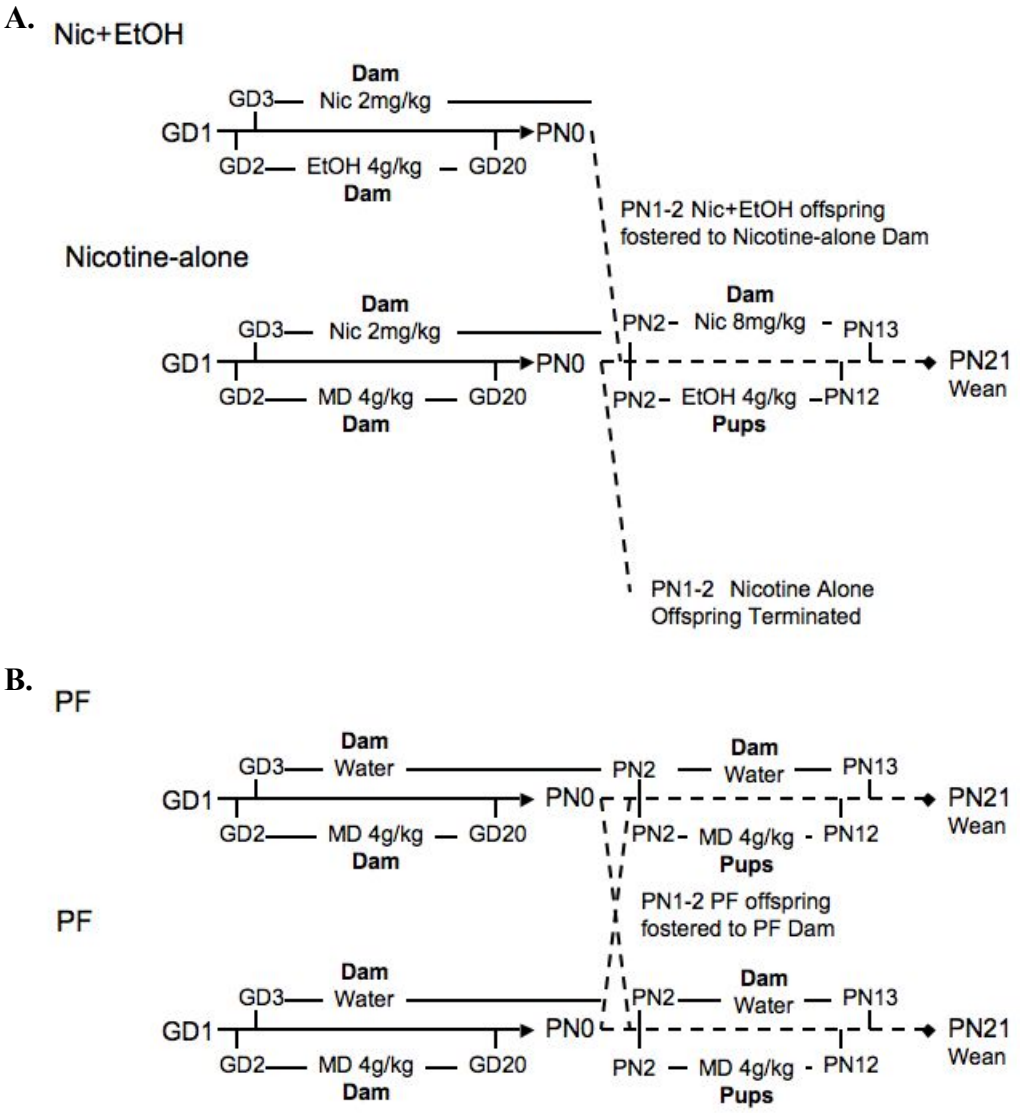


Figure 2.2: Protocol for drug treatments in fostered offspring. (A) Dams designated as Nic+EtOH received EtOH gavages (4g/kg) beginning on GD2 and a nicotine mini-osmopump (2mg/kg/day) implantation on GD3. Offspring were fostered on PN1-2 to a surrogate dam that received prenatal nicotine exposure (Nicotine-alone group) and whose pups were terminated at fostering. Postnatal exposure to EtOH began on PN2 with twice daily gavages of EtOH (4g/kg total) to each offspring. Nicotine exposure was administered by replacing the nicotine pump (8g/kg) in the surrogate dam, so that the pups received nicotine through the dams' milk. (**B)** PF offspring were fostered to different PF dams on PN1-2. Postnatal exposure for the offspring was given by twice daily gavages of MD (4g/kg total) beginning on PN2 and a replacement control mini-osmopump on PN2 in the surrogate.

was returned to its dam within 5-6 minutes at which time every dam recollected and covered her litter regardless of treatment group. All procedures were conducted in accordance with the NIH Guidelines for the Use and Care of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center.

Fostering: The fostering of the offspring was completed between PN1-2. Surrogate dams were timed to deliver at the same time as Nic+EtOH or PF litters. Since the experimental paradigm requires a 3-trimester equivalent drug exposure, postnatal Nic+EtOH offspring could not be fostered to a drug-naïve dam because the drug-naïve dam would have adverse reactions to the sudden exposure to an 8mg/kg/day nicotine mini-osmopump on PN2, which is required to deliver the appropriate dose of nicotine to the pups via the dams breast milk (Matta and Elberger 2007). Therefore, the fostered Nic+EtOH pups were placed with the Nicotine-alone surrogate dam that had been exposed to 2mg/kg nicotine prenatally (GD1-21) and gavaged daily with MD; these Nic+EtOH pups were gavaged with EtOH daily (**Figure 2.2A**). All the offspring from these designated Nicotine-alone surrogates were sacrificed at the time that the experimental Nic+EtOH offspring were fostered to those dams. Fostered PF offspring were placed with a new PF dam that received a new water mini-osmopump on PN2 and the pups received MD from P2-P12 (**Figure 2.2B**).

Litter Parameters: In addition to those previously reported (Matta and Elberger 2007), additional litter parameters were measured on PN1 to determine if there were developmental alterations due to gestational drug treatment. Pups with *in utero* exposure to Nic+EtOH or PF (n=9 each) from 5 Nic+EtOH litters and 4 PF litters were used to measure body weight (Bwt), volume distribution, crown-rump length, and brain weight. After Bwt measurement, the volume of water displaced when the pup was placed in a cylinder of water was determined, followed by measuring the length from the crown of the head to the beginning of the tail, and then the brain was removed and weighed. Since there were no sex differences in these (or the previous) parameters, the data were pooled.

Locomotor Activity: Activity in a novel environment was measured for adolescent male offspring (PN45) with a Micromax monitoring system (Accuscan Instruments, Columbus, OH, USA). Rats were placed in individual chambers (45×24×19cm) with dividers preventing each rat from observing the others. Data were collected in 5-minute increments during 1 hour with the lights off.

Nicotine Self-Administration: Young adult male offspring (PN60-75) were allowed chronic, almost unlimited (i.e., 23 h/d) access to bar press for nicotine without prior training, shaping, or food deprivation, using our established model (Valentine et al. 1997). Briefly, after implantation of a jugular cannula, rats were placed into individual operant chambers (Coulbourn Instruments, Allentown, PA, USA) enclosed in ventilated,

sound-attenuating environmental boxes. The jugular line was connected to a microinjection pump (Med Associates, St. Albans, VT, USA) by a single channel swivel (Instech, Plymouth Metting, PA, USA). Rats were given 3 d to recover from surgery; thereafter, rats were provided free access for 23 h daily (1 h daily is required for animal husbandry and downloading data) to two randomly assigned levers positioned 5 cm above the floor, each with a green light above signaling the availability of the drug. Pressing the active lever ("active") signaled the computer (Graphic State Notation software, Coulbourn Instr.) to activate a pump that delivered 30µg/kg nicotine in a 50-µl heparinized saline bolus over 0.81 s on a fixed ratio (FR1) schedule with a 7 second delay; pressing the inactive lever ("inactive") had no programmed consequence. Self-administration day 1 (SA d1) was the first day nicotine was made available and the offspring were given 10 days free access to acquire nicotine SA on an FR1 schedule.

As per our previously reported criteria for this open access model (Valentine et al. 1997), acquisition of nicotine self-administration behavior to maintenance criteria was defined by: (1) stabilization of the number of active bar presses on the final 3 d to within 15% variation, (2) active bar presses greater than inactive presses, and (3) active presses greater than 12/day.

Statistics: Three-way ANOVA analyses were completed for SA, locomotor activity and the offspring growth curve to examine the interaction between cohort and fostering. One-way ANOVA analysis was completed for litter parameters with a Scheffe *post-hoc* comparison using SPSS software; significance was set at $p<0.05$. To reduce the number of animals used, once statistical significance was achieved, further rats were not tested. Values are mean $\pm$ SEM and the number of animals/group is indicated within parentheses in the text and figures.

Results

Litter Parameters: Litter parameters were measured on PN1 using 2 pups per treatment group for Nic+EtOH (n=9) and PF cohorts (n=9) (**Table 2.1**). These parameters were chosen because they are indicators of exposure to doses of gestational EtOH, used in models of FAS; such doses are greater than that administered in the current study. In marked contrast to reports on FAS, there were no differences in volume distribution, crown-rump length, or brain weight for Nic+EtOH or their PF controls. Similar to previous observations (Matta and Elberger 2007), there was no significant difference in the birth weight between the Nic+EtOH and PF offspring. In addition, peak BECs in Nic+EtOH dams and their offspring (154 ± 6 and 151 ± 6 mg/dl, respectively) were comparable to those commonly reported in humans cited for a DWI (i.e., driving while intoxicated) violation (Matta and Elberger 2007). Also, peak plasma nicotine levels (30 ± 3 and 21 ± 2 ng/ml, respectively) were lower than those eliciting nicotine-induced hypoxia (Slotkin et al. 1987). These data demonstrate that offspring were exposed to moderate doses of EtOH similar to those frequently imbibed by human binge drinkers

Table 2.1: Litter parameters.

Cohort	Birth Weight PN1 (g)	Volume Displacement (ml)	Crown-Rump Length (cm)	Brain Weight (mg)
Nic+EtOH (n=9)	6.2 ± 0.3	5.8 ± 0.4	4.2 ± 0.1	255.3 ± 6.9
PF (n=9)	6.5 ± 0.2	5.9 ± 0.2	4.3 ± 0.1	246.3 ± 8.4

No significant differences were seen for birth weight (PN1), volume distribution, crown-rump length, and brain weight. This demonstrates that the gestational drug exposure did not have any overt developmental effect on the offspring (Results are reported as average ± SEM).

and moderate smokers. Taken together, these data indicate that gestational drug treatment did not alter general indices of offspring health.

Offspring Growth Curve: **Figure 2.3** illustrates postnatal body weight gain among treatment groups. Fostering had no effect on body weight gain in the Nic+EtOH offspring, nor did it alter weight gain in the PF offspring; weight gain was similar in both treatment groups ($p=0.27$). These data demonstrate that neither fostering nor drug treatments altered growth during postnatal drug administration.

Locomotor Activity: Fostered Nic+EtOH and PF offspring had a higher total activity over the 1 hour session on PN45 compared to non-fostered Nic+EtOH and PF offspring ($F_{1,348}=4.1$; $p<0.05$) (**Figure 2.4**). Similarly, the non-fostered offspring (Nic+EtOH and PF) did not differ significantly from each other nor did the fostered Nic+EtOH and PF offspring behaviors differ.

Nicotine Self-Administration: To demonstrate that offspring learned the SA behavior active vs. inactive lever presses were compared. Both non-fostered and fostered PF rats expressed significant higher active lever presses compared to inactive lever presses for the entire 10-day period of nicotine SA behavior; PF non-fostered offspring ($F_{1,100}=97.36$ $p<0.01$); PF fostered ($F_{1,100}=150.68$, $p<0.01$). Surprisingly, the fostered PF offspring had significantly increased nicotine SA compared to non-fostered PF offspring ($F_{1,100}=16.55$, $p<0.01$) (**Figure 2.5A**). Furthermore, fostered offspring acquired the behavior by d4 ($p<0.05$, active vs. inactive bar presses by day), while non-fostered required a longer time interval (d8; $p<0.05$, active vs. inactive presses by day). Fostered PF offspring also exhibited significantly more bar presses than the non-fostered group during the last 3 d: 77±8 vs. 50±8 fostered vs. non-fostered, respectively ($F_{1,34}=5.7$, $p=0.02$).

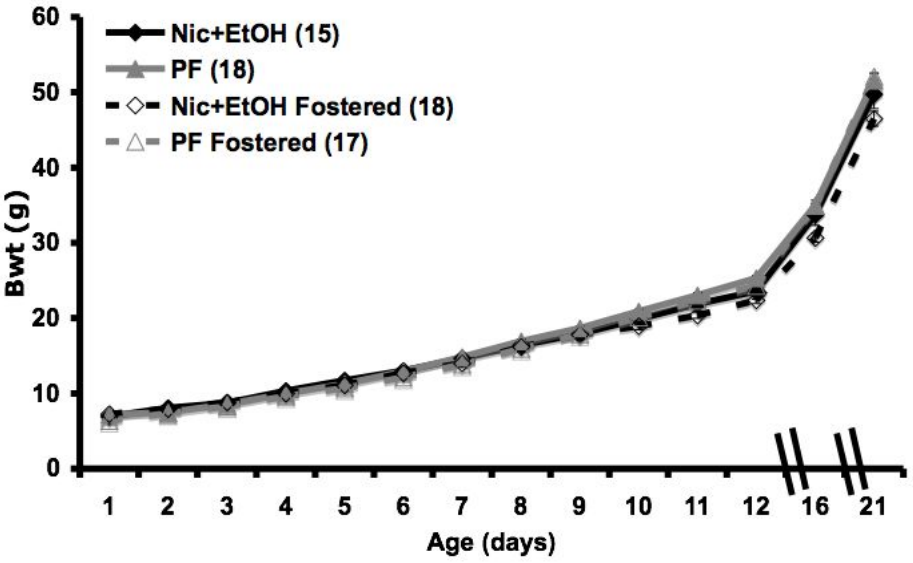


Figure 2.3: Postnatal Bwt gain in fostered and non-fostered Nic+EtOH and PF offspring. Bwt gain was not influenced by full gestational exposure nor by postnatal fostering.

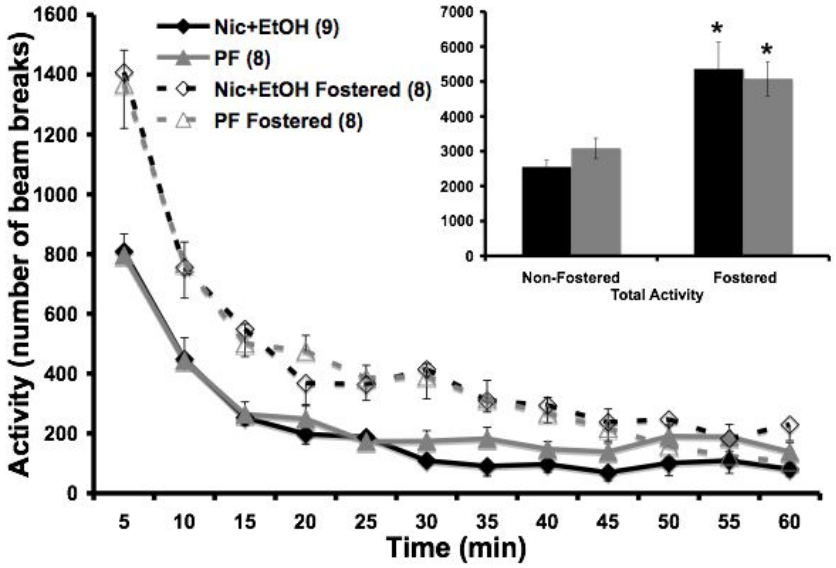
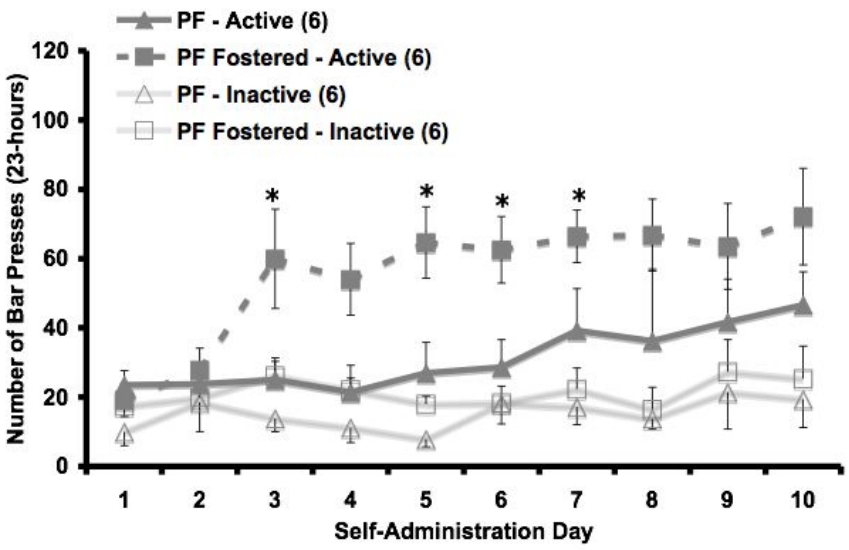


Figure 2.4: Locomotor activity for fostered and non-fostered Nic+EtOH and PF offspring at PN45. Fostered offspring demonstrated a higher number of beam breaks compared to the non-fostered offspring, regardless of full gestational drug treatment. This demonstrates that fostering *itself* can increase activity in a novel environment (Fostered vs. Non-fostered offspring *p=<.05).

A.



B.

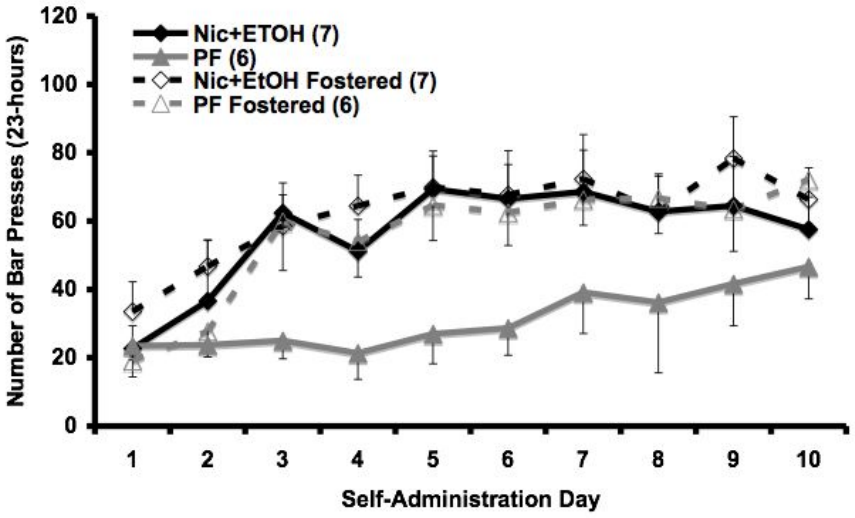


Figure 2.5: Nicotine SA of fostered and non-fostered PF and Nic+EtOH offspring. **(A)** In the PF control young-adult offspring, fostering alone significantly increased nicotine SA**.** PF offspring, both fostered and non-fostered, were given 23-hour access to acquire self-administration of 30ug/kg/inj of nicotine without priming, shaping, or training. Fostering increased lever presses on the active bar delivering nicotine 30µg/kg/inj (solid line), without affecting behavior on the inactive bar (no consequences lever; dashed line); *$p<0.05$, Active bar presses, PF non-fostered vs. PF fostered. (**B**) Fostering had no effect on the nicotine SA by the Nic+EtOH young adult offspring. Full gestational exposure (GD2-PN12) to Nic+EtOH resulted in increased levels of nicotine SA, as well as a shortening in the onset of initiation of nicotine SA, regardless of fostering status. Surprisingly, PF controls that had been fostered, but had no gestational drug exposure, demonstrated comparable levels of nicotine SA to Nic+EtOH offspring.

In contrast, **Figure 2.5B** shows that fostering Nic+EtOH offspring did not alter their SA behavior ($p=0.99$ for Nic+EtOH vs. Nic+EtOH Fostered). Active bar presses were greater than inactive presses in both groups: non-fostered Nic+EtOH offspring ($F_{1,120}=136.91$ $p<0.01$); Nic+EtOH Fostered ($F_{1,120}=70.67$ $p<0.01$; data not shown). Non-fostered Nic+EtOH, fostered Nic+EtOH, and fostered PF offspring showed significantly higher levels of nicotine SA in comparison to non-fostered PF offspring (active bar presses: $F_{1,220}=6.62$, $p<0.01$).

Discussion

Our data show that early postnatal fostering *per se* markedly increased nicotine SA in young adult PF offspring – the control group for Nic+EtOH full gestational treatment. These results indicate that the alteration of postnatal environment (i.e. maternal behavior; maternal grooming, nursing, etc.) increases the long-term drug taking behavior of the offspring.

There is a limitation of the three-trimester model of full gestational drug exposure, which was designed to approximate full three-trimester exposure in humans. During the third trimester (i.e. postnatally) in this model, we have attempted to approximate the continuous exposure of the postnatal pups to the blood nicotine levels they had while in utero. In contrast, since the EtOH was administered to the pregnant dams as a binge dose, we also administered the EtOH to the postnatal pups as a binge dose in order to control for BEC. That is, even if we had gavaged the nursing dam, there was no way we could guarantee that each individual pup would drink sufficient breast milk with EtOH within the timeframe of EtOH metabolism in the dam.

Full gestational exposure to relatively moderate amounts of both nicotine and EtOH did not alter birth parameters (PN1) or body weight gain in offspring (PN1-PN21) compared to PF controls. These results are in agreement with our previous report in which sex ratio, stillbirths, righting reflex, and the day of eye opening were similar between all treatment groups (Matta and Elberger 2007). Previous studies have shown that gestational EtOH exposure (4g/kg and 6g/kg) was associated with low birth weight and behavioral abnormalities in the offspring (Abel 1978; Abel and Dintcheff 1978). However, the outcome of low birth weight was inconsistent, even following relatively high doses of 6g/kg (Caul et al. 1979; Matta and Elberger 2007; Osborne et al. 1980); this could reflect differences in the mode of EtOH delivery or rat strain. Prenatal nicotine exposure has also been associated with low birth weight of pups, however this is seen only at higher doses (>6mg/kg/day) and not at the lower dose used herein (i.e., 2mg/kg/day) (Eppolito and Smith 2006; Navarro et al. 1989; Slotkin et al. 1987). Depending on drug and dose, prenatal drug exposure also may modify aspects of the postnatal environment. It has been shown that offspring with prenatal EtOH exposure fail to gain weight at the same rate as control offspring after being born with low birth weight and this lack of weight gain was thought to be attributed to factors in the postnatal environment (i.e. the drug exposed dam) and not to the prenatal EtOH exposure itself (Abel and Dintcheff 1978). In contrast, cross-fostering of control offspring to EtOH exposed dams did not alter the

weight gain of these offspring, demonstrating that the EtOH-treated dams themselves did not adversely affect the growth of the offspring (Osborne et al. 1980). Despite the lack of any correlation between drug-exposure in dams and offspring behavior, the authors of that early report still strongly recommended fostering to drug naïve-dams. However, our results show that regardless of gestational treatment (Nic+EtOH or PF), the postnatal growth curves for non-fostered offspring were consistent with our previous report (Matta and Elberger 2007) and did not differ from each other. Taken together, these data indicate that neither fostering nor gestational drug treatment altered the overall bodily growth and gross CNS maturation of offspring.

Fostering at different postnatal time points can exert divergent effects on behavior (Barbazanges et al. 1996; Darnaudery et al. 2004). It has been reported that maternal separation stress in early human life dysregulates the hypothalamic-pituitary-adrenal (HPA) stress axis, thus increasing the risk for drug taking behavior (Andersen and Teicher 2009; Vallee et al. 1999). Also, rat offspring fostered at PN5 or PN12 displayed enhanced stress responses, similar to those detected in offspring exposed to maternal separation at the same time intervals (Barbazanges et al. 1996). However, offspring fostered at PN1 did not display these increased stress responses (Darnaudery et al. 2004). In rodents, prolonged maternal separation stress (3h) at different postnatal ages increased subsequent dopamine responses to stress or stimulants in the mesocorticolimbic reward pathway (Brake et al. 2004). This reward pathway, which includes the ventral tegmental area, nucleus accumbens, and medial prefrontal cortex, is essential to the acquisition of drug self-administration (Koob and Nestler 1997). In addition to reported alterations in the HPA axis, altered maternal-offspring interactions also can affect components of this reward pathway. For example, repeated or acute separation stress (1-3h/daily from PN3-21), altered multiple neuronal circuits. Specifically, 1-hour separation from PN1-20 increased serotonin levels and serotonin turnover (5-HIAA/5HT ratio) in medial prefrontal cortex and hippocampus (Jezierski et al. 2006), while 1-hour separation from PN1-21 increased GABA in the medial prefrontal cortex (Helmeke et al. 2008). Finally, a recent report (Champagne and Curley 2009) showed that cross-fostering of drug naïve WKY pups to an spontaneous hypertensive rats (SHR) surrogate rat caused a shift in phenotype to that of SHR. Studies in progress are centered on modulation of GABAergic inhibition and glutamatergic excitation in the VTA of the Nic+EtOH offspring, as well as specific epigenetic changes, such as DNA methylation in these neuronal types.

The fostering/cross-fostering of offspring and the resultant locomotor activity has been reported to produce varied results. While offspring fostered on PN1 did not differ from controls, groups with maternal separation from the dam had higher locomotor response (Barbazanges et al. 1996). Adult rats fostered at later neonatal time points (PN5 or PN12) had a significantly higher locomotor responses compared to non-fostered offspring (Barbazanges et al. 1996). Also, it has been reported that neither fostering nor cross-fostering of Wistar and Fisher rats altered total motor activity for open field behavior. In contrast, a report on the cross-fostering of Lewis and Fisher strains demonstrated significant differences in locomotor activity for both males and females, whereas in-strain fostering of the offspring produced no differences in activity (Gomez-Serrano et al. 2001). Locomotor activity to a novel environment has been used in the past to separate

animals into higher anxiety vs. lower anxiety responder groups (Gancarz et al. 2011). High responder groups of younger rats (4 months old) have been associated with increased HPA axis activity in response to stress (Dellu et al. 1996). Responding for nicotine, methamphetamine, and even visual stimulus, has also been correlated with high locomotor response to novelty (Gancarz et al. 2011; Suto et al. 2001). Our locomotor results show that only fostered offspring, regardless of full gestational treatment group, had an increased response to a novel environment in adolescence. In addition, the inactive lever presses during self-administration did not increase with prenatal drug exposure or fostering, therefore demonstrating that offspring were not hyperactive in the SA chambers. We conclude from this that increased locomotor activity in a novel environment is not necessarily a predictor of a high nicotine self-administration.

In our study, we did not expect to see behavioral changes, since the PF control offspring were fostered on PN1 and there were no differences in litter parameters. Yet, we found dramatic increases in the nicotine SA behavior of fostered PF offspring that was comparable to the behavior of experimental offspring with full gestational exposure to Nic+EtOH. Self-administration behavior of the non-fostered PF offspring remained consistent with data previously reported (Matta and Elberger 2007). Active bar presses were less in non-fostered than fostered PF (e.g. 50 ± 8 vs. 77 ± 8) and acquisition time was longer (i.e. 8 days compared to 4 days for Nic+EtOH). Therefore, fostering alone, even in the absence of gestational exposure to drugs, is sufficient to alter nicotine SA behavior. In contrast, fostering itself did not change the enhanced nicotine SA behavior of Nic+EtOH pups. There were no differences in the number of active bar presses, in the ratio of active to inactive presses or in the number of days to acquire stable nicotine SA. The lack of any effect of fostering in these pups could be due, at least in part, to a maximal or "ceiling" effect of gestational drug treatment on the developing brain of Nic+EtOH offspring.

Variability in the effect of fostering has been reported for various drugs. Rats with prenatal methamphetamine exposure (5mg/kg/day) show improvement in maturation tests when fostered to drug-naïve dams (Pometlova et al. 2009). Similarly, while non-fostered rat offspring with *in utero* saline or morphine exposure (5mg/kg injection GD11-13, 10mg/kg GD14-18) did not exhibit higher operant response rates for cocaine (0.5mg/kg/inj) compared to controls, fostering *itself* increased responding for cocaine regardless of prenatal treatment group (Vathy et al. 2007). In our previous study, cross-fostering of offspring with prenatal nicotine exposure (3mg/kg/day) did not affect the increase in the dopaminergic response to a nicotine injection. However, cross-fostering of controls resulted in an increase in accumbal dopamine similar to that seen in offspring with prenatal nicotine exposure (Kane et al. 2004). These studies, as well as our data herein, support the idea that the fostering procedure itself, assumed to reduce negative maternal effects by many, is contraindicated and may actually introduce new sources of variance, subsequently changing pup behavior (Darnaudery et al. 2004).

In summary, fostering of PF offspring significantly increased the nicotine SA behavior in young adult rats without altering the enhanced SA behavior of the Nic+EtOH offspring. Indeed, the experimental practice of fostering *alone* elevated the nicotine self-

administration behavior of PF offspring to levels comparable to these Nic+EtOH rats. Therefore, in the current model of unlimited (i.e. 23-hour) nicotine access, fostering introduces a major confound that precludes its use as a control for environmental interactions during the neonatal period for our full gestational model of Nic+EtOH exposure. Thus, the dogmatic assertion that fostering is a crucial control is inaccurate, rather fostering is its own experimental variable.

CHAPTER 3. GESTATIONAL AND NEONATAL EXPOSURE TO NICOTINE AND ETHANOL RESULTS IN LONG-TERM GABA DYSREGULATION IN THE VENTRAL TEGMENTAL AREA

Introduction

Drug exposure during pregnancy has long been known to exert deleterious effects on the fetal brain. For example, maternal smoking has been correlated with low birth weight, behavioral problems, and nicotine dependence (Buka et al. 2003; Cornelius and Day 2000). Alcohol exposure during pregnancy is characterized by fetal alcohol syndrome and fetal alcohol spectrum disorder, which also include behavioral problems and poor social skills (Buka et al. 2003; Cornelius and Day 2000; Greenbaum et al. 2009; Kandel et al. 2007). Since women of child-bearing age frequently use nicotine and alcohol together (Substance Abuse and Mental Health Services Administration 2012), these disorders may be the result of the combined effects of both drugs. Despite the high frequency of concurrent use of both drugs, most preclinical investigations on the neurological consequences of gestational drug exposure studied either nicotine or alcohol--missing the potential synergistic effects of these two drugs.

Our lab has reported a model of 3-trimester gestational exposure to nicotine and ethanol (Nic+EtOH) (Matta and Elberger 2007). To expose the developing rodent brain to Nic+EtOH throughout 3 trimesters equivalent to human gestation, this model continues drug treatment during the first 12 postnatal days; this is the rodent equivalent of the human third trimester, a period that is critical for brain development. We have reported that full gestational exposure to Nic+EtOH enhanced the acquisition of nicotine self-administration (SA) behavior (i.e. faster acquisition and higher level of responding) .

The mesocorticolimbic pathway central to drug-seeking behavior is comprised of the midbrain ventral tegmental area (VTA), the forebrain nucleus accumbens (NAcc), and the medial prefrontal cortex (mPFC) (Koob and Nestler 1997; Tolu et al. 2013). Dopaminergic (DA) neurons projecting from the VTA to NAcc are modulated, in part, by afferent inputs containing excitatory glutamate from cortex, laterodorsal tegmental nucleus, and local VTA neurons or inhibitory GABA modulators from ventral pallidum and local inhibitory GABA interneurons (Laviolette and van der Kooy 2004). During development, GABA neurotransmission changes from excitatory to inhibitory, and nicotinic activity is responsible for this transition (Liu et al. 2006). Modulation of cholinergic, glutamatergic, and GABAergic neurotransmission during development by nicotine and alcohol exposure could alter brain development, resulting in long lasting changes in brain circuitry that affect behavior such as the enhanced acquisition of nicotine SA (Tolu et al. 2013).

In the VTA, both DA and GABA neurons express inhibitory GABA-B receptors, and intra-tegmental GABA exerts a tonic inhibitory effect on DA neurons (Kalivas 1993). Altered regulation by the GABA-B receptors expressed by GABA and DA neurons would affect this inhibition, particularly since DA neurons have few GABA-A receptors

(Kalivas 1993). Since it has been shown that elevations in VTA GABA levels block nicotine SA (Corrigall et al. 2000), a decrease in GABA modulation of DA neurons would be expected to increase nicotine-stimulated DA release and enhance nicotine SA. Given the enhanced nicotine SA seen in offspring with Nic+EtOH exposure, we focused on clarifying the GABAergic regulation of dopamine projections from the VTA to NAcc shell. We postulate that GABAergic dysregulation due to full gestational exposure to Nic+EtOH is one key mechanism contributing to the vulnerability to acquire nicotine SA in these offspring.

Materials and Methods

Animals and Gestational Exposure: Sprague-Dawley rats (Harlan Laboratories) were group housed on a reverse light cycle (11AM off and 11PM on) for all experiments. The model of gestational exposure was developed on site and described (Matta and Elberger 2007). Briefly, female rats were housed with male breeders overnight until sperm positive. Then the females were single housed and placed into one of four groups; the Nic+EtOH, nicotine alone (Nic), ethanol alone (EtOH), or pair-fed (PF) group. All dams were fed according to the amount of feed consumed by the Nic+EtOH dam they paired to. The EtOH prenatal exposure (**Figure 3.1A** and **3.1C**) was given by daily gavage of 4g/kg from gestational day 2 (GD2) until GD20. The model utilizes a low dose to mimic moderate drinking. Nicotine was administered prenatally by implantation (**Figure 3.1A** and **3.1B**) of a mini-osmopump (Alzet 2ML4; Durect, Cupertino, CA, USA) delivering a constant 2mg/kg dose (pH = 7.2; calculated as free base; (−)-Nicotine hydrogen tartrate salt; Sigma Aldrich Co., St. Louis, MO, USA). The dose chosen approximates 1-2 packs of cigarettes per day. For the Nic (**Figure 3.1B**) and PF (**Figure 3.1D**) groups, dams were gavaged with maltose dextran to equal the calories given to Nic+EtOH and EtOH groups. After the pups were born they received Nic+EtOH treatments (**Figure 3.1**) from postnatal day 3 (PND3) until PND12, this was to mimic the third trimester of pregnancy. The nicotine mini-osmopump in the dam was replaced on PND 2 with an 8mg/kg dose. The nicotine is delivered to the pups through the breast milk. Ethanol was given by two pup gavages of 2g/kg each, 30 minutes apart. Pups not receiving ethanol gavages were instead given matched gavages of maltose dextran (MD). The pups were weaned at PND21. The offspring were given full access to food and water and used at PND60 for the proposed experiments. All procedures were conducted in accordance with the NIH Guidelines for the Use and Care of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Brain Cannulae Implantation: Animals were anesthetized with a cocktail containing 43 mg/kg ketamine and 8.6 mg/kg xylazine before being placed in a David Kopf stereotaxic instrument. Animals received two implantations of 20 gauge (20 mm) guide cannulae. One was placed in the posterior VTA (A.P, -5.6 mm; M/L, -0.2 mm; D/V -8.0 mm) and the other in the NAcc shell (A/P, +1.4 mm; M/L, +0.5 mm; D/V, -6.0 mm). The cannulae were implanted ipsilaterally in the microdialysis experiments. Microinjection

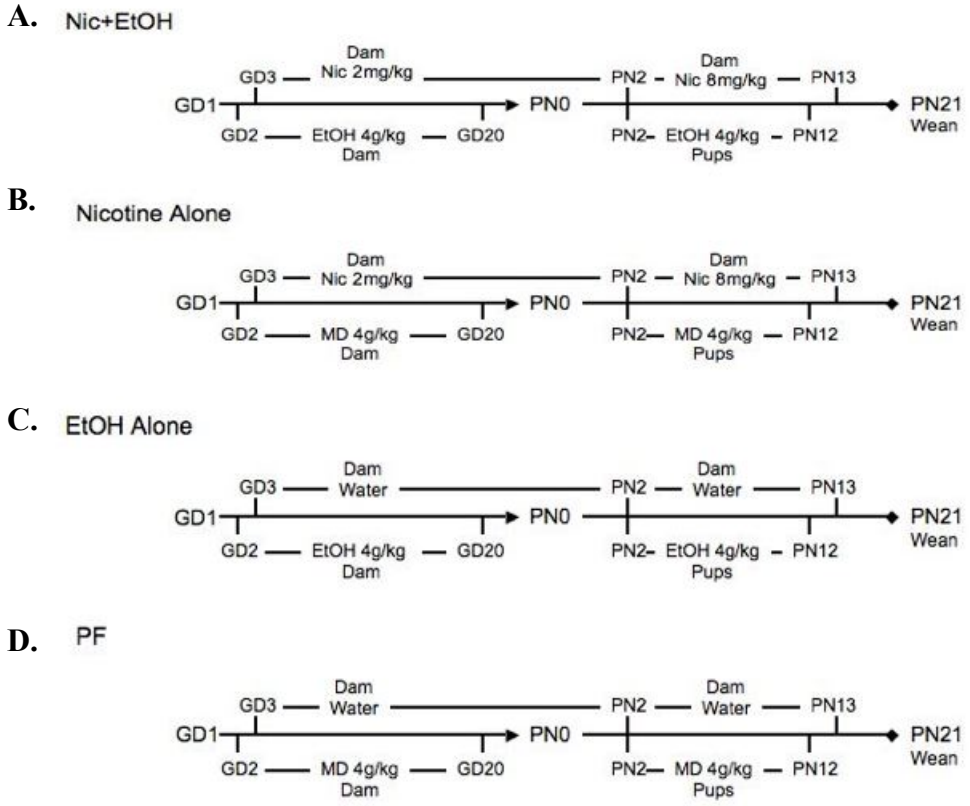


Figure 3.1: Protocol for gestational drug treatments of offspring. (A) Dams designated as Nic+EtOH on GD1, received EtOH gavages (4g/kg) beginning on GD2 and a nicotine mini-osmopump (2mg/kg/day) implantation on GD3. Postnatal exposure to EtOH began on PN2 with twice daily gavages of EtOH (4g/kg total) to each pup. Nicotine exposure was administered by replacing the nicotine pump (8g/kg) in the dam, so that the pups received nicotine through the dams' milk. (**B**) Nic dams received daily gavages beginning GD2 of MD (4g/kg) and a nicotine mini-osmopump (2mg/kg/day) on GD3. Postnatal pup exposure with twice daily gavages of MD (4g/kg total) began on GD2 and a replacement nicotine mini-osmopump (8mg/kg/day) was inserted in their dam on GD3. (**C**) EtOH dams received EtOH gavages (4g/kg) beginning on GD2 and a control mini-osmopump of water implanted on GD3. Postnatal exposure to EtOH began on PN2 with twice daily gavages of EtOH (4g/kg total) to each pup. The control mini-osmopump was replaced on GD3. (**D)** PF dams received daily gavages beginning GD2 of MD (4g/kg) and a control mini-osmopump of water on GD3. Postnatal pup exposure with twice daily gavages of MD (4g/kg total) began on GD2 and a replacement control mini-osmopump was inserted in their dam on GD3.

experiments require only one cannula in the VTA. Each cannula was anchored with 2 stainless steel screws and was covered with acrylic cement. Animals recovered for 5 days before implantation of a jugular cannula.

Jugular Cannula Implantation: Animals were anesthetized with a cocktail containing 43mg/kg ketamine and 8.6 mg/kg xylazine before being implanted with a catheter (PE 90 and silastic tubing) into the right jugular vein and a plastic button (Instech, Plymouth Meeting, Pa., USA) posterior to the shoulder blades. Animals for microdialysis experiments recovered for 2 days prior to experimentation.

In Vivo Microdialysis: Concentric microdialysis probes (2 mm for VTA and NAcc; MW cutoff 13,000 Da, outer diameter 235 um) were constructed in our laboratory. Microdialysis was performed at PND65-75; rats were placed into the alert-rat microdialysis chambers (CMA, Chelmsford, MA) located within an isolated room. Microdialysis probes were inserted into both NAcc and VTA guide cannulae. Following insertion, probes were perfused (2ul/min) with artificial cerebral spinal fluid (αCSF; 147mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, and 0.85 mM $MgCl_2$ in polished water; 0.2 mm filter sterilized and degassed) for 2 hours. Subsequently, 15 min microdialysate samples are collected into plastic vials; 3 consecutive samples are collected to measure basal dopamine or GABA levels prior to drug administration. Following baseline sampling the microdialysis experiments were conducted as follows. For Nicotine-stimulated neurotransmitter release measuring GABA in the VTA and DA in the NAcc, nicotine was administered after the third baseline sample. Including the sample during the nicotine infusion, four 15 min samples were collected.

HPLC and Electrochemical Detection of Neurotransmitters: The procedure for DA detection was as previously described (Fu et al. 2000). Briefly, mobile phase contained 80mM sodium dihydrogen phosphate monohydrate, 5nM diamino ethanetetaacetic acid, 2mM 1-octanesulfonic acid, 100ul/L triethylamine, and 10% Acetonitrile with pH 3.0 (adjusted by phosphoric acid). An ESA 580 pump was used to perfuse mobile phase through an ODS C18 column (ESA, Chelmsford, MA) at a flow rate of 0.25ml/min. Electrochemical detection was performed at 220 mV with the gain at 1 nA and guard cell at 350 mV. Elution time for DA was approximately 5 minutes. Peak height was used for relative quantitation of DA.

GABA was measured with a mobile phase containing 0.1 M disodium hydrogen phosphate in 10% methanol and 10% acetonitrile, pH 4.4 (adjusted by phosphoric acid). An ESA 580 pump was used to perfuse mobile phase through a LMS C18 column (ESA, Chelmsford, MA) at a flow rate of 2.0 ml/min. The derivatization stock solution was made by dissolving 22 mg of phthaldialdehyde (OPA) in 0.5 ml of 100% EtOH, and then adding 0.5 ml of 0.1M sodium sulfite and 9 ml of 0.1 M sodium tetraborate, pH 10.0. The working OPA solution was prepared daily by 1:30 diultion with Millipore water. For automated derivatization of a sample (with the ESA 542 Autosampler), 10 μl from the

VTA dialysate was mixed with 10 μl of working OPA solution. After 2 min, 17 μl of the mixture was injected onto the HPLC column and analyzed with an ESA Coulochem II 5200A electrochemical detector, with ESA 5011A analytical cell. Elution time for GABA was approximately 6 min. Peak height was used for relative quantitation of GABA.

Nicotine Self-Administration: Adult male and female offspring (PND60-75) were allowed chronic, 23 h/d access to bar press for nicotine without prior training, shaping, or food deprivation, using our established model (Chen et al. 2007; Fu et al. 2001; Matta and Elberger 2007; Valentine et al. 1997). Briefly, after implantation of a jugular cannula, rats were placed into individual operant chambers (Coulbourn Instr., Allentown, PA, USA) enclosed in ventilated, sound-attenuating environmental boxes. The jugular line was connected to an injection pump (Med Associates, St. Albans, VT, USA) by a swivel (Instech, Plymouth Metting, PA, USA). Rats were given 3 d to recover from surgery while housed in individual operant chambers. Thereafter, rats were provided free access for 23 h daily (1 h daily is required for animal husbandry and downloading data) to two randomly assigned bars positioned 5 cm above the floor, each with a green light above signaling the availability of an injection. Pressing the active lever signaled the computer (Graphic State Notation software, Coulbourn Instr.) to activate a pump that delivered 30 μg/kg nicotine in a 50 μl heparinized saline bolus over 0.81 s on an FR1 with a 7 s delay schedule; pressing the inactive lever had no programmed consequence. Self-administration day 1 was the first day when nicotine was made available, and the offspring were given 10 days unlimited free access to acquire nicotine self-administration on an FR1 schedule.

As per our previously reported criteria for this open access model (Fu et al. 2001; Matta and Elberger 2007; Valentine et al. 1997), acquisition of nicotine self-administration behavior to maintenance criteria was defined as: (1) stabilization of the number of active bar presses on the final 3 d to within 15% variation, (2) active bar presses greater than inactive presses, and (3) active presses greater than 12/day.

Microinjections of baclofen or vehicle (αCSF) into the VTA were completed 30 minutes prior to access to nicotine on the first 5 days of self-administration. Injections of 50 ng/250 nl of baclofen were made into the VTA over the course of 1 min and then the needle stayed in place for 2 minutes before withdrawal.

Statistics: Statistical analyses were completed with repeated measures ANOVA, one-way ANOVA, and t-test where appropriate using SPSS software, with significance set at $p<0.05$. Values are presented as the mean $\pm$ standard error of the mean (SEM) and the number of animals in each group was indicated within parentheses in the text and figures.

Results

NAcc Shell DA Response to 30μg/kg i.v. Nicotine : Baseline DA levels were not different between the treatment groups (p=0.34). **Figure 3.2A** shows that combined gestational exposure to Nic+EtOH increased nicotine-stimulated (30 μg) DA release in the NAcc shell of offspring compared to the groups treated with nicotine alone or ethanol and PF controls ($F_{3,14}$: 4.26, p=.03). Baseline GABA levels were similar in all treatment groups (p=0.92). **Figure 3.2B** shows that Nic+EtOH offspring released less nicotine-stimulated GABA in the VTA compared to PF offspring ($F_{3,20}$: 4.5, p=0.01).

Nicotine Self-Administration with Intra-VTA Baclofen Microinjection: In agreement with our previous findings (Matta and Elberger 2007), gestational exposure to Nic+EtOH significantly augmented nicotine SA in the offspring compared to gestational PF controls ($F_{1,100}$: 71.35, p<0.001; **Figure 3.3**). However, intra-VTA baclofen significantly reduced nicotine SA in Nic+EtOH offspring ($F_{1,180}$: 16.89 and p<0.001) without affecting PF offspring. Indeed, baclofen reduced nicotine SA in Nic+EtOH offspring to the level observed in PF offspring, and there was no significant difference between the Nic+EtOH rats that received baclofen and the two control groups (i.e. PF offspring administered baclofen or vehicle). Therefore, the augmentation of nicotine SA induced by exposure to Nic+EtOH throughout gestation was abolished by intra-VTA baclofen.

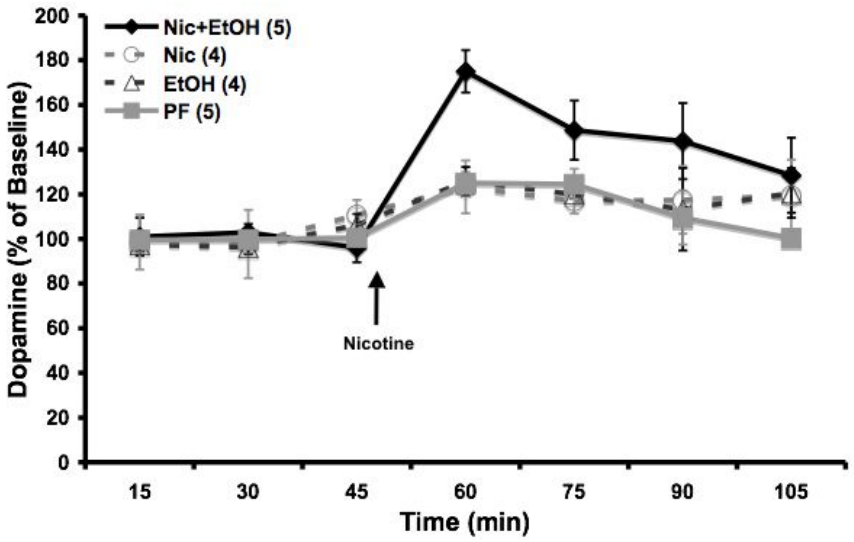
Discussion

We determined whether VTA GABAergic neurotransmission is involved in the enhanced nicotine SA that is a consequence of gestational co-exposure to Nic+EtOH. The results of the *in vivo* microdialysis experiment demonstrated that nicotine stimulated a 20% increase in DA levels within the NAcc shell of offspring previously exposed to only Nicotine, EtOH or PF, whereas, DA increased by 75% in Nic+EtOH offspring. In addition, VTA GABA levels declined by 20% in Nicotine, EtOH, and PF offspring, while Nic+EtOH offspring showed a 50% reduction. Additionally, Intra-VTA administration of baclofen abolished the enhanced nicotine SA seen in Nic+EtOH offspring, without affecting nicotine SA in PF offspring.

Our lab had previously reported that adult male and female offspring with full-gestational exposure to the combination of nicotine and ethanol (Nic+EtOH) showed an increased nicotine SA compared to control offspring (i.e. those receiving gestational exposure to nicotine alone, EtOH alone, or PF) (Matta and Elberger 2007). Our data suggest that the greater reduction in GABA release seen in Nic+EtOH offspring might be necessary to produce the greater increase of accumbal dopamine release also seen in these offspring.

The reduction of VTA GABA release seen in Nic+EtOH offspring could occur because of a change in the sensitivity of GABA receptors following gestational drug exposure. It

A.



B.

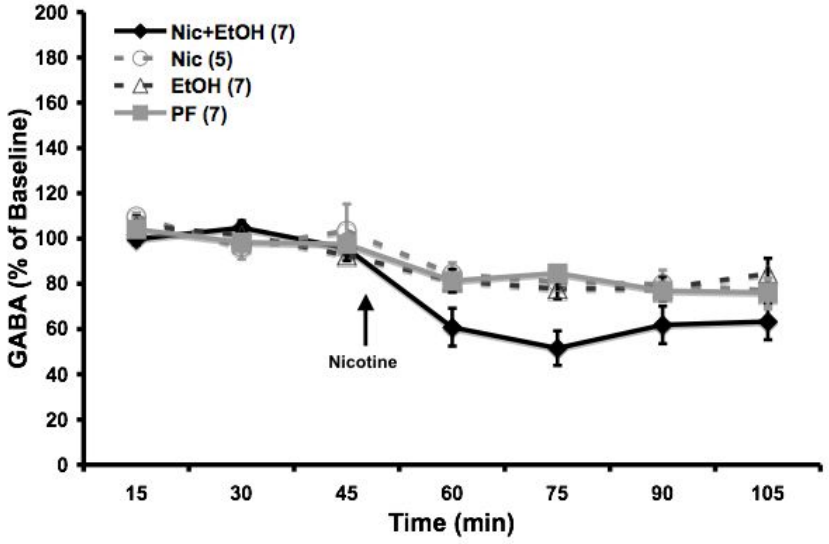


Figure 3.2: Neurotransmitter release in response to 30µg of nicotine. (A) Nicotine-induced dopamine release in NAcc shell was significantly greater in PND60 Nic+EtOH offspring than PF (pair-fed). After 3 baseline microdialysate samples were collected, nicotine (30 µg/kg/10ul, i.v.) was injected into the jugular vein of freely moving rats. Dopamine was measured by HPLC with electrochemical detection. Dopamine levels after nicotine injection were significantly increased in Nic+EtOH offspring compared to controls; $F_{3,14}$: 4.26, p=.03. Baseline dopamine levels were unaffected by gestational treatment [PF vs. Nic+EtOH (mean±SEM): 1.33±0.087 pg/15ul and 1.54±0.086]. (**B**) Nicotine-induced GABA release in VTA was significantly greater in PND60 Nic+EtOH and EtOH offspring than PF (pair-fed). After 3 baseline microdialysate samples were collected, nicotine (30 ug/kg/10ul, i.v.) was injected into the jugular vein of freely moving rats. GABA was measured by HPLC with electrochemical detection. GABA levels after nicotine injection of Nic+EtOH offspring were decreased to a significantly greater level compared to controls; $F_{3,20}$: 4.5, p=0.01.

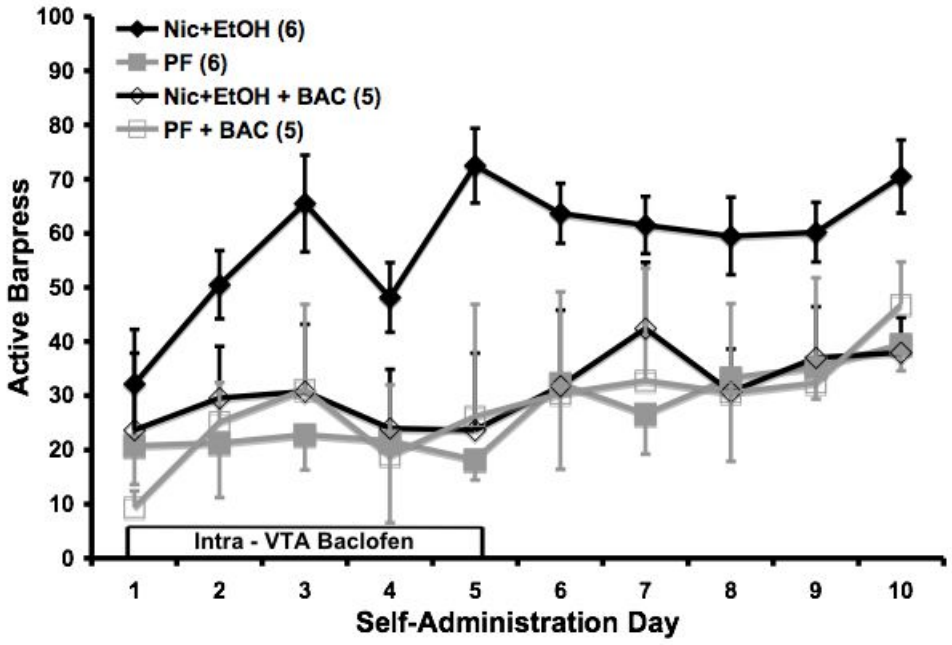


Figure 3.3: Nic+EtOH and PF offspring self-administration of 30µg/kg/inj of nicotine with and without microinjections of 50ng/250nl of baclofen. Nic+EtOH offspring without baclofen had significantly higher responding for nicotine ($F_{1,180}$: 16.89 and $p<0.001$) over 10 days.

has been shown that evoked dopamine release in tissue after chronic subcutaneous nicotine injections (0.4 mg/kg) did not change with the application of a baclofen bath, whereas control animals had a reduction in dopamine release with a baclofen bath (Amantea and Bowery 2004). Therefore, it is possible that the gestational nicotine exposure produced a long-term change in the sensitivity of VTA GABA receptors as part of their role to modulate NAcc DA release.

The dopamine and GABA release in response to 30 μg/kg nicotine of gestational nicotine alone and EtOH alone offspring did not differ from PF control offspring. Therefore, these groups were not included in the SA experiment. However, these data indicate that at the low nicotine and alcohol doses that we used, the synergistic effect between gestational EtOH and nicotine exposure is responsible for the long-term consequence of neuroadapation and behavioral changes. Given the greater reduction of GABA release seen in Nic+EtOH offspring we hypothesized a possible reduction of GABA receptors in the VTA. Studies have shown that prenatal ethanol exposure have reduced the number of GABA positive cells in rat and guinea pig (Bailey et al. 2004; Moore et al. 1998).

The GABA-B agonist baclofen was chosen for the self-administration experiment since there is evidence-linking baclofen as a possible therapy for those attempting to quite smoking or for other drugs of abuse (Bowery 2006; Cousins et al. 2002). Baclofen (50 μM) infused into the VTA was previously shown to decrease accumbal dopamine release by 35% compared to saline (Westerink et al. 1996). Experiments using animal models have displayed the ability of baclofen to lower nicotine self-administration responding (Corrigall et al. 2000; Fattore et al. 2002; Paterson et al. 2004). Using our gestational model we were able to demonstrate that a low dose of baclofen was only able to disrupt the self-administration behavior of the Nic+EtOH offspring without altering the responding of PF offspring. Baclofen microinjection into the VTA was given during the 1 hour allotted for husbandry prior to the beginning of the 23-hour self-administration session. The half-life of baclofen is 1-5 hours in humans (Sallerin-Caute et al. 1991), which would argue that most of the reduction in SA is likely attributable to reduced nicotine intake early on during SA. The finding that intra-VTA baclofen did not affect nicotine responding in PF offspring suggested that the lowered responding of Nic+EtOH offspring with baclofen microinjections was not due to the sedative effects of baclofen. It has previously been shown that microinjection of baclofen into the VTA reduces dopamine in the NAcc (Kalivas 1993). Other studies have also shown that low doses of baclofen do not alter responding for food (Le Foll et al. 2008; Paterson et al. 2004). Therefore, our data suggested that enhanced GABA neurotransmission in VTA is a potential mechanism to ameliorate the enhanced vulnerability to nicotine resulting from gestational drug exposure.

In addition to the studies using animal models baclofen has also been involved in clinical studies for smoking cessation. In one study, taking baclofen before smoking changed the individuals' sensory association with a cigarette. Those who took baclofen were more likely to associate smoking as negative or "harsh" (Cousins et al. 2001). Preliminary studies have also shown that baclofen was able to reduce smoking compared to those taking a placebo (Franklin et al. 2009). The clinical studies that demonstrate the possible

effectiveness that baclofen can alter smoking behavior may be connected with our experimental data that nicotine decreases GABA release in the VTA by 20% in control offspring. Further reduction in normal VTA GABA inhibition at DA neurons would contribute to the increased smoking (SA) behavior. Such dysinhibition would be offset by the baclofen by activation of GABA-B receptors.

In conclusion, the offspring with full gestational Nic+EtOH exposure exhibited a greater reduction of GABA release in response to nicotine compared to PF offspring. This marked reduction of VTA GABA in Nic+EtOH offspring in response to nicotine is not due to lack of GABA-B receptors. Rather, it is the hypo-GABAergic modulation of DA neurons that contributed to elevated accumbal DA release and subsequent enhanced nicotine SA behavior.

CHAPTER 4. FULL-GESTATIONAL EXPOSURE TO NICOTINE AND ETHANOL AUGMENTS NICOTINE SELF-ADMINISTRATION BY ALTERING VENTRAL TEGMENTAL DOPAMINERGIC FUNCTION DUE TO NMDA RECEPTORS IN ADOLESCENT RATS[‡]

Introduction

Tobacco is used by 26.5% of the US population over age 12 (Substance Abuse and Mental Health Services Administration 2012); 90% of habitual smokers begin before age 18 (Kandel et al. 2007). During pregnancy, maternal smoking and ethanol intake negtively impact fetal brain development, resulting in behavioral disorders and smoking in offspring (Cornelius *et al.* 2007). During teenage pregancy, 58% of smokers also drink alcohol (Cornelius et al. 2007). Because of such concurrent usage throughout pregnancy and its impact on offspring, we developed a model of 3-trimester gestational exposure to both drugs (Matta and Elberger 2007); adult offspring exposed during gestation self-administer significantly more nicotine.

The mesocorticolimbic pathway, essential for motivated drug-taking, includes ventral tegmental area (VTA), nucleus accumbens (NAcc), and medial prefrontal cortex (Koob and Nestler 1997). Critical VTA dopaminergic (DA) neurons projecting to NAcc are regulated by glutamatergic inputs primarily from prefrontal cortex (Mansvelder et al. 2003). N-methyl-D-aspartate (NMDA) receptors on DA neurons stimulate burst firing, increasing accumbal DA release (Amantea and Bowery 2004).

Glutamatergic neurocircuitry, including VTA NMDA receptors essential for acquisition of appetitive behaviors, is developmentally regulated (Zellner et al. 2009). For example, in early postnatal brain development, the predominant NR2B subunits are replaced by NR2A, altering NMDA receptor kinetics (Liu et al. 2004). This is delayed in cerebellar granule neurons by early postnatal exposure to ethanol (Snell et al. 2001). Additionally, peak expression of NR3A subunits, affecting synapse formation and synaptic spines, occurs during early postnatal (PN7-14) development (Henson et al. 2010). Based on this exquisite timing of subunit expression and tuning of NMDA receptors and their role in addiction (Henson et al. 2010; Zellner et al. 2009), we hypothesized that concurrent gestational exposure to ethanol and nicotine would disrupt the control of VTA dopaminergic circuitry by NMDA receptors, augmenting nicotine self-administration (SA) in adolescent offspring.

In vivo microdialysis was used to determine the responsiveness of accumbal DA and VTA glutamate to intra-VTA NMDA and systemic nicotine in adolescent offspring exposed to nicotine and ethanol *in utero* and early post-partum (i.e., during the human 3rd

[‡]Roguski EE, Sharp BM, Chen H, Matta SG. Full-Gestational Exposure to Nicotine and Ethanol Augments Nicotine Self-Administration by Altering Ventral Tegmental Dopaminergic Function due to NMDA Receptors in Adolescent Rats. J Neurochem (2013) *In Press*

trimester equivalent of rat brain development) vs. pair-fed controls (PF). We also determined the efficacy of intra-VTA NMDA receptor blockade of nicotine-stimulated DA release and nicotine SA in both groups of offspring. Lastly, the expression of NMDA receptor mRNA transcripts and protein in VTA was measured.

Materials and Methods

Materials: (−)-Nicotine hydrogen tartrate salt (all doses calculated as free base) was purchased from Sigma (St Louis, MO). NMDA was also purchased from Sigma (St Louis, MO). SKF-83566 hydrobromide and DL-2-Amino-5-phosphonopentanoic acid sodium salt (AP5) were purchased from Tocris Bioscience (Bristol, UK).

Animals and Gestational Exposure: The model of gestational exposure to nicotine and ethanol in Sprague-Dawley rats was developed on site and described (Matta and Elberger 2007). Briefly, female rats were group housed in order to acclimate to a reverse light cycle (11a.m. off; 11p.m. on) and then housed overnight (2 females to 1 male) until sperm positive. On the sperm positive day, dams were randomly assigned to either the Nic+EtOH or pair fed (PF) group (**Figure 4.1**). PF dams were matched and fed according to the nutrition of the Nic+EtOH dam. Gestational drug treatments are illustrated in **Figure 4.1A** and **4.1B**. The EtOH prenatal exposure was delivered by daily gavage of 4g/kg from gestational day 2 (GD2) until GD20. The model utilizes a low dose of EtOH to mimic moderate binge drinking. Nicotine was administered prenatally by implantation of a mini-osmopump delivering a constant 2 mg/kg dose (pH = 7.2-7.4). This dose simulates the blood levels achieved by 1-2 packs of cigarettes per day. PF dams were gavaged with an amount of maltose dextran (MD) that is equivalent to the calories of the EtOH gavage consumed by Nic+EtOH dams. After the pups were born they received nicotine and ethanol treatments from postnatal day 2 (PN2) until PN12, in order to simulate drug exposure during the third trimester of human pregnancy. The nicotine mini-osmopumps were replaced with another delivering 8mg/kg to the dams, whose breast milk transferred nicotine to the suckling pups. Ethanol was administered to pups by 2 gavages of 2g/kg each 30 minutes apart, whereas PF pups were gavaged twice daily with calorically matched maltose dextran. All pups were weaned at PN21 and, thereafter, received standard amounts of food and water. They were used in the proposed experiments at PN41-45. All procedures conducted in accordance with the NIH Guidelines for the Use and Care of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Cannula Implantation: Using minor modifications of methods previously described (Fu *et al.* 2000, Kane *et al.* 2004), animals were anesthetized with a mixture of ketamine 43mg/kg and xylazine 8.6 mg/kg and then stereotaxically implanted with two 20 GA (20mm) guide cannula targeted at the posterior VTA (A.P, -5.6 mm; M/L, -0.2 mm; D/V

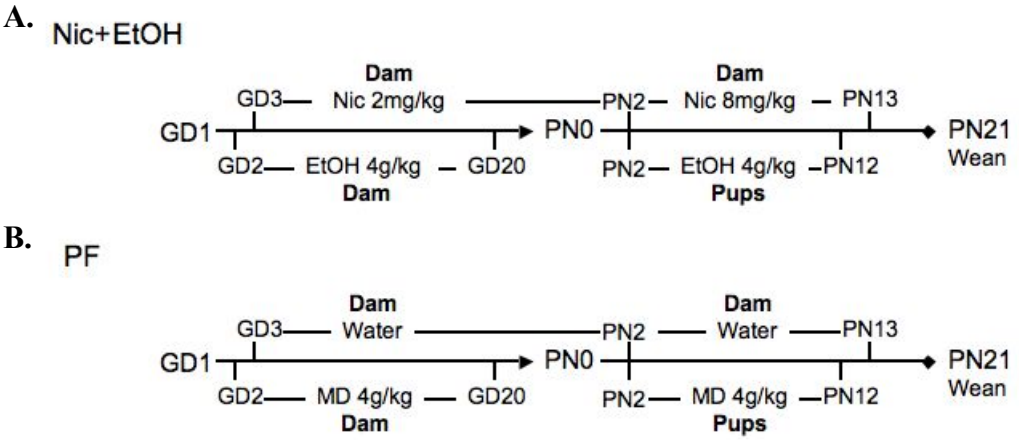


Figure 4.1: Protocol for offspring gestational drug treatments. (A) Dams designated as Nic+EtOH on GD1, received EtOH gavages (4g/kg) beginning on GD2 and a nicotine mini-osmopump (2mg/kg/day) implantation on GD3. Postnatal exposure to EtOH began on PN2 with twice daily gavages of EtOH (4g/kg total) to each pup. Nicotine exposure was administered by replacing the nicotine pump (8g/kg) in the dam, so that the pups received nicotine through the dams' milk. **(B)** PF dams received daily gavages beginning GD2 of MD (4g/kg) and a control mini-osmopump of water on GD2. Postnatal pup exposure with twice daily gavages of MD (4g/kg total) began on PN2 and a replacement control mini-osmopump was inserted in their dam on PN2. All offspring are weaned on PN21.

-8.0 mm) and the NAcc shell (A/P, +1.4 mm; M/L, +0.5 mm; D/V, -6.0 mm). The coordinates were adjusted for adolescent animals by body weight (AP=adultAP-0.005×(300-body weight); ML=adultML/2; DV=adultDV-(approximately 0.5-1.0). For microinjection experiments, only VTA was cannulated. Animals recovered for 5 d before implantation of a jugular cannula.

In Vivo Microdialysis: As previously described (Fu et al. 2000; Kane et al. 2004), concentric microdialysis probes (2 mm for VTA and NAcc; MW cutoff 13,000 Da, outer diameter 235 um) were constructed in our laboratory. For microdialysis experiments, performed at PN45-55, rats were placed into alert-rat microdialysis chambers (CMA, Chelmsford, MA), located within an isolation room, and microdialysis probes were inserted into both NAcc and VTA guide cannulae. Probes were then perfused (2μl/min) with artificial cerebrospinal fluid (αCSF; 147mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, and 0.85 mM $MgCl_2$ in polished water; pH to 7.2-7.4, sterilized through 0.2 mm filters and degassed) for 2 hours. After acclimation to the microdialysis probes, samples were collected into plastic vials every twenty minutes until the end of the experiment. Up to 4 rats were run concurrently with an equal number of Nic+EtOH and PF offspring. For reverse dialysis, syringes were changed 20 minutes prior to drug infusion into the VTA (length of time at 2ul/min for the solution to fill the microdialysis probe).

Protocols for Microdialysis Experiments:

1. NMDA Dose Response Experiment: NMDA and KCl were dissolved in aCSF (NMDA: 200μM and 500μM; KCl: 100mM) and reverse dialyzed into VTA during the experiment. Each concentration of agent was set to pH 7.4, degassed, and then drawn into a 2.5mL gastight Hamilton syringe. DA samples were analyzed from 5 Nic+EtOH and 6 PF offspring. Glutamate samples were analyzed from 9 Nic+EtOH and 7 PF offspring. Three consecutive baseline samples (20 min each) were collected during reverse dialysis of aCSF, followed by 3 samples during reverse dialysis of 200uM NMDA, 2 samples during delivery of aCSF, 3 samples during reverse dialysis of 500uM NMDA, 2 samples during αCSF, and finally 3 samples during reverse dialysis of 100mM KCl.

2. Antagonism of D1 Receptors in the VTA followed by NMDA: D1 receptor antagonist, SKF-83566 (100μM), and NMDA (200μM and 500μM) were dissolved in αCSF for reverse dialysis. DA samples were analyzed from 7 Nic+EtOH and 5 PF offspring at 200μM NMDA and 8 Nic+EtOH and 10 PF offspring at 500μM NMDA. Glutamate samples were analyzed from 9 Nic+EtOH and 13 PF offspring at 200μM NMDA and 5 Nic+EtOH and 8 PF offspring at 500μM NMDA. Three baseline samples (20 min each) were collected during reverse dialysis of αCSF, followed by 2 samples during reverse dialysis of SKF-83566, and then 3 samples during delivery of NMDA at either the 200μM or 500μM concentration.

3. 30μM Nicotine i.v. with NMDA Receptor Antagonism: Nicotine was dissolved in saline to deliver 30μg/kg (free base) in 10μL (pH of 7.4). AP5 for reverse dialysis was dissolved in αCSF at 75μM and 200μM (pH 7.4). DA samples were analyzed from 6 Nic+EtOH and 6 PF offspring at 0μM AP5 (αCSF), 4 Nic+EtOH and 5 PF offspring at 75μM AP5, and 5 Nic+EtOH and 7 PF offspring at 200μM AP5. Glutamate samples were analyzed from 5 Nic+EtOH and 4 PF offspring at 0μM AP5, 4 Nic+EtOH and 5 PF offspring at 75μM AP5, and 8 Nic+EtOH and 3 PF offspring at 200μM AP5. Three baseline samples were collected during reverse dialysis of αCSF and then the last 6 samples were collected during reverse dialysis of AP5 (0μM, 75μM, or 200μM). At the start of the 5th sample, nicotine (30μg in 10μL) was injected i.v.

HPLC with Electrochemical Detection: The procedure for detection of dopamine (DA) and excitatory amino acids (e.g., glutamate) was previously described (Fu et al. 2000). Briefly, for DA, the mobile phase contained 80mM sodium dihydrogen phosphate monohydrate, 5nM diamino ethanetetraacetic acid, 2mM 1-octanesulfonic acid, 100μl/L triethylamine, and 10% acetonitrile, pH 3.0 (adjusted with phosphoric acid). An ESA 580 pump perfused mobile phase through a 15 cm 3 x 4.6 mm ODS C18 column (ESA, Chelmsford, MA) at 0.25ml/min, and DA was detected at 220 mV with gain at 1 nA and guard cell at 350 mV. For glutamate, a mobile phase containing 0.1 M disodium hydrogen phosphate in 10% methanol and 10% acetonitrile, pH 4.4. Perfused into a 15 cm 3 x 4.6 mm ODS C18 column (ESA, Chelmsford, MA) at 1.0 ml/min. Phthaldialdehyde (OPA) stock solution was prepared from 22 mg OPA in 0.5 ml of 100% EtOH, followed by the addition of 0.5 ml sodium sulfite 0.1M and 9 ml sodium tetraborate 0.1 M, pH 10.0. A working OPA solution (1:30 dilution in water) was prepared daily and used for automated sample derivatization (ESA 542 Autosampler), in which 2 μl VTA dialysate was diluted with 8ul polished HPLC grade water and mixed with 10 μl of working OPA for 2 min, and then 17 μl was injected on column and analyzed with an ESA Coulochem II 5200A detector (5011A analytical cell).

Brain Punches and Tissue Preparation: Offspring at PN45 were anesthetized with isoflurane and decapitated to obtain brain tissues for qRT-PCR (Nic+EtOH n=7, PF n=7) and Western blot analysis (Nic+EtOH n=15, PF n=9). The brain punch procedure and tissue preparation were performed according to our established protocol (Wang et al. 2007). Briefly, rats were anesthetized with isoflurane, decapitated, and brains were removed and stored at -80^{0}C. Frozen brain sections, 0.7 mm thick, were obtained using razor blades and a stage, designed and assembled in our laboratory. The VTA was punched according to the atlas of Paxinos and Watson (1986), using a 20-gauge syringe adapter. Dissected tissues were frozen on dry ice and stored at –80°C.

RNA Isolation, cDNA Synthesis, and qtPCR.: Total RNA was extracted, using RNAeasy Lipid Tissue Mini Kit (Qiagen), and reverse transcribed into cDNA with Superscript III (Invitrogen), using oligo(dT) 18 primers according to the manufacturer's instructions. qtPCR was performed by using the following primers: NR1-Forward: acatatgtgagaaatacaacctttgg, NR1-Reverse: cttccgggacatgagcag, NR2A-Forward: agcagccgggtagagagg, NR2A-Reverse: tccccctttcagagtacacc, NR3A-Forward: gctttgccgtcactagact, NR3A-Reverse: atgcacaacagcacgaagag, HPRT-Forward: gaccggttctgtcatgtcg, HPRT-Reverse: acctggttcatcatcactaatcac, YWHAZ-Forward: gcagttactgagagacaacttgaca, YWHAZ-Reverse: tggaaggccggttaatttt, PGK1-Forward: gcaaagactggccaagctac, PGK1-Reverse: gcctcagcatatttcttactgct. Quantification was performed using the comparative CT method (ΔΔ CT). Results are expressed relative to the levels of housekeeping genes: HPRT, YWHAZ and PGK1.

Western Blot Analysis: To determine ionotropic glutamate receptor expression levels after gestational drug exposure, alternating samples from same brain region of each treatment group (i.e., Nic+EtOH vs. PF) were loaded on the same gel.

The procedure for Western immunoblotting was previously described by us (Wang et al. 2007) . Briefly, tissue samples were sonicated on ice water in 2% sodium dodecyl sulfate (SDS) for 30s with 10 s bursts and then centrifuged at 800 × g for 5 min. Protein concentrations were measured using the bicinchoninic acid assay (Pierce, Rockford, IL). Samples were diluted in Laemmli buffer (Bio-Rad, Hercules, CA) at a ratio of 1:2, boiled for 5 min, loaded, and then separated on 10% Tris-HCl ready gels (Bio-Rad, Hercules, CA) in a buffer containing 0.1% SDS, 192mM glycine, and 25mM Tris, pH 8.3. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) in a buffer containing 192mM glycine, 20% methanol, and 25mM Tris, pH 8.3. Membranes were then washed in Tris-buffered saline with Tween-20 (TBST) and blocked overnight with agitation in 5% nonfat dried milk at 4°C. Based on molecular weight (MW) differences between NMDA receptor subunits and b-actin, blots were cut into 2 sections by visualizing the MW markers: upper blots were used to detect NMDA receptor subunits and lower blots for b-actin. Each section contained alternating samples from the Nic+EtOH and PF offspring. Blots were incubated in blocking solution for 2h at RT or overnight at 4C with antibodies against b -actin (1:1000; Sigma, St Louis, MO), NMDA receptor subunit 1 (NR1; 1:300 ; Santa Cruz, CA), NR2A (1:200; Santa Cruz) and NR2B (1:200; Santa Cruz), washed in TBST and then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000, Santa Cruz, CA). Target proteins were visualized by Supersignal (Pierce, Rockford, IL) followed by imaging with a Bio-Rad ChemiDoc and quantitation by Quantity One software (Bio-Rad, Hercules, CA).

Nicotine Self-Administration: Adolescent offspring (PN 41-55) were allowed chronic, almost unlimited (i.e., 23 h/d) access to bar press for nicotine without prior training, shaping, or food deprivation, using our established model (Matta and Elberger 2007; Valentine et al. 1997). Briefly, after implantation of a jugular cannula, rats were placed into individual operant chambers (Coulbourn Instr., Allentown, PA, USA) enclosed in

ventilated, sound-attenuating environmental boxes. The jugular line was connected to a microinjection pump (Med Associates, St. Albans, VT, USA) by a single channel swivel (Instech, Plymouth Meeting, PA, USA). Rats were given 3 d to recover from surgery while housed in individual operant chambers. Thereafter, rats were provided free access for 23 h daily (1 h daily is required for animal husbandry and downloading data) to two randomly assigned bars positioned 5 cm above the floor, each with a green light above signaling the availability of an injection. Pressing the active lever signaled the computer (Graphic State Notation software, Coulbourn Instr.) to activate a pump that delivered 30μg/kg nicotine in a 50-μl bolus, containing heparin, over 0.81 s on an FR1 schedule with a 7 s time-out; pressing the inactive lever had no programmed consequence. Self-administration day 1 (SA d1) was the first day when nicotine was made available, and the offspring were given 10 d unlimited free access to acquire nicotine SA on an FR1 schedule.

As per our previously reported criteria for this prolonged access model (Fu et al. 2001; Matta and Elberger 2007; Valentine et al. 1997), acquisition of stable nicotine SA behavior was defined as: (1) <15% variation in active bar presses during the final 3 d of SA, (2) active bar presses > inactive presses, and (3) active presses > 12/day.

Nicotine Self-Administration Experimental Protocol: Nic+EtOH offspring were placed into individual operant chambers following surgery to insert a cannula into the left jugular vein. After surgery offspring were randomly designated to receive an intra-VTA microinjection of either AP5 or αCSF. AP5 was injected into the VTA of 7 Nic+EtOH and 6 PF offspring, while αCSF was injected into 6 Nic+EtOH and 8 PF offspring. Microinjections into VTA were completed 30 min prior to access to nicotine [30μg/kg dissolved in heparin-saline (200units/mL) pH 7.2-7.4] on the first 5 d of SA. AP5 (500ng AP5/250nl αCSF) or αCSF was injected over 1 min and the needle remained in place for 2 more min. The rats remained in the operant chambers and were undisturbed except for animal husbandry.

Statistics: Statistical analysis, using SPSS, was performed with three-way ANOVA, two-way ANOVA, one-way ANOVA, and repeated measures ANOVA where appropriate, with significance set at $p<0.05$. Values are mean $\pm$ standard error (SEM). HPLC values were calculated using peak heights obtained from chromatograms using PowerChrom (eDAQ). The analyte levels present in the first 3 baseline samples from each animal were averaged and the levels of analyte in all subsequent samples were expressed as a percentage of this baseline level.

Results

Effects of Intra-VTA NMDA on DA in NAcc Shell and Glutamate in VTA : Peak accumbal DA levels in response to reverse dialysis of 200μM NMDA, 500μM NMDA,

and 100mM KCl were detected 40-60 min (i.e., 3rd sample) after the administration of each agent (**Figure 4.2A**). NMDA stimulated significantly greater peak DA levels in Nic+EtOH vs. PF offspring (**Figure 4.2B**; 200μM and 500μM NMDA, $F_{1,9}$:6.29 p=0.03 and $F_{1,9}$:6.69 p=0.03 respectively), whereas the effects of KCl were similar in both groups.

Peak VTA glutamate levels followed the same pattern (**Figure 4.2C**), in that Nic+EtOH offspring had significantly higher peak levels (**Figure 4.2D**) in response to 200μM ($F_{1,14}$:6.64 p=0.02) and 500μM NMDA ($F_{1,14}$:15.93 p=0.001), while glutamate responses to KCl were not different between the two cohorts. Baseline levels of both DA (Nic+EtOH vs. PF: 1.40±0.24 vs. 1.26±0.16 pg/20μl) and glutamate (Nic+EtOH vs. PF: 47.58±4.39 vs. 43.00±5.39 pg/μl) were similar in the two cohorts.

Effects of Intra-VTA D1 Receptor Antagonist SKF-83566 on NMDA-Stimulated Release of DA in NAcc Shell and Glutamate in VTA: In **Figure 4.3A**, following administration of D1 antagonist, SKF-83566, reverse dialysis of NMDA (200μM or 500μM) into the VTA did not stimulate NAcc DA release compared to baseline. Baseline DA levels in Nic+EtOH and PF groups were unaffected by the intra-VTA infusion of SKF-83566 (1.37±0.09 vs. 1.43±0.18 pg/20μl, respectively).

Figure 4.3B displays the levels of VTA glutamate, which were unchanged compared to baselines following infusion of SKF-83566 into the VTA. Subsequent, intra-VTA delivery of NMDA (200μM or 500μM) did not stimulate glutamate release compared to baselines in both gestational treatment groups. The baseline levels of glutamate in Nic+EtOH offspring were similar to PF (43.92±5.83 vs. 46.57±3.64 pg/μl, respectively).

Effects of NMDA Receptor Antagonist AP5 on DA and Glutamate Levels Stimulated by Nicotine: In the absence of AP5 (**Figure 4.4A**), nicotine (30ug, i.v.) significantly stimulated NAcc DA release compared to baselines in both Nic+EtOH and PF offspring ($F_{1,40}$: 8.55, p=0.006 and $F_{1,34}$: 6.76, p=0.014, respectively). DA release was greater in Nic+EtOH offspring (approximately 50% increase over baseline) compared to PF offspring (approximately 20% increase over baseline; $F_{1,11}$:8.36; p=0.02). In contrast, VTA glutamate levels in both cohorts were unaffected by this dose of nicotine (**Figure 4.4B**).

Figure 4.5 shows the effects of intra-VTA AP5 on NAcc DA and VTA glutamate following i.v. nicotine. Microinjections of AP5 into the VTA did not significantly alter baseline NAcc DA levels. Compared to AP5 0μM (same data as in Fig. 4A), both 75μM and 200μM AP5 inhibited nicotine-stimulated DA release in Nic+EtOH offspring ($F_{2,13}$:13.87, p=0.001; **Figure 4.5A**). Although DA levels in the 3 PF treatment groups shown in **Figure 4.5B** were not significantly different from each other, nicotine-stimulated DA release was only significant in the absence of AP5 (i.e., AP5 AP5 0μM; same data as in **Figure 4.4A**). In addition, baseline DA levels were not different in the 3 PF treatment groups (p=0.96).

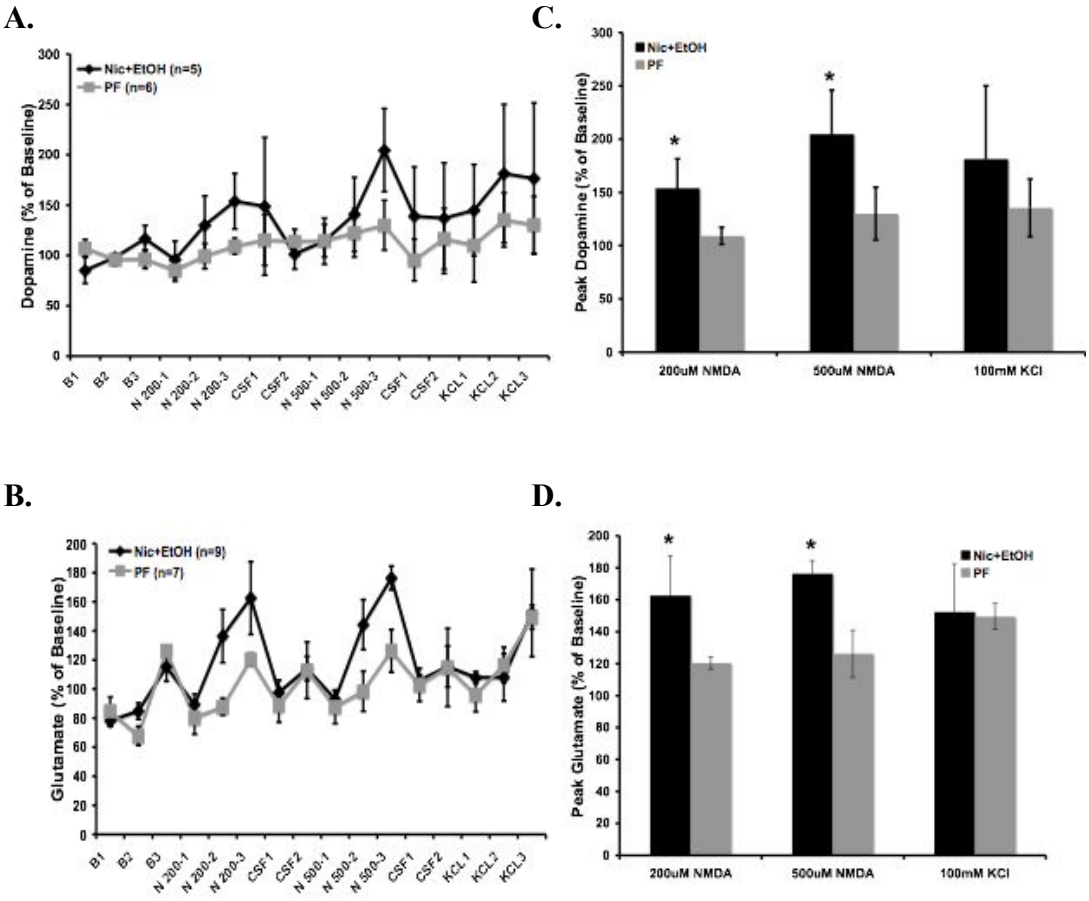


Figure 4.2: Effects of intra-VTA NMDA on NAcc shell DA and VTA glutamate levels. NAcc shell DA **(A)** and VTA glutamate **(C)** were measured following reverse dialysis of 200µM NMDA (N 200-1,2,3), 500µM NMDA (N 500-1,2,3) and 100mM KCl (KCl 1,2,3), each for 60 min into the VTA. Sequential 20 min-samples were collected (i.e., beginning at each time mark in **Figure 4.2A** and **4.2C**) until the end of the experiment. Neither baseline DA nor glutamate levels were significantly different between Nic+EtOH and PF offspring (DA: 1.40±0.4 vs. 1.26±0.16 pg/20µl, respectively; glutamate: 47.58±4.39 vs. 43.00±5.39 pg/µl, respectively). Peak levels of DA **(B)** present in the third sample collected after initiating reverse dialysis with each agent were significantly higher for Nic+EtOH (n=6) compared to PF (n=5) at 200µM ($F_{1,9}$:6.29 p=0.03) and 500µM ($F_{1,9}$:6.69 p=0.03) of NMDA. Peak glutamate levels **(D)** were significantly greater in Nic+EtOH (n=9) offspring compared to PF (n=7) in response to NMDA 200µM ($F_{1,14}$:6.64 p=0.02) and 500µM ($F_{1,14}$:15.93 p=0.001). There were no significant differences in responses to 100mM KCl between the treatment groups.

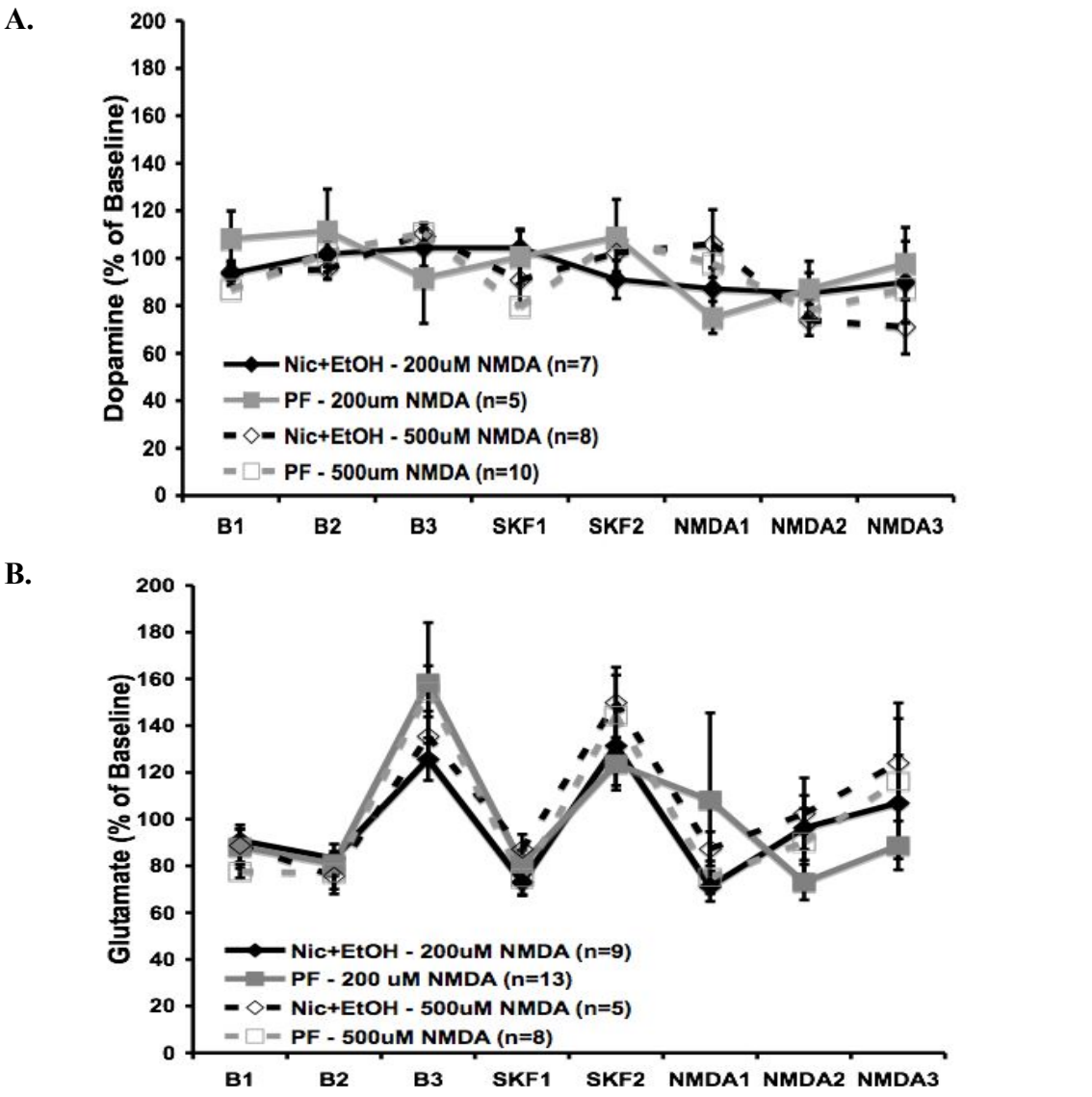


Figure 4.3: Effects of NMDA exposure following pretreatment with D1 receptor antagonist SKF-83566 on NAcc shell DA and VTA glutamate levels. NAcc DA **(A)** and VTA glutamate **(B)** were measured during reverse dialysis of 100uM SKF-83566 (SKF1,2) followed by either 200μM or 500μM NMDA (NMDA1,2,3) into the VTA. **(A)** Following NMDA, DA did not increase for either treatment group. Baseline DA levels were not significantly different between Nic+EtOH (200μM: n=7 and 500μM: n=8) and PF (200μM: n=5 and 500μM: n=10) offspring (1.37±0.09 vs. 1.43±0.18 pg/20μl, respectively). **(B)** Similarly, VTA glutamate was unaffected by NMDA in Nic+EtOH (200μM: n=9 and 500μM: n=5) and PF (200μM: n=13 and 500μM: n=8), additionally baseline glutamate levels were not different (43.92±5.83 vs. 46.57±3.64 pg/μl, respectively).

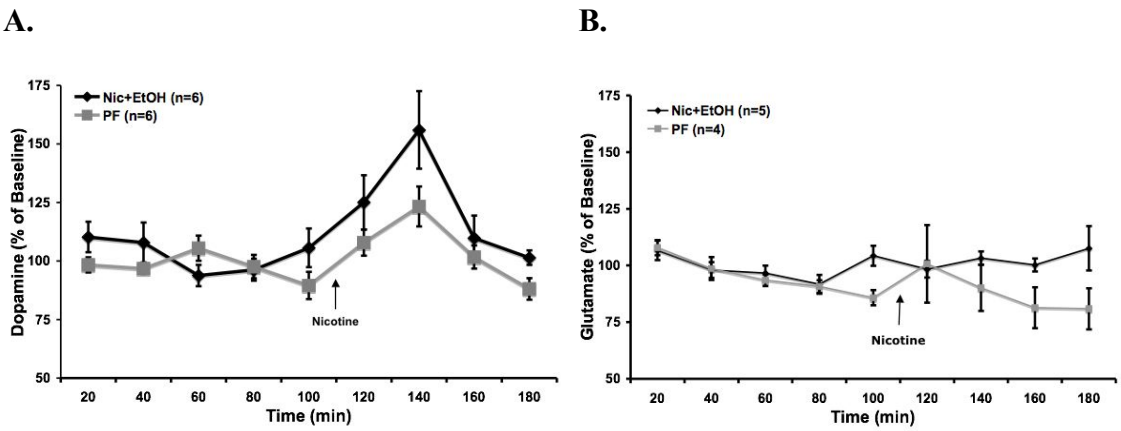


Figure 4.4: Effects of nicotine on NAcc DA and VTA glutamate in Nic+EtOH and PF offspring. Nicotine 30ug was injected i.v. at the beginning of the 5th sample collection (120min). **(A)** Nic+EtOH (n=6) offspring had a significantly greater accumbal DA response to nicotine compared to PF (n=6; $F_{1,11}$:8.36; p=0.02). Baseline DA levels were not significantly different between Nic+EtOH and PF (1.50±0.21 vs. 1.56±0.05 pg/20µl, respectively). Accumbal DA release in response to nicotine was significantly increased from baseline levels for both Nic+EtOH ($F_{1,40}$: 8.55, p=0.006) and PF ($F_{1,34}$: 6.76, p=0.014). **(B)** VTA glutamate was unaffected by nicotine in both Nic+EtOH (n=5) and PF (n=4) offspring, and baseline glutamate levels were not different (37.68±6.97 vs. 40.59±8.33 pg/µl, respectively).

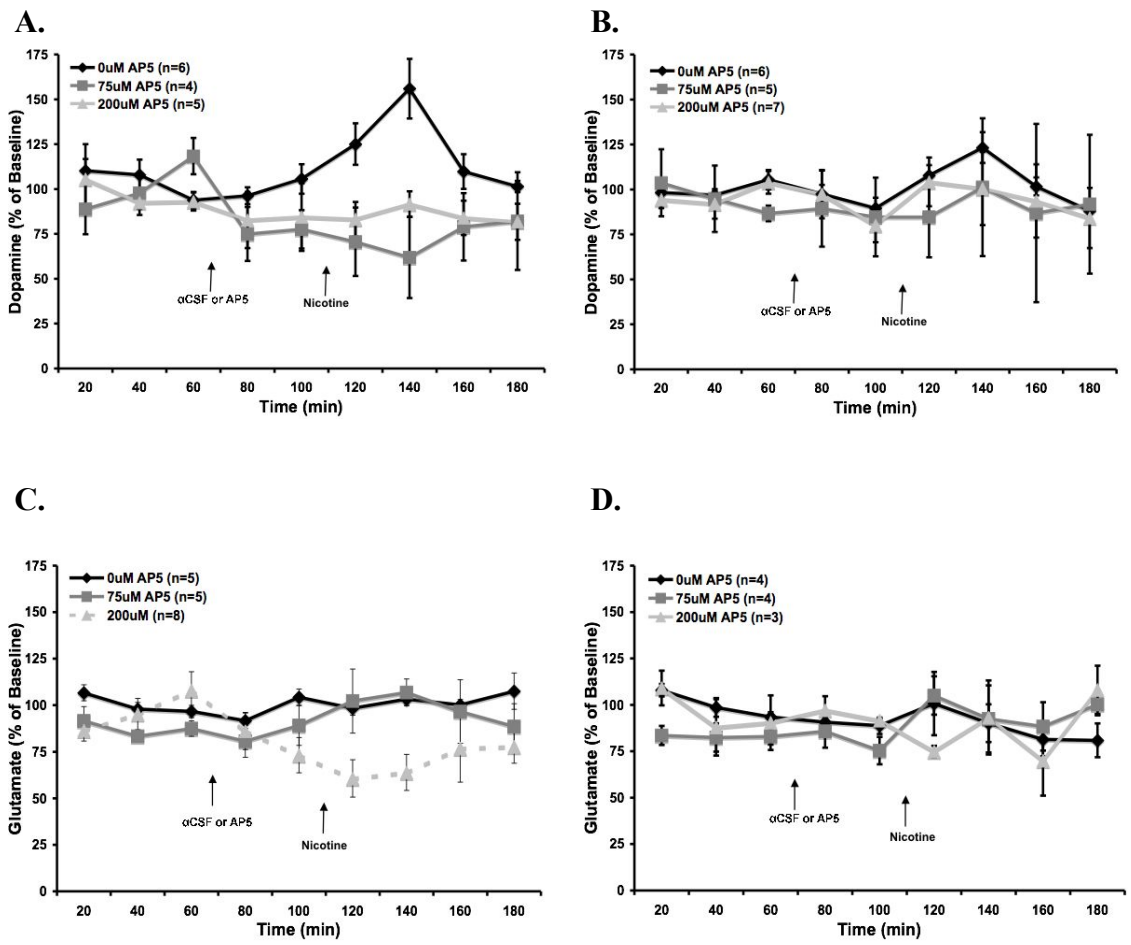


Figure 4.5: Effects of AP5 on nicotine-stimulated NAcc DA and VTA glutamate release in Nic+EtOH and PF offspring. Nic+EtOH and PF offspring received various doses of AP5 prior to 30µg nicotine i.v. **(A)** DA release from Nic+EtOH offspring was significant from baseline $F_{1,40}$: 8.55, p=0.006 without infusion of AP5 (0µM n=6). AP5 75µM (n=4) and 200µM (n=5) blocked nicotine-stimulated DA release in Nic+EtOH offspring. Nicotine-stimulated DA release **(B)** in PF treated with AP5 0µM (n=6), 75µM (n=5), and 200µM (n=7) did not differ significantly. However, only accumbal DA release in PF offspring produced a significant response from baseline ($F_{1,34}$: 6.76, p=0.014). Baseline DA levels were not significantly different between Nic+EtOH and PF offspring (1.53±0.18 vs. 1.38±0.20 pg/20µl). Nicotine did not stimulate significant glutamate release in VTA of Nic+EtOH **(C).** In the Nic+EtOH group, treatment with AP5 200µM (n=8) followed by i.v. nicotine significantly reduced glutamate levels ($F_{2,17}$:6.98; p=0.01) compared to 0µM (n=5) and 75µM (n=5). PF offspring **(D)** did not show a change in glutamate in response to nicotine with AP5 infusion at 0µM (n=4), 75µM (n=4), or 200µM (n=3). Baseline glutamate levels were not significantly different between Nic+EtOH and PF offspring (41.28±3.73 vs. 41.96±5.98 pg/µl, respectively).

In Nic+EtOH offspring, neither dose of AP5 *per se* affected VTA glutamate levels, although glutamate significantly decreased after nicotine in the group receiving 200μM AP5 ($F_{2,17}$:6.98; p=0.01; **Figure 4.5C**). Similarly, neither AP5 nor nicotine affected VTA glutamate levels in PF offspring (**Figure 4.5D**).

Effects of Gestational Nicotine and Ethanol Exposure on mRNA Transcript Expression of NMDA Receptor Subunits in VTA: Since gestational drug exposure occurred during critical phases of brain development, NMDA receptor gene expression was measured in VTA from adolescent offspring (PN45). **Figure 4.6** demonstrates that gestational exposure to Nic+EtOH did not alter gene expression levels of VTA NMDA receptor subunits compared to PF controls (NR1: p=0.42, NR2A: p=0.81, NR2B: p=0.7, and NR3A: p=0.55).

Effects of Gestational Nicotine and Ethanol Exposure on NMDA Receptor Subunit Protein Expression in VTA: Western immunoblottting of VTA extracts from PN45 offspring was done to determine if exposure to Nic+EtOH altered the relative amount of NMDA receptor subunits during adolescence. **Figure 4.7** shows that NMDA receptor subunit expression in Nic+EtOH and PF offspring did not differ (NR1: p=0.37, NR2A: p=0.57, and NR2B: p=0.53).

Effect of NMDA Receptor Antagonist AP5 on Nicotine Self-Administration: We have previously shown that nicotine SA is amplified in adult Nic+EtOH compared to PF offspring (Matta and Elberger 2007). In the experiment shown in **Figure 4.8**, our aim was to determine whether AP5 would differentially inhibit the acquisition of nicotine SA by altering this behavior in Nic+EtOH offspring without affecting PF offspring. Adolescent Nic+EtOH offspring that received a daily intra-VTA microinjection of vehicle (i.e., α-CSF) showed enhanced nicotine SA behavior compared to PF offspring (Two-way ANOVA: gestational treatment, $F_{1,120}$: 51.27, p=0.000001). Daily microinjections of AP5 reduced the level of nicotine SA acquired by Nic+EtOH offspring, without affecting PF rats (Three-way ANOVA: gestational treatment × AP5, $F_{1,220}$: 18.04, p=0.000032). Indeed, nicotine SA behavior in Nic+EtOH offspring receiving AP5 was not different from vehicle-treated PF offspring. Therefore, AP5 specifically prevented acquisition of the component of nicotine SA behavior that was enhanced by gestational exposure to Nic+EtOH.

Discussion

Glutamatergic innervation of VTA is vital to the regulation of DA neurons projecting to nucleus accumbens that mediate the rewarding properties of nicotine and other abused drugs (Geisler and Wise 2008). These experiments demonstrate that adolescent offspring with gestational exposure to Nic+EtOH are more sensitive to intra-VTA NMDA than PF offspring. Thus, NMDA induced greater increases in accumbal DA and VTA glutamate

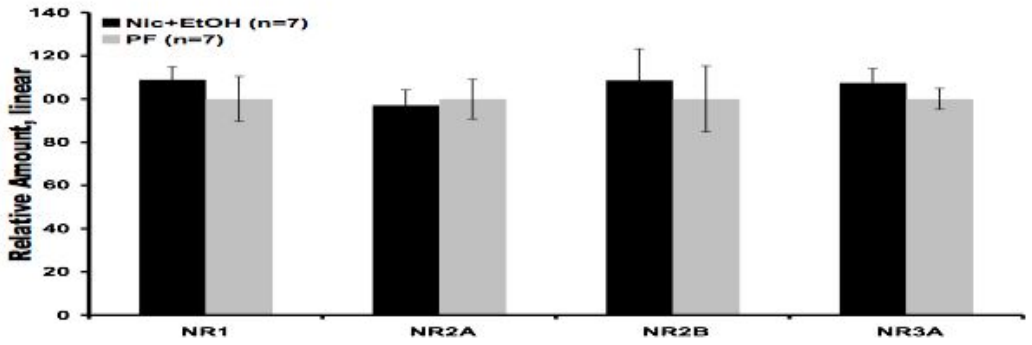


Figure 4.6: Effects of gestational drug exposure on NMDA receptor subunit transcript expression by qt-PCR. VTA brain punches were taken from adolescent (P45) Nic+EtOH (n=7) and PF offspring (n=7). Gestational Nic+EtOH exposure did not alter the levels of mRNA transcripts encoding NMDA receptor subunits (NR1: p=0.42, NR2A: p=0.81, NR2B: p=0.7, and NR3A: p=0.55).

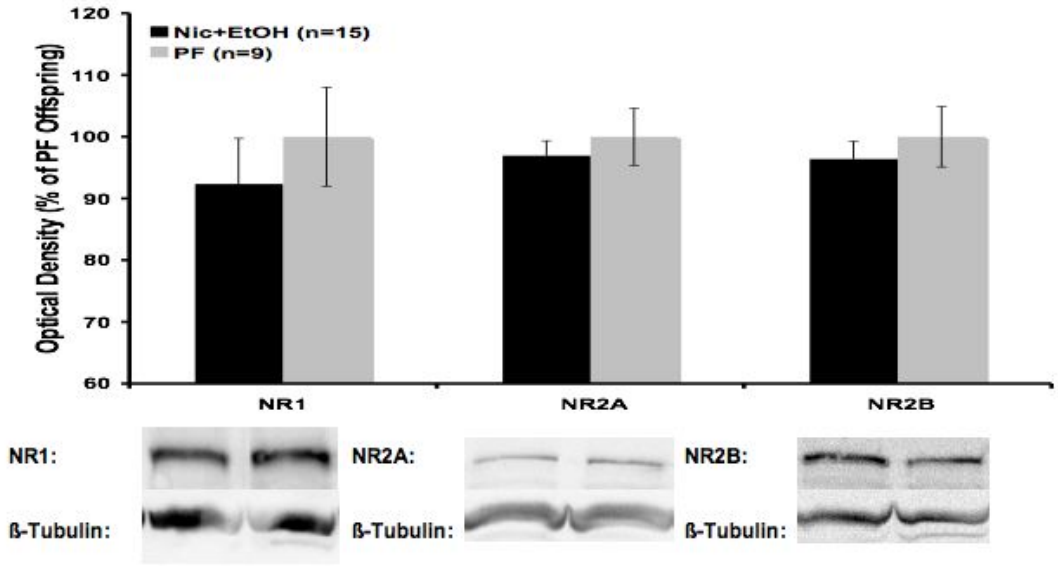


Figure 4.7: Effects of gestational Nic+EtOH exposure on NMDA receptor subunit protein expression. VTA brain punches were taken from adolescent (P45) Nic+EtOH (n=15) and PF (n=9) offspring. NMDA receptor subunit protein levels were not changed in Nic+EtOH offspring compared to PF (NR1: p=0.37, NR2A: p=0.57, and NR2B: p=0.53.

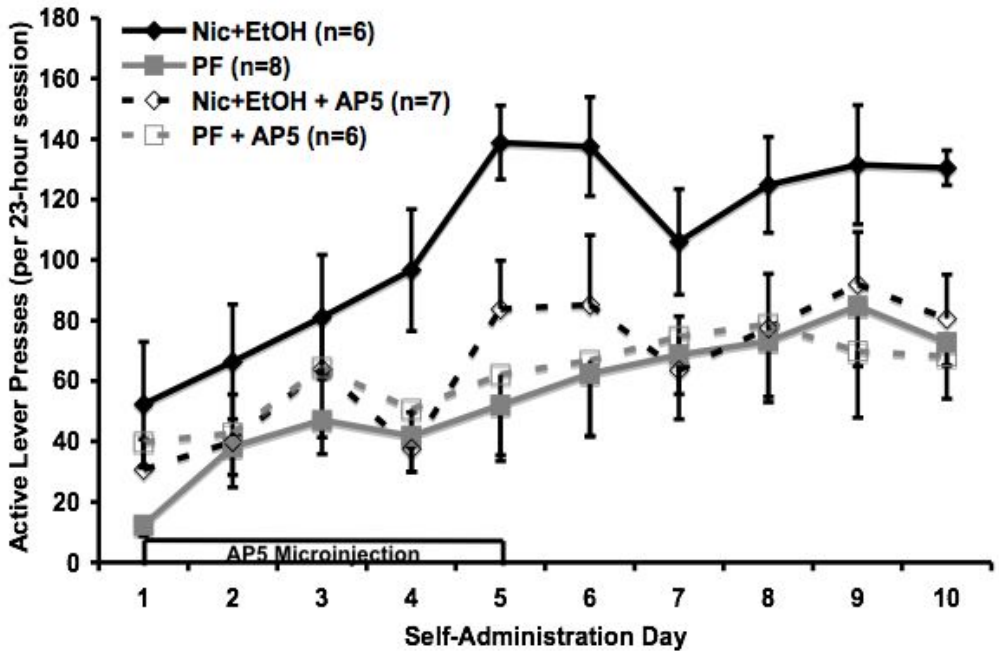


Figure 4.8: Effects of AP5 on nicotine SA in adolescent Nic+EtOH and PF offspring. Nicotine SA (30μg/kg/inj) was acquired by both groups of adolescent offspring that received AP5 (500ng/250nl) or vehicle microinjections into VTA at the beginning of each SA session on d 1-5. In the absence of AP5, Nic+EtOH offspring showed significantly higher responding for nicotine over the entire 10 days compared to PF, Nic+EtOH+AP5, and PF+AP5 offspring ($F_{1,220}$:18.04, p=0.000032; interaction between gestational drug treatment and AP5 microinjection).

in adolescent rats exposed to Nic+EtOH *in utero*. In addition, nicotine stimulated higher levels of accumbal DA in these offspring and they acquired more robust nicotine SA. Consistent with their enhanced sensitivity to NMDA, nicotine SA by adolescent Nic+EtOH offspring was reduced by an NMDA receptor antagonist, whereas PF controls were unaffected. Indeed, intra-VTA AP5 completely reversed the enhanced nicotine SA, resulting in levels observed in untreated PF adolescents. Therefore, during adolescence, NMDA receptor-dependent activation of VTA DA neurons projecting to NAcc shell is altered by gestational exposure to Nic+EtOH.

The role that NMDA receptors play in the mesocorticolimbic reward pathway has long been recognized (Jain et al. 2008; Mansvelder et al. 2003; Zellner and Ranaldi 2010). We previously demonstrated that VTA glutamate release induced by nicotine is correlated with accumbal DA release in untreated rats (Fu et al. 2000). However, relatively high dose i.v. nicotine was necessary to detect a significant increase in VTA glutamate release (Fu et al. 2000). Therefore, in the current study, we expected a relatively nominal release of glutamate after 30μg nicotine, although this dose robustly reinforces nicotine SA behavior (Valentine et al.), especially in Nic+EtOH offspring. Thus, by utilizing this dose of nicotine (i.e., 30μg), the effects of nicotine on neurotransmitters and behavior can be directly compared.

We found that 30μg nicotine stimulated significantly higher levels of accumbal DA in Nic+EtOH than PF offspring. In both of these cohorts, the efficacy of AP5 showed that signaling through NMDA receptors in VTA is required for nicotine-induced DA release, even though VTA glutamate levels were unaffected by nicotine. This may indicate that tonic synaptic levels of VTA glutamate are sufficient to activate dopamine neurons via NMDA receptors. Alternatively, it may simply indicate that stimulation by this low dose of nicotine was not sufficient to detect the synaptic overflow of glutamate. In previous studies, (Fu et al. 2000), we also demonstrated the role of NMDA receptors by infusing an NMDA antagonist into the VTA, which diminished accumbal dopamine release in response to nicotine. Another study showed that nicotine enhanced intracranial self-stimulation and this enhancement was blocked by an NMDA receptor antagonist (Kenny et al. 2009). In VTA slices, AP5 also was able to reduce but not completely block the excitatory response of nicotine (Grillner and Svensson 2000).

As to the mechanism underlying the release of VTA glutamate by NMDA, It is known that activation of VTA D1 receptors stimulates the local release of glutamate (Kalivas and Duffy 1995). Therefore, we hypothesized that elevation of accumbal DA by NMDA would be accompanied by release of VTA DA and activation of D1 receptors. The release of VTA DA by nicotine has been reported ((Erhardt et al. 2002). The current studies show that antagonism of VTA D1 receptors blocked the VTA glutamate response to NMDA, implicating the release of VTA DA by NMDA in the stimulation of D1 receptors. Unexpectedly, D1 receptor antagonism also inhibited accumbal DA release in response to NMDA, suggesting that activation of VTA NMDA receptors is insufficient to stimulate DA release and that other populations of glutamate receptors are targeted by glutamate released in response to VTA DA. AMPA receptors are potential targets of this glutamate. One study, using VTA slices, showed that 6-cyano-7-nitroquinoxaline-2,3-

dione (i.e., CNQX), an AMPA receptor antagonist, reduced DA neuron firing induced by nicotine (Grillner and Svensson 2000). Moreover, the AMPA receptor antagonist [1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(fluoro-methyl) quinoxalin-1-yl] methylphosphonate (i.e., ZK200775; 3.0mg/kg, i.p.) blocked nicotine-stimulated NAcc dopamine release in a dose dependent manner (Kosowski et al. 2004). In contrast, we have previously shown by microdialysis that intra-VTA CNQX did not alter nicotine-stimulated NAcc DA release (Fu et al. 2000). In addition, it was found that AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) did not alter nicotine SA behavior (Kenny et al. 2009). In face of this conflicting data, the role of AMPA receptors in nicotine-stimulated NAcc dopamine release and nicotine SA remains unclear.

The expression of NMDA receptor subunits in VTA was measured to gain further insight into the function of NMDA receptors. The expression of the NR2A and NR2B receptor subunits, in particular, demonstrate a delayed "developmental switch" induced by *in vitro* exposure to ethanol (Snell et al. 2001). In another study, chronic exposure to ethanol followed by withdrawal induced long-term facilitation in the dorsomedial striatum of adult rats that was mediated by NMDA receptors containing NR2B subunits (Wang et al. 2010). Although withdrawal after ethanol exposure increased NMDA receptor activity (i.e., facilitation) in dorsomedial but not dorsolateral striatum, the expression of NR2B subunits did not differ between these two brain regions. In alignment with this report, our results demonstrate that both the number of transcripts encoding specific NMDA subunits and receptor subunit expression, detected by immunoblotting, were unaffected by gestational exposure to Nic+EtOH. Our lab has also shown that expression of NR2A and NR2B receptor subunits was unchanged after chronic nicotine SA in adult Lewis rats (Wang et al. 2007). Taken together, these studies indicate that chronic exposure to ethanol followed by withdrawal can alter the long-term function of NMDA receptors containing specific receptor subunits (e.g., NR2B), without affecting expression levels of the receptor subunits.

These experiments show that AP5 antagonism of VTA NMDA receptors only blocked the fraction of nicotine SA behavior that was augmented by gestational exposure to Nic+EtOH. The unblocked fraction of nicotine SA was equivalent to nicotine SA in PF, which itself was unaffected by AP5. Despite these clear behavioral differences in responsiveness to AP5, accumbal DA release to a single dose of nicotine was abolished in both gestational cohorts. This discrepancy most likely reflects the relatively limited efficacy of a single daily intra-VTA injection of AP5 in our prolonged access model (i.e., 23 h access to nicotine SA). Indeed, AP5 inhibited the acquisition of food-reinforced behavior in 1 h operant sessions (Zellner et al. 2009). However, a single daily dose of AP5 apparently was sufficient to disrupt the function of those NMDA receptors with a gain of function due to gestational exposure to Nic+EtOH. However, the mechanism underlying this susceptibility to AP5 is not understood.

In summary, adolescent offspring exposed to Nic+EtOH during gestation show markedly increased vulnerability to become dependent on nicotine. These offspring acquire significantly higher levels of nicotine SA during adolescence. This reflects the enhanced

function of a subpopulation of VTA NMDA receptors that confer greater nicotine-induced DA release in NAcc.

CHAPTER 5. CONCLUSIONS

Nicotine and ethanol each exert effects on the developing brain that result in long-lasting behavioral alterations. Our full-gestational exposure to Nic+EtOH results in an enhanced acquisition to nicotine SA in both adult and adolescent offspring compared to PF controls. We have shown that the model of gestational drug exposure is responsible for the alteration in SA behavior, not a change in maternal-offspring interactions. The enhancement of nicotine SA by Nic+EtOH offspring can be reversed with the treatment of a GABA-B receptor agonist or a NMDA receptor antagonist. Nic+EtOH offspring display an alteration in the modulation of VTA DA neurons in response to nicotine by GABA, this is not seen in PF offspring or offspring with exposure to Nic, or EtOH alone. The resulting increased DA response to nicotine in the NAcc is also only seen in Nic+EtOH offspring, which indicates that the gestational exposure to both drugs is necessary to produce the enhanced SA behavior. The Nic+EtOH offspring display a hypersensitivity to intra-VTA NMDA compared with PF and a greater reduction of GABA release in response to 30μg/kg nicotine i.v. Offspring born with gestational exposure do not differ in basic physical characteristics (growth curve and birth parameters: birth weight (PN1), volume displacement, crown-rump length, and brain weight) from PF offspring. Taken together these results indicate that the enhanced acquisition of nicotine SA as seen in Nic+EtOH offspring is at least, in part, due to a change in modulation of VTA DA neurons by glutamate and GABA in the mesocorticolimbic pathway.

The experiments show that intra-VTA microinjection of either baclofen or AP-5 was able to disrupt the Nic+EtOH offspring SA behavior and alter responding for nicotine to PF levels. It should be noted that the microinjection was not able to block the acquisition of nicotine of these offspring or the PF offspring. Previous studies have shown that both microinjection of AP5 (Zellner *et al.* 2009) and baclofen (Paterson *et al.* 2004) was able to disrupt learning behavior in untreated animals. The difference in the results could be due to the dose of the microinjections or possibly a result of the use of our 23-hour SA model because baclofen or AP-5 would not be in the animals system for the entire session. However, adding a second microinjection would disrupt the SA session and would add another variable to which there would be no control.

It is important to note that the VTA as part of the mesocorticolimbic pathway is a complex area containing inputs that were not mentioned in these experiments that include: Acetylcholine (ACh), Noradrenaline, and Serotonin (5-HT) (Adell and Artigas 2004). In addition the VTA has DA neurons that project to the prefrontal cortex. Each of the experiments completed focused on only one VTA modulator in the mesocorticolimbic pathway.

Further investigation is needed to determine the full extent of alteration in the VTA as part of the mesocorticolimbic pathway of Nic+EtOH offspring. One possible alteration to be examined is serotonin (5-HT) from the midbrain raphe nuclei (Adell and Artigas 2004). Serotonin neurons are located on both dopamine and GABA neurons and there

are several 5-HT receptor subtypes in the VTA (Adell and Artigas 2004). For example, the 5-HT_{1A} receptor subunit exerts biphasic control in the VTA, increasing dopamine release at low doses, while at high doses dopamine release is reduced (Adell and Artigas 2004).

Prenatal exposure to nicotine has been indicated in changes in the 5-HT system whose interactions might indicate predisposition of cognitive impairments (Muneoka et al. 1997). The prenatal exposure produces long-lasting changes in 5-HT synaptic function, these changes have demonstrated a relationship between maternal smoking and nicotine self-administration by offspring (Slotkin et al. 2007). The study by Slotkin et al. (2007) also showed that the initial response to nicotine produces adjustments in baseline levels of the 5-HT system. Prenatal nicotine has also been identified in a decrease in 5-HT transporters (Xu et al. 2001). However, the study also found, upon examination of cell bodies the marker for transporter synthesis was increased in the brainstem of animals with exposure to nicotine indicating regrowth in response to neural damage (Xu et al. 2001). In addition, prenatal ethanol exposure in mice has been shown to hinder the development of 5-HT neurons causing them to appear abnormal (Zhou et al. 2001). Studies in both mice and rats have demonstrated a reduction in 5-HT neurons in the dorsal raphe of offspring with prenatal exposure to ethanol (Sari and Zhou 2004; Tajuddin and Druse 2001).

Gestational drug exposure (to either nicotine or EtOH) in animal models has previously shown changes in 5-HT. Therefore, possible changes 5-HT input of VTA function as part of the mesocorticolimbic pathway in Nic+EtOH offspring should be examined. Another possible contributor to the enhancement in Nic+EtOH SA behavior is ACh. Both DA and GABA neurons in the VTA are modulated by ACh with inputs from PPTg and LTDg (Tolu et al. 2013). It has been shown that intra-VTA methyllycaconitine (MLA), an $\alpha 7$ nicotinic acetylcholine (nACh) receptor antagonist was able to block the increased NAcc DA release (Schilstrom et al. 1998). A study has shown that $\alpha 7$ nACh receptors are located on glutamatergic axon terminals in the VTA (Jones and Wonnacott 2004). The placement of the nACh receptors enhances the release of glutamate onto VTA DA neurons (Jones and Wonnacott 2004).

The understanding of ACh and 5-HT in the mesocorticolimbic pathway may provide insight into the effectiveness of current smoking cessation treatments. One of the popular treatments is varenicline, acting as a partial nACh receptor agonist and by blocking nACh receptors (Polosa and Benowitz 2011; Syed and Chaudhari 2013). Varenicline is also an agonist for the 5-HT_3 receptors (Lummis et al. 2011). Other smoking cessation treatments include nicotine replacement therapy (NRT) and the use of NRT with buproprion, an antidepressant (Syed and Chaudhari 2013). However, the mechanism of action for buproprion is unknown (Syed and Chaudhari 2013).

The co-morbid effects to gestational nicotine and ethanol investigated using our gestational model provide some insight into the possible alteration of mesocorticolimbic mechanisms responsible for the increased correlation between maternal smoking as an indicator of their children smoking (Buka et al. 2003). Our results also show that the

increase in nicotine intake is present in both adolescent and adult animals, suggesting that the alteration in the mesocorticolimbic pathway involved in drug reward is long-lasting. It has been shown that most smokers try their first cigarette before the age of 18 (Kandel et al. 2007) and they tend to continue to smoke into adulthood (Colby et al. 2000). In addition, those who begin to smoke as an adolescent require less exposure to cigarettes before showing signs of dependence when compared to adult smokers (Colby et al. 2000).

Epidemiological studies have shown that children of smoking mothers are more likely to experiment early with cigarettes (Cornelius et al. 2000). The early availability of cigarettes due to legal purchase at age 18 is found to possibly lead to the use of other drugs of abuse (Myers and Kelly 2006). The experiments completed provide some insight into the deleterious effects of gestational Nic+EtOH exposure and how they resulted in the enhanced nicotine SA. It is possible that there is subset of the population that is more likely to use drugs of abuse because of alterations in their mesocorticolimbic reward pathway because of their gestational drug exposure. Documentation of gestational drug exposure and the outcomes of the children, specifically drug use, that could provide much needed information on whether this behavioral phenomenon observed in our experiments is present in the human population.

LIST OF REFERENCES

Abel EL (1978) Effects of ethanol on pregnant rats and their offspring. Psychopharmacology (Berl) 57: 5-11.

Abel EL, Dintcheff BA (1978) Effects of prenatal alcohol exposure on growth and development in rats. J Pharmacol Exp Ther 207: 916-21.

Adell A, Artigas F (2004) The somatodendritic release of dopamine in the ventral tegmental area and its regulation by afferent transmitter systems. Neurosci Biobehav Rev 28: 415-31.

Amantea D, Bowery NG (2004) Reduced inhibitory action of a GABAB receptor agonist on [3H]-dopamine release from rat ventral tegmental area in vitro after chronic nicotine administration. BMC Pharmacol 4: 24.

Andersen SL, Teicher MH (2009) Desperately driven and no brakes: developmental stress exposure and subsequent risk for substance abuse. Neurosci Biobehav Rev 33: 516-24.

Anthony JC, Echeagaray-Wagner F (2000) Epidemiologic analysis of alcohol and tobacco use. Alcohol Res Health 24: 201-8.

Bailey CD, Brien JF, Reynolds JN (2004) Chronic prenatal ethanol exposure alters the proportion of GABAergic neurons in layers II/III of the adult guinea pig somatosensory cortex. Neurotoxicol Teratol 26: 59-63.

Barbazanges A, Vallee M, Mayo W, Day J, Simon H, Le Moal M, Maccari S (1996) Early and later adoptions have different long-term effects on male rat offspring. J Neurosci 16: 7783-90.

Bobo JK, Husten C (2000) Sociocultural influences on smoking and drinking. Alcohol Res Health 24: 225-32.

Bowery NG (2006) GABAB receptor: a site of therapeutic benefit. Curr Opin Pharmacol 6: 37-43.

Brake WG, Zhang TY, Diorio J, Meaney MJ, Gratton A (2004) Influence of early postnatal rearing conditions on mesocorticolimbic dopamine and behavioural responses to psychostimulants and stressors in adult rats. Eur J Neurosci 19: 1863-74.

Breslau N, Peterson EL (1996) Smoking cessation in young adults: age at initiation of cigarette smoking and other suspected influences. Am J Public Health 86: 214-20.

Brodie MS (2002) Increased ethanol excitation of dopaminergic neurons of the ventral tegmental area after chronic ethanol treatment. Alcohol Clin Exp Res 26: 1024-30.

Buka SL, Shenassa ED, Niaura R (2003) Elevated risk of tobacco dependence among offspring of mothers who smoked during pregnancy: a 30-year prospective study. Am J Psychiatry 160: 1978-84.

Caul WF, Osborne GL, Fernandez K, Henderson GI (1979) Open-field and avoidance performance of rats as a function of prenatal ethanol treatment. Addict Behav 4: 311-22.

Champagne FA, Curley JP (2009) Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neurosci Biobehav Rev 33: 593-600.

Chen H, Matta SG, Sharp BM (2007) Acquisition of nicotine self-administration in adolescent rats given prolonged access to the drug. Neuropsychopharmacology 32: 700-9.
Colby SM, Tiffany ST, Shiffman S, Niaura RS (2000) Are adolescent smokers dependent on nicotine? A review of the evidence. Drug Alcohol Depend 59 Suppl 1: S83-95.
Cornelius MD, Day NL (2000) The effects of tobacco use during and after pregnancy on exposed children. Alcohol Res Health 24: 242-9.
Cornelius MD, Goldschmidt L, DeGenna N, Day NL (2007) Smoking during teenage pregnancies: effects on behavioral problems in offspring. Nicotine Tob Res 9: 739-50.
Cornelius MD, Leech SL, Goldschmidt L, Day NL (2000) Prenatal tobacco exposure: is it a risk factor for early tobacco experimentation? Nicotine Tob Res 2: 45-52.
Corrigall WA, Coen KM, Adamson KL, Chow BL, Zhang J (2000) Response of nicotine self-administration in the rat to manipulations of mu-opioid and gamma-aminobutyric acid receptors in the ventral tegmental area. Psychopharmacology (Berl) 149: 107-14.
Cousins MS, Roberts DC, de Wit H (2002) GABA(B) receptor agonists for the treatment of drug addiction: a review of recent findings. Drug Alcohol Depend 65: 209-20.
Cousins MS, Stamat HM, de Wit H (2001) Effects of a single dose of baclofen on self-reported subjective effects and tobacco smoking. Nicotine Tob Res 3: 123-9.
Darnaudery M, Koehl M, Barbazanges A, Cabib S, Le Moal M, Maccari S (2004) Early and later adoptions differently modify mother-pup interactions. Behav Neurosci 118: 590-6.
Dellu F, Mayo W, Vallee M, Maccari S, Piazza PV, Le Moal M, Simon H (1996) Behavioral reactivity to novelty during youth as a predictive factor of stress-induced corticosterone secretion in the elderly--a life-span study in rats. Psychoneuroendocrinology 21: 441-53.
DiFranza JR, Rigotti NA, McNeill AD, Ockene JK, Savageau JA, St Cyr D, Coleman M (2000) Initial symptoms of nicotine dependence in adolescents. Tob Control 9: 313-9.
DiFranza JR, Savageau JA, Rigotti NA, Fletcher K, Ockene JK, McNeill AD, Coleman M, Wood C (2002) Development of symptoms of tobacco dependence in youths: 30 month follow up data from the DANDY study. Tob Control 11: 228-35.
DiFranza JR, Wellman RJ, Sargent JD, Weitzman M, Hipple BJ, Winickoff JP (2006) Tobacco promotion and the initiation of tobacco use: assessing the evidence for causality. Pediatrics 117: e1237-48.
Dwyer JB, McQuown SC, Leslie FM (2009) The dynamic effects of nicotine on the developing brain. Pharmacol Ther 122: 125-39.
Eppolito AK, Smith RF (2006) Long-term behavioral and developmental consequences of pre- and perinatal nicotine. Pharmacol Biochem Behav 85: 835-41.
Ernst M, Moolchan ET, Robinson ML (2001) Behavioral and neural consequences of prenatal exposure to nicotine. J Am Acad Child Adolesc Psychiatry 40: 630-41.
Fattore L, Cossu G, Martellotta MC, Fratta W (2002) Baclofen antagonizes intravenous self-administration of nicotine in mice and rats. Alcohol Alcohol 37: 495-8.

Franklin TR, Harper D, Kampman K, Kildea-McCrea S, Jens W, Lynch KG, O'Brien CP, Childress AR (2009) The GABA B agonist baclofen reduces cigarette consumption in a preliminary double-blind placebo-controlled smoking reduction study. Drug Alcohol Depend 103: 30-6.

Fu Y, Matta SG, Brower VG, Sharp BM (2001) Norepinephrine secretion in the hypothalamic paraventricular nucleus of rats during unlimited access to self-administered nicotine: An in vivo microdialysis study. J Neurosci 21: 8979-89.

Fu Y, Matta SG, Gao W, Brower VG, Sharp BM (2000) Systemic nicotine stimulates dopamine release in nucleus accumbens: re-evaluation of the role of N-methyl-D-aspartate receptors in the ventral tegmental area. J Pharmacol Exp Ther 294: 458-65.

Gallegos RA, Lee RS, Criado JR, Henriksen SJ, Steffensen SC (1999) Adaptive responses of gamma-aminobutyric acid neurons in the ventral tegmental area to chronic ethanol. J Pharmacol Exp Ther 291: 1045-53.

Gancarz AM, San George MA, Ashrafioun L, Richards JB (2011) Locomotor activity in a novel environment predicts both responding for a visual stimulus and self-administration of a low dose of methamphetamine in rats. Behav Processes 86: 295-304.

Geisler S, Wise RA (2008) Functional implications of glutamatergic projections to the ventral tegmental area. Rev Neurosci 19: 227-44.

Golub M, Kornetsky C (1975) Effects of testing age and fostering experience on seizure susceptibility of rats treated prenatally with chlorpromazine. Dev Psychobiol 8: 519-24.

Gomez-Serrano M, Tonelli L, Listwak S, Sternberg E, Riley AL (2001) Effects of cross fostering on open-field behavior, acoustic startle, lipopolysaccharide-induced corticosterone release, and body weight in Lewis and Fischer rats. Behav Genet 31: 427-36.

Greenbaum RL, Stevens SA, Nash K, Koren G, Rovet J (2009) Social cognitive and emotion processing abilities of children with fetal alcohol spectrum disorders: a comparison with attention deficit hyperactivity disorder. Alcohol Clin Exp Res 33: 1656-70.

Grillner P, Svensson TH (2000) Nicotine-induced excitation of midbrain dopamine neurons in vitro involves ionotropic glutamate receptor activation. Synapse 38: 1-9.

Grucza RA, Bierut LJ (2006a) Cigarette smoking and the risk for alcohol use disorders among adolescent drinkers. Alcohol Clin Exp Res 30: 2046-54.

Grucza RA, Bierut LJ (2006b) Co-occurring risk factors for alcohol dependence and habitual smoking: update on findings from the Collaborative Study on the Genetics of Alcoholism. Alcohol Res Health 29: 172-8.

Helmeke C, Ovtscharoff W, Jr., Poeggel G, Braun K (2008) Imbalance of immunohistochemically characterized interneuron populations in the adolescent and adult rodent medial prefrontal cortex after repeated exposure to neonatal separation stress. Neuroscience 152: 18-28.

Hendrickson LM, Zhao-Shea R, Tapper AR (2009) Modulation of ethanol drinking-in-the-dark by mecamylamine and nicotinic acetylcholine receptor agonists in C57BL/6J mice. Psychopharmacology (Berl) 204: 563-72.

Henson MA, Roberts AC, Perez-Otano I, Philpot BD (2010) Influence of the NR3A subunit on NMDA receptor functions. Prog Neurobiol 91: 23-37.
Jain R, Mukherjee K, Balhara YP (2008) The role of NMDA receptor antagonists in nicotine tolerance, sensitization, and physical dependence: a preclinical review. Yonsei Med J 49: 175-88.
Jezierski G, Braun K, Gruss M (2006) Epigenetic modulation of the developing serotonergic neurotransmission in the semi-precocial rodent Octodon degus. Neurochem Int 48: 350-7.
Jones IW, Wonnacott S (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. J Neurosci 24: 11244-52.
Kahler CW, Strong DR, Papandonatos GD, Colby SM, Clark MA, Boergers J, Niaura R, Abrams DB, Buka SL (2008) Cigarette smoking and the lifetime alcohol involvement continuum. Drug Alcohol Depend 93: 111-20.
Kalivas PW (1993) Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Res Brain Res Rev 18: 75-113.
Kalivas PW, Duffy P (1995) D1 receptors modulate glutamate transmission in the ventral tegmental area. J Neurosci 15: 5379-88.
Kandel D, Chen K, Warner LA, Kessler RC, Grant B (1997) Prevalence and demographic correlates of symptoms of last year dependence on alcohol, nicotine, marijuana and cocaine in the U.S. population. Drug Alcohol Depend 44: 11-29.
Kandel DB, Hu MC, Griesler PC, Schaffran C (2007) On the development of nicotine dependence in adolescence. Drug Alcohol Depend 91: 26-39.
Kane VB, Fu Y, Matta SG, Sharp BM (2004) Gestational nicotine exposure attenuates nicotine-stimulated dopamine release in the nucleus accumbens shell of adolescent Lewis rats. J Pharmacol Exp Ther 308: 521-8.
Kenny PJ, Chartoff E, Roberto M, Carlezon WA, Jr., Markou A (2009) NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-administration: role of the ventral tegmental area and central nucleus of the amygdala. Neuropsychopharmacology 34: 266-81.
Koob GF, Nestler EJ (1997) The neurobiology of drug addiction. J Neuropsychiatry Clin Neurosci 9: 482-97.
Kosowski AR, Cebers G, Cebere A, Swanhagen AC, Liljequist S (2004) Nicotine-induced dopamine release in the nucleus accumbens is inhibited by the novel AMPA antagonist ZK200775 and the NMDA antagonist CGP39551. Psychopharmacology (Berl) 175: 114-23.
Kuczkowski KM (2007) The effects of drug abuse on pregnancy. Curr Opin Obstet Gynecol 19: 578-85.
Lavezzi AM, Matturri L, Del Corno G, Johanson CE (2013) Vulnerability of fourth ventricle choroid plexus in sudden unexplained fetal and infant death syndromes related to smoking mothers. Int J Dev Neurosci 31: 319-27.
Laviolette SR, van der Kooy D (2004) The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. Nat Rev Neurosci 5: 55-65.
Le Foll B, Wertheim CE, Goldberg SR (2008) Effects of baclofen on conditioned rewarding and discriminative stimulus effects of nicotine in rats. Neurosci Lett 443: 236-40.

Liu XB, Murray KD, Jones EG (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. J Neurosci 24: 8885-95.
Liu Z, Neff RA, Berg DK (2006) Sequential interplay of nicotinic and GABAergic signaling guides neuronal development. Science 314: 1610-3.
Lummis SC, Thompson AJ, Bencherif M, Lester HA (2011) Varenicline is a potent agonist of the human 5-hydroxytryptamine3 receptor. J Pharmacol Exp Ther 339: 125-31.
Mansvelder HD, De Rover M, McGehee DS, Brussaard AB (2003) Cholinergic modulation of dopaminergic reward areas: upstream and downstream targets of nicotine addiction. Eur J Pharmacol 480: 117-23.
Maritz GS, Mutemwa M (2012) Tobacco smoking: patterns, health consequences for adults, and the long-term health of the offspring. Glob J Health Sci 4: 62-75.
Matta SG, Elberger AJ (2007) Combined exposure to nicotine and ethanol throughout full gestation results in enhanced acquisition of nicotine self-administration in young adult rat offspring. Psychopharmacology (Berl) 193: 199-213.
Mattson SN, Schoenfeld AM, Riley EP (2001) Teratogenic effects of alcohol on brain and behavior. Alcohol Res Health 25: 185-91.
Moore DB, Quintero MA, Ruygrok AC, Walker DW, Heaton MB (1998) Prenatal ethanol exposure reduces parvalbumin-immunoreactive GABAergic neuronal number in the adult rat cingulate cortex. Neurosci Lett 249: 25-8.
Muneoka K, Ogawa T, Kamei K, Muraoka S, Tomiyoshi R, Mimura Y, Kato H, Suzuki MR, Takigawa M (1997) Prenatal nicotine exposure affects the development of the central serotonergic system as well as the dopaminergic system in rat offspring: involvement of route of drug administrations. Brain Res Dev Brain Res 102: 117-26.
Myers MG, Kelly JF (2006) Cigarette smoking among adolescents with alcohol and other drug use problems. Alcohol Res Health 29: 221-7.
Navarro HA, Seidler FJ, Schwartz RD, Baker FE, Dobbins SS, Slotkin TA (1989) Prenatal exposure to nicotine impairs nervous system development at a dose which does not affect viability or growth. Brain Res Bull 23: 187-92.
Osborne GL, Caul WF, Fernandez K (1980) Behavioral effects of prenatal ethanol exposure and differential early experience in rats. Pharmacol Biochem Behav 12: 393-401.
Paterson NE, Froestl W, Markou A (2004) The GABAB receptor agonists baclofen and CGP44532 decreased nicotine self-administration in the rat. Psychopharmacology (Berl) 172: 179-86.
Polosa R, Benowitz NL (2011) Treatment of nicotine addiction: present therapeutic options and pipeline developments. Trends Pharmacol Sci 32: 281-9.
Pometlova M, Hruba L, Slamberova R, Rokyta R (2009) Cross-fostering effect on postnatal development of rat pups exposed to methamphetamine during gestation and preweaning periods. Int J Dev Neurosci 27: 149-55.
Puglia MP, Valenzuela CF (2010) Repeated third trimester-equivalent ethanol exposure inhibits long-term potentiation in the hippocampal CA1 region of neonatal rats. Alcohol 44: 283-90.

Roguski EE, Chen H, Sharp BM, Matta SG. Fostering *itself* increases nicotine self-administration in young adult male rats. Psychopharmacology 2013 Sept;229(2)227-34.
Roguski EE, Sharp BM, Chen H, Matta SG. Full-Gestational Exposure to Nicotine and Ethanol Augments Nicotine Self-Administration by Altering Ventral Tegmental Dopaminergic Function due to NMDA Receptors in Adolescent Rats. J Neurochem (2013) *In Press*
Sallerin-Caute B, Lazorthes Y, Monsarrat B, Cros J, Bastide R (1991) CSF baclofen levels after intrathecal administration in severe spasticity. Eur J Clin Pharmacol 40: 363-5.
Sari Y, Zhou FC (2004) Prenatal alcohol exposure causes long-term serotonin neuron deficit in mice. Alcohol Clin Exp Res 28: 941-8.
Schilstrom B, Svensson HM, Svensson TH, Nomikos GG (1998) Nicotine and food induced dopamine release in the nucleus accumbens of the rat: putative role of alpha7 nicotinic receptors in the ventral tegmental area. Neuroscience 85: 1005-9.
Slotkin TA, Orband-Miller L, Queen KL, Whitmore WL, Seidler FJ (1987) Effects of prenatal nicotine exposure on biochemical development of rat brain regions: maternal drug infusions via osmotic minipumps. J Pharmacol Exp Ther 240: 602-11.
Slotkin TA, Ryde IT, Tate CA, Seidler FJ (2007) Lasting effects of nicotine treatment and withdrawal on serotonergic systems and cell signaling in rat brain regions: separate or sequential exposure during fetal development and adulthood. Brain Res Bull 73: 259-72.
Snell LD, Bhave SV, Tabakoff B, Hoffman PL (2001) Chronic ethanol exposure delays the 'developmental switch' of the NMDA receptor 2A and 2B subunits in cultured cerebellar granule neurons. J Neurochem 78: 396-405.
Stanwood GD, Levitt P (2004) Drug exposure early in life: functional repercussions of changing neuropharmacology during sensitive periods of brain development. Curr Opin Pharmacol 4: 65-71.
Steffensen SC, Walton CH, Hansen DM, Yorgason JT, Gallegos RA, Criado JR (2009) Contingent and non-contingent effects of low-dose ethanol on GABA neuron activity in the ventral tegmental area. Pharmacol Biochem Behav 92: 68-75.
Substance Abuse and Mental Health Services Administration OoAS The NSDUH Report - - Alcohol Use among Pregnant Women and Recent Mothers: 2002 to 2007. Rockville, MD.
Substance Abuse and Mental Health Services Administration OoAS The NSDUH Report - - Cigarette Use among Pregnant Women and Recent Mothers. Rockville, MD.
Substance Abuse and Mental Health Services Administration S (2012) Results from the 2011 National Survey on Drug Use and Health: Summary of National Findings. NSDUH Series H-44, HHS Publication No (SMA) 12-4713 Rockville, MD: Substance Abuse and Mental Health Services Administration.
Suto N, Austin JD, Vezina P (2001) Locomotor response to novelty predicts a rat's propensity to self-administer nicotine. Psychopharmacology (Berl) 158: 175-80.
Syed BA, Chaudhari K (2013) Smoking cessation drugs market. Nat Rev Drug Discov 12: 97-8.

Tajuddin NF, Druse MJ (2001) A persistent deficit of serotonin neurons in the offspring of ethanol-fed dams: protective effects of maternal ipsapirone treatment. Brain Res Dev Brain Res 129: 181-8.

Tizabi Y, Bai L, Copeland RL, Jr., Taylor RE (2007) Combined effects of systemic alcohol and nicotine on dopamine release in the nucleus accumbens shell. Alcohol Alcohol 42: 413-6.

Tolu S, Eddine R, Marti F, David V, Graupner M, Pons S, Baudonnat M, Husson M, Besson M, Reperant C, Zemdegs J, Pages C, Hay YA, Lambolez B, Caboche J, Gutkin B, Gardier AM, Changeux JP, Faure P, Maskos U (2013) Co-activation of VTA DA and GABA neurons mediates nicotine reinforcement. Mol Psychiatry 18: 382-93.

Valentine JD, Hokanson JS, Matta SG, Sharp BM (1997) Self-administration in rats allowed unlimited access to nicotine. Psychopharmacology (Berl) 133: 300-4.

Vallee M, MacCari S, Dellu F, Simon H, Le Moal M, Mayo W (1999) Long-term effects of prenatal stress and postnatal handling on age-related glucocorticoid secretion and cognitive performance: a longitudinal study in the rat. Eur J Neurosci 11: 2906-16.

Vathy I, Slamberova R, Liu X (2007) Foster mother care but not prenatal morphine exposure enhances cocaine self-administration in young adult male and female rats. Dev Psychobiol 49: 463-73.

Wang F, Chen H, Steketee JD, Sharp BM (2007) Upregulation of ionotropic glutamate receptor subunits within specific mesocorticolimbic regions during chronic nicotine self-administration. Neuropsychopharmacology 32: 103-9.

Wang J, Lanfranco MF, Gibb SL, Yowell QV, Carnicella S, Ron D (2010) Long-lasting adaptations of the NR2B-containing NMDA receptors in the dorsomedial striatum play a crucial role in alcohol consumption and relapse. J Neurosci 30: 10187-98.

Westerink BH, Kwint HF, deVries JB (1996) The pharmacology of mesolimbic dopamine neurons: a dual-probe microdialysis study in the ventral tegmental area and nucleus accumbens of the rat brain. J Neurosci 16: 2605-11.

Xu Z, Seidler FJ, Ali SF, Slikker W, Jr., Slotkin TA (2001) Fetal and adolescent nicotine administration: effects on CNS serotonergic systems. Brain Res 914: 166-78.

Zellner MR, Kest K, Ranaldi R (2009) NMDA receptor antagonism in the ventral tegmental area impairs acquisition of reward-related learning. Behav Brain Res 197: 442-9.

Zellner MR, Ranaldi R (2010) How conditioned stimuli acquire the ability to activate VTA dopamine cells: a proposed neurobiological component of reward-related learning. Neurosci Biobehav Rev 34: 769-80.

Zhou FC, Sari Y, Zhang JK, Goodlett CR, Li T (2001) Prenatal alcohol exposure retards the migration and development of serotonin neurons in fetal C57BL mice. Brain Res Dev Brain Res 126: 147-55.

VITA

Emily Elizabeth Roguski was born in Pittsburgh, Pennsylvania in 1983. She graduated from Bethel Park High School in May of 2001. In May of 2005 she graduated from Allegheny College with a Bachelor of Science degree in Psychology. Following graduation she began work at the University of Tennessee Health Science Center as a Research Assistant in the laboratory of Shannon G. Matta. In August 2007, she entered the Integrated Program in Biomedical Sciences program at the University of Tennessee Health Science Center. She completed her Doctor of Philosophy in Neuroscience in December of 2013.