IV Prenatal Nicotine Exposure Modulates α6, α7, and α4β2 Nicotinic Acetylcholinergic Receptor Subunit Expression In Male And Female Neonatal And Adolescent Rat Offspring: An Autoradiographical Analysis

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IV Prenatal Nicotine Exposure Modulates α6, α7, and α4β2 Nicotinic Acetylcholinergic Receptor Subunit Expression in Male and Female Neonatal and Adolescent Rat Offspring: An Autoradiographical Analysis

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DEDICATION

To my dearest Clayton. Without you, none of this would be possible. Thank you for your dedicated partnership for success in my life.

Several academic mentors have provided guidance. Thank you, Dr. Steven Harrod, for making my journey and its conclusion possible. I’ve gained valuable, lifelong knowledge about the importance of integrity in science, and how only the most careful work can produce valid results. Thank you Dr. Charles Mactutus. You are a genius and I am proud to have worked with you and your wife, Dr. Rose Booze. You’ve both inspired me with unparalleled passion and devotion to the pursuit of knowledge, using rigorous standards. Thank you Dr. Richard Metzger, my Master’s advisor—your insight and foresight shaped my love of neuroscience.

Last but most certainly not least, I owe everything up to this point to my mother. You gave me life, and during my youth you instilled the values needed to work this through to the end, despite so many bumps and detours along the road. To the departed: granny Cleo, I dedicate this and my entire body of work, to you. You loved me beyond measure. Dad, I miss you and wish you were here to see my accomplishments.
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Drs. Ryan Lacy and Steven Harrod made this study possible, procuring the animals needed, and investing days, hours, weeks, months and years to perfecting the IV nicotine treatment procedure. A special thanks to Dr. Jim Pauly, who performed the autoradiography methods and radioligands in this study. Thank you to Dr. Jim Fadel, as a collaborator and committee member. Your collaboration and mentorship has been critical for my success. Finally, funding from 5 T32 GM091740 (USC) and NIDA Grant R01 – DA021287 (SBH) made this work, as well as my entire body of work, possible.
Maternal smoke exposure produces long-term adverse cognitive and behavioral outcomes in offspring, including an increased likelihood of attention problems (e.g., attention deficit hyperactivity disorder; ADHD) and drug abuse. Preclinical research shows that gestational exposure to nicotine, the primary psychoactive compound in tobacco smoke, influences the neurodevelopment of attention and reward neuronal circuits. This study investigated hypotheses about five brain regions, to determine if prenatal nicotine (PN) exposure altered expression of nicotinic acetylcholine receptors (nAChRs). A low dose, intravenous nicotine (IV) exposure method was used to administer nicotine (0.05 mg/kg/injection) or saline, 3x/day on gestational days 8–21 (Treatment). Brain tissue was collected from both male and female offspring (Sex), on postnatal days (PND) 10 and 35 (Age). Autoradiography quantified $^{125}$I-Epibatidine, $^{125}$I-$\alpha$-Bungarotoxin, $^{125}$I-$\alpha$-Conotoxin MII binding, to measure putative $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 6$-containing nAChR subunit expression levels. It was hypothesized that $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 6$-subunit-containing nAChRs in the Ventral Tegmental Area (VTA), Nucleus Accumbens Core (NAc), prefrontal cortex (PFC), Pendunculopontine Tegmental Nucleus (PPTg), and Hippocampus, would have significantly different expression levels between PN and prenatal saline (PS) groups (Treatment). Males were expected to have higher levels of nAChR expression across regions, and tissue from older (PND35; adolescent) subjects was hypothesized to also have higher levels of nAChR expression, compared to neonates.
Following autoradiography, receptor expression levels were analyzed with the MicroBrightField software Densita, measuring receptor density as Luminance value, (a lower Luminance value indicates greater density of receptors). A total of 10 rodent dams contributed four offspring each (n=40), and the obtained Luminance values underwent preliminary statistical screening, to determine if variance analyses should proceed with Luminance as a Random Effect variable. If Litter did not have a significant effect (conservative p>0.250), a between-subjects Analysis of Variance (ANOVA) was used; if Litter had a significant effect on the shape of Luminance distribution (conservative p<0.250), statistical analyses continued with Litter as a Random Effect in a Mixed Models Linear (MML) analysis. Post-hoc analyses determined the retrospective power of each observed significant effect, based on sample size and parameter estimates derived from either ANOVA or MML. Statistically significant, sufficiently powered, main effects of Treatment were found for α4β2 nAChR expression levels in the VTA and NAcc, but no Treatment effect was found in the PPTg. As hypothesized, α7 nAChRs expression levels were lower for PN rats; the effect was only seen in the NAcc, with no significant Treatment effect found in the VTA or PFC. In the Hippocampus, Treatment was a significant main effect, but the PN group expressed more α7 nAChRs than the PS group. A statistically significant, sufficiently powered, main effect of Sex was found only in the PFC; as hypothesized, Males expressed greater density of α4β2 nAChRs. Statistically significant, sufficiently powered, main effects of Age were found for α4β2 nAChR expression in the PFC and PPTg; in the PFC, the effect was opposite: older rats expressed fewer α4β2 nAChRs. No age differences were found in the VTA or NAcc. For nα7 nAChRs, Age had a significant main effect in the Hippocampus, with adolescents
expressing lower density of receptors, compared to neonates. No statistically significant, sufficiently powered effects for α6-containing nAChRs for Treatment, Sex, or Age were found. In addition to the five hypothesized regions, 12 more brain areas were analyzed for α4β2, α7, and α6-subunit containing nAChR expression levels. Those results are presented in tandem with the hypothesized findings in the Results section of this report.
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LIST OF ABBREVIATIONS

ACh .................................................................................................................. Acetylcholine
AChE .................................................................................................................. Acetylcholinesterase
ADHD ................................................................................................................. Attention Deficit Hyperactivity Disorder
ChAT .................................................................................................................. Choline Acetyltransferase
DA ....................................................................................................................... Dopamine
GD ....................................................................................................................... Gestational Day
ICC ...................................................................................................................... IntraCluster Correlation
IPN ....................................................................................................................... Interpeduncular Nucleus
IV ......................................................................................................................... Intravenous
MLM .................................................................................................................. Multilevel Modeling
MML .................................................................................................................. Mixed Models Linear
NAcc ................................................................................................................... Nucleus Accumbens Core
nAChRs .............................................................................................................. Nicotine Acetylcholine Receptors
ODC .................................................................................................................... Ornithine Decarboxylase
OMP .................................................................................................................... Osmotic Minipump
PCSE .................................................................................................................. Prenatal Cigarette Smoke Exposed
PFC ....................................................................................................................... Prefrontal Cortex
PN ......................................................................................................................... Prenatal Nicotine
PND ...................................................................................................................... Postnatal Day
PPTg ............................................................. Pedunculopontine Tegmental Nucleus
PS ...................................................................................... Prenatal Saline
SC ...................................................................................... Subcutaneous
VTA ................................................................. Ventral Tegmental Area
CHAPTER I

INTRODUCTION

1 Maternal smoking during pregnancy: Clinical and Epidemiological Findings

Mothers who smoke cigarettes while pregnant increase the risk of health complications for themselves and their offspring. Among pregnant American women, the most recent reports indicate that 15.4% continue to smoke while pregnant (SAMHSA, 2014). Maternal smoking during pregnancy is correlated with several adverse outcomes in offspring, including increased behavioral problems, cognitive impairments, and substance use. Tobacco smoke is a neuroteratogen, and offspring of mothers who smoke while pregnant may exhibit any number of a constellation of problematic effects.

The physical phenotype of exposure to maternal smoking during gestation can include low birth weight (Delpisheh et al., 2006; Dunn et al., 1976; Ernst et al., 2009; Fenercioglu et al., 2009; Kramer et al., 1990), as a result of restricted intrauterine growth (Horta et al., 1997; Kramer, 1987; Nordentoft et al., 1996; Quinton et al., 2008). These newborns are more likely to be born prematurely (Cooke, 1998; Cooperstock et al., 2000), and have a higher risk of sudden infant death syndrome (Burguet et al., 2004; Fleming & Blair, 2007; Haglund & Cnattingius, 1990).

Neonates subjected to maternal tobacco smoke during gestation have significantly decreased head circumference, reductions of brain volume in some prefrontal cortical
areas, and anisotropic distribution of white matter in areas responsible for attention processing (DiFranza et al., 2004; Cornelius & Day, 2009; Ernst et al., 2009; Fenercioglu et al., 2009). These early life biological challenges may foreshadow a myriad of behavioral and cognitive effects following tobacco smoke exposure during gestation.

1. **Maternal smoking during pregnancy: Clinical and Epidemiological Findings**

1.1 Cognitive and behavioral outcomes

Problems with attention and behavior/conduct are some of the most common maladaptive developmental trajectories following maternal smoking during pregnancy. A dose-dependent relationship between amount of maternal cigarettes smoked per day during pregnancy, and incidence of conduct disorder, has been reported among PCSE (prenatal cigarette smoke exposed) offspring. PCSE children exposed to ≥20 cigarettes daily are most likely to display conduct disorder (Fergusson et al., 1993).

When problematic child behaviors germane to the clinical “conduct disorder” diagnosis are assessed, these individual actions fall under the umbrella term “externalizing behaviors.” These component aggressive and antisocial behaviors include fighting, screaming a lot, destroying possessions, arguing, lying, and throwing temper tantrums (Achenback & Edelbrock, 1981).

PCSE children whose mothers smoked ≥20 cigarettes daily were significantly more likely to exhibit the maximum amount of externalizing behaviors, compared to PCSE offspring whose mothers smoked between 1-9 daily cigarettes during pregnancy (Williams et al., 1998). This population also shows long-term increases in delinquency,
behavior problems, impulsivity, and aggression (Fried et al., 1998; 2003; Brennan et al., 1999, 2002; Weissman et al., 1999; DiFranza et al., 2004; Button et al., 2007; Cornelius & Day, 2009).

Clinical reports of the neurocognitive teratogenicity of PCSE show that soon after birth, abnormal emotional reactivity becomes evident, and is consequent to PCSE. PCSE neonates are less able to self-regulate, are more distressed by novelty, and react slower than non-PCSE neonates (Martin et al., 2006). As infants develop, additional dimensions of dysfunction promulgated by PSCE emerge. Heavy smoking of ≥20 daily cigarettes is predictive of reduced offspring intelligence at ages 4 and 7, as well as poor academic performance at age 7 (Gilman et al., 2008; Polańska et al., 2015).

At six months, infants show deficits in focused attention, information processing, and greater distractibility (Fenercioglu et al., 2009), and these early deficiencies are followed by childhood increases in hyperactivity and inattention (Langley et al., 2005; Jacobson et al., 2007; Cornelius & Day, 2009), as well as a three-fold increase in likelihood of a clinical diagnosis of ADHD (Linnet et al., 2003; Thapar et al., 2003; Button et al., 2007; Yoshimasu et al., 2009; Kovess et al., 2015). Academically, this population performs worse on intelligence tests and is slower to learn language, reading, and mathematics than non-PCSE children (DiFranza et al., 2004).

Therapeutic countermeasures are targeted for children and adolescents, but PCSE exposed individuals face consequences for decades. One prospective longitudinal study (Brennen et al., 1999) found that PCSE can predict adult male criminal behavior, and has a significant positive relationship with lifetime criminal activity. This finding among
adult males supports an extension of those atypical behaviors (seen in childhood and adolescence) including conduct disorder, impulse control, and aggression.

1.2 Drug use and obesity

Recent research indicates that at least one dimension of motivation is appreciably altered among PSCE children. Wiebe and colleagues (2015) obtained self-report measures of smoking behavior and cotinine levels from mothers at 14 and 28 weeks of pregnancy. Cotinine is a metabolite and biomarker for tobacco smoke exposure—the word itself is an anagram for ‘nicotine’. At three years of age, PCSE offspring were assessed for self-regulation. Participants were tested on cognitive and motivational self-regulation tasks; each task measured the ability to hold information in mind while inhibiting a pre-potent response behavior.

For example, one test required children to perform a go/no-go task. Each child sat at a computer, and when the image of a fish appeared on the screen, they had to “catch it,” by pressing a button within 1500ms. When a shark appeared, subjects had been instructed to “let it go,” and refrain from pressing the button. This task is meant to measure the cognitive construct of inhibition, which is a component of executive function (Keeler & Robbins, 2011; Wiebe et al., 2015). The 3-year-olds also completed assessments to determine motivational self-regulation.

In one task, the “goody shelf task,” children were brought into a testing room and seated at a table. On a nearby shelf, two tempting toys were unveiled—one a flashing
wand, the other a jelly bean dispenser. The children were told that the toys were only for ‘looking at,’ and not to be touched. Subjects were given three crayons and a piece of paper to draw on, while an examiner sat in the corner of the room (Wiebe et al., 2015). Interestingly, no significant differences were found among non-PCSE and PCSE offspring on the cognitive self-regulation tasks.

However, there were significant differences between non-PCSE children and PCSE children on the motivational self-regulation tasks. The children prenatally exposed to maternal tobacco smoke performed significantly worse on the tasks measuring motivational self-regulation. These children were less likely to refrain from touching the toys, more likely to sustain contact with the toys after the examiner reminded them not to, and more likely to openly defy or ignore the examiner all together, and do whatever they wanted with the toys (Wiebe et al., 2015).

Thus, these clinical reports offer a compelling indication of the risks for dysfunctional motivated behavior patterns following PCSE.

These behaviors are primarily driven by the brains motivational neurocircuitry, but are notably distinct from behaviors that are cognitive per se. Keeler & Robbins (2011) discuss how motivation is inherent within a constellation of cognitive behaviors, including inhibitory control (measured by the “go fishing” task), decision making, flexibility, planning, long-term and working memory, attention, and perception. Keeler & Robbins (2011) ascribe that the processes deemed “cognitive,” are essentially ways of manipulating information in the mind, designed to detect sensory input and produce motor output. All of these cognitive processes are couched within, and influenced by, the organism’s motivational state (Keeler & Robbins, 2011).
The findings by Wiebe et al. (2015) indicate that PCSE toddlers perform differently on tasks measuring inhibitory control and motivation for hedonic reward (flashing toy, candy in a dispenser). These findings concur with further evidence that PCSE can influence motivation for high-fat, sugary foods in children.

Offspring exposed to tobacco smoke during pregnancy are more likely to be clinically overweight at age 7, and PCSE children are significantly more likely to be obese (Al Mamun et al., 2006; Jaddoe et al., 2008; Møller et al., 2014; Power & Jefferis, 2002). PCSE is significantly more likely to predict obesity than paternal smoking, household smoking, or postnatal environmental tobacco smoke exposure. The motivated behaviors of seeking out and obtaining natural rewards (e.g., high-fat, high-sugar foods) engage the same neurocircuitry as behaviors involved in obtaining drug rewards and reinforcers (Berridge & Robinson, 1998; Tomasi et al., 2015; Volkow et al., 2011).

Later in development, PCSE individuals have an increased likelihood of substance abuse generally, (Brennan et al., 2002; Ellis et al., 2012; Goldschmidt et al., 2012; Weissman et al., 1999); including tobacco use (Cornelius et al., 2000; Buka et al., 2003). Cornelius and Day (2009) found that 10-year-olds with a history of PCSE are 5 times more likely to experiment with tobacco; in this study, the tendency of PCSE offspring to smoke also continued throughout the lifespan.

At 14 years of age, PSCE offspring smoke significantly more cigarettes than non-PCSE offspring (Cornelius et al., 2000), and are twice as likely to smoke throughout adolescence (Kandel et al., 1994; Button et al., 2007). In adulthood, PCSE exposed individuals are classified as clinically nicotine dependent more often than non-PCSE adults (Fergusson et al., 1998; Buka et al., 2003).
According to Goldschmidt et al., (2012), exposure to tobacco smoke during gestation is significantly positively related to an increased likelihood of early alcohol use (initiation <16 years of age), with 63% of PCSE adolescents initiating alcohol use early, compared to 45% non-PCSE. The PCSE adolescents also initiated marijuana use earlier, and this clinical report indicates that 56% of PCSE adolescents initiated tobacco smoking early in adolescence, compared to 43% non-PCSE.

Overall, there is a significant positive relationship between amount of cigarettes smoked by mothers during the first trimester, and early initiation of multiple substance usage, (including tobacco, alcohol and marijuana) in adolescence (Goldschmidt et al., 2012). The odds of an adolescent with first trimester PCSE engaging in multiple substance use (polysubstance use) are nearly twice as high as non-PCSE adolescents. Environmental and genetic factors don't contribute to maternal smoking and drug-seeking behavior(s) in the Goldschmidt study, and when treated as covariates, the relationship between PCSE and drug-seeking remains statistically strong.

2. Nicotine acts as a neuroteratogen

2.1 Nicotine

Clinical reports provide evidence for the teratogenicity of tobacco smoke. Smoking tobacco exposes the individual, and parenthetically the fetal offspring, to 4000 disparate constituents (Rodgman & Perfetti, 2013). Existing data concedes that several toxic compounds, (e.g., monoamine oxidase inhibitors, carbon monoxide, nitric oxide)
contribute to adverse developmental outcomes following PCSE (Franke et al., 2007; Mactutus & Fetchner 1984, 1985).

However, when studied alone, nicotine is a well-known neurotertatogen, with a complex and profound influence in the developing brain (Dwyer et al., 2009; Heath & Piccioto 2009; Roy et al., 1998; Slotkin 1998). Nicotine is the primary psychoactive compound in cigarettes that maintains tobacco smoking addiction in humans (Benowitz 2009; Collins 1990). Changes in the trajectory of development for multiple neural systems is associated with prenatal nicotine exposure (Dwyer et al., 2008; Harrod et al., 2011; Kane et al., 2004; Muneoka et al., 1999; Morgan et al., 2013).

Smoking a cigarette causes plasma nicotine levels to peak rapidly, due to the pharmacokinetic profile of inhaled tobacco smoke. About 1-2 mg of nicotine is absorbed systemically during smoking (Benowitz & Jacob, 1984). The surface of the lungs has a large alveolar blanket (Benowitz, 1990), which allows rapid distribution from the lungs to blood, with nicotine reaching the brain in about 7-10 seconds (NIDA Research Report, 1998).

The rapid rise of nicotine in the brain is temporary, and the ‘bolus’ of nicotine is an effective reinforcer for the smoker (Russell & Feyerabend, 1978). Because nicotine is highly lipophilic, it easily absorbs in mucosa, the gastrointestinal tract, and readily crosses the fetal placenta, where it enters fetal serum and permeates the blood brain barrier (Luck et al., 1985). During maternal smoking, nicotine acts as an exogenous ligand for neuronal nicotinic acetylcholine receptors (nAChRs) in the developing fetal brain. Nicotine is an agonist and can bind nAChRs with a range of affinities, depending on receptor stoichiometry.
2.2 Acetylcholine (ACh)

Acetylcholine (ACh) is the endogenous neurotransmitter for nAChRs. ACh is synthesized from choline, found in circulation. The first step in ACh synthesis is rate-limiting, when choline is taken into the presynaptic cell by a high affinity choline uptake transporter (CHT) system, where choline acetyltransferase (ChAT) catalyzes the synthesis of ACh (Wu et al., 2015).

ACh is loaded into vesicles by the vesicular ACh transporter (VACHT), and released from the presynaptic neuron. In the synaptic cleft, acetylcholinesterase (AChE) hydrolyzes ACh back into choline and acetate. About 50% of hydrolysis-derived choline is reacquired for ACh production (Amenta & Tayebati, 2008).

Ionic flow across the cell membrane is a function of ACh release and nAChR binding. An influx of cations through channel pores increases cell excitability by depolarizing the membrane, with Ca$^{2+}$ in particular triggering a substantial series of intracellular signaling cascades (Colombo et al., 2013). nAChRs are found in abundance throughout the CNS, and belong to a cys-loop superfamily of ligand-gated ion channels that includes GABA, glycine, and 5-HT$_3$ receptors (Betz, 1990).

2.2.1 nAChRs

Two forms of AChRs exist in mammalian species, nicotinic and muscarinic (mAChR) AChRs. nAChRs are widely expressed in the CNS where they qualitatively, if
not quantitatively, modulate neurotransmitter release via actions in pre-, post-, and extrasynaptic membranes (Amenta & Tayebati, 2008; Vizi et al., 2004).

Symmetrically pentameric, nAChRs are cation selective (including Na⁺, K⁺, and Ca²⁺) and each has five subunits characterized by their composition around a core ion-conducting pore (Fucile, 2004; Wu et al., 2015). A single nAChR subunit is typically 600 amino acids long (Hogg et al., 2003). There are 12 putative nAChR subunits, (α2 -α10 with the α8 subunit primarily functional in avian species) and (β2-β4), and receptors exist with some plasticity in convertible states, whose constitution is primarily driven by the binding of agonists and antagonists (Role & Berg, 1996). Stoichiometry regulates the neurophysiological characteristics of nAChRs, with each receptor either homomeric (α7)₅ or heteromeric, such as α4β2.

The heteromeric nAChRs have two ACh binding sites (per pentamer), found where certain subunits come together—where an α2, α3, α4 or α6 subunit meets a β2 or β4 subunit. The most common nAChR conformations are (α4)₂(β2)₃ and (α4)₃(β2)₂ (Lindstrom, 2000). In contrast, α7 homomeric receptors have five identical ligand binding sites at the interface between each subunit (Gotti et al., 2009). Nicotine is powerful as an nAChR agonist, and acts to promote binding of nAChRs that have a high affinity for nicotine, particularly the sensitive α4β2 nAChR (Kuryatov et al., 2005).

Chronic nicotine acts to desensitize nAChRs. Desensitization is a standard term describing the loss of receptor activity after sustained stimulation (Ochoa et al., 1989). nAChRs don’t naturally desensitize to ACh, because endogenous ACh normally doesn’t reach high enough concentrations, and the amount of time ACh interacts with nAChRs to cause activation is negligible (nanoseconds).
However, chronic exposure to nicotine drives receptors into desensitized, non-conducting conformational states (Lukas, 1991; Marks et al., 1993; Peng et al., 1994). Smoking tobacco leads to the prolonged temporal exposure of receptors to nicotine, and increased concentration of nicotine in the brain.

During tobacco dependence, chronic exposure causes protracted nAChR desensitization, and nAChRs in the brains of smokers are said to depress into “deeply desensitized states,” and these states of deep desensitization cause an upregulation in nAChR receptor number (Dani & Heinemann, 1996; Fenster et al., 1999).

Following this, nicotine can induce upregulation and actually promote intracellular assembly of high-affinity nAChRs. Individual α and β subunits exist in a neuron’s cellular endoplasmic reticulum (ER), and during chronic nicotine exposure, an upregulation of high-affinity membrane nAChRs reflects nicotine-driven assembly of nAChRs from pools of subunits in the ER. Moreover, when comparing the effects of nicotine, to prolonged ACh exposure, (achieved by incubating cells with 300µM of ACh and with an AChE inhibitor) only during chronic nicotine do high-affinity α4β2 nAChRs preferentially assemble and upregulate from the ER (Kuryatov et al., 2005).

nAChRs are distributed widely within the CNS, and regulate a constellation of developmental and neurocognitive functions. In the earliest stages of life, these receptors and their binding ligands modulate cell proliferation, survival, differentiation, gene expression, synapse formation and maturation, axonal pathfinding, and neurotransmitter release (Dwyer et al., 2009). The α6 and α7 subunit-containing nAChRs, along with the α4β2 nAChRs, peak within the first three weeks of birth, and then begin to decrease in density, with expression levels plateauing and stabilizing throughout adulthood and aging.

2.2.2 ACh during neurodevelopment

Acetylcholine has a fundamental role in the development of fetal brain tissue. ACh is active in the fetus as early as the gastrulation phase, beginning gestational day (GD) 6, as evidenced by the appearance of the synthesis enzyme ChAT as well as the hydrolyzing enzyme AChE, which appears when ACh begins guiding morphogenic cell organization (Hohmann, 2003; Kassam et al., 2008; Dwyer et al., 2009).

In the spinal cord and brain stem, nAChRs appear by GD 11, and can be desensitized by chronic exogenous nicotine in the brain. After initial gestational nAChR innervation, these receptors continue to aggregate all over the brain, regionally and transiently (indicating certain “critical periods” in typical brain development, when nAChRs appear, along with excitatory ACh, to stimulate and provide guidance for extensive heterogeneous brain region development). nAChRs appear in multiple cortical layers, where ACh regulates neurotransmission and information flow, influencing neurons of various classes (Xiang et al., 1998).

Prenatal nAChRs are flexible and multifunctional, with responsibilities including the formation of rudimentary sensory and neural circuitry. The appearance of nicotine as exogenous agonist during gestation can have wide-ranging effects. For example, the $\alpha_7$ homomeric nAChR induces $\text{Ca}^{2+}$ entry immediately after formation of the neuroepithelium, making nAChRs responsible for very early neuronal pathfinding and survival. During prenatal development, stimulating the $\alpha_7$ receptor causes neurite
retraction (Pugh & Berg, 1994; Small et al., 1995); when α7 is antagonized, neurites extend (Lipton et al., 1988). In the normally developing brain, bursts of ACh procure symmetrical, balanced gradients to guide the direction of forthcoming neurite growth (Zheng et al., 1994).

The α7 subunit is also responsible for orchestrating the processes of apoptosis, cell projection, and maintaining the balance of cell production and recycling (Opanashuk et al., 2001; Hauser et al., 2003). The α6 nAChR mRNA appears at GD 15, and regulates the development of growing DA neurons (Azam et al., 2007). Introducing a high-affinity ligand such as nicotine during these critical periods changes brain growth during critical phases.

Brain formation relies on cell differentiation from stem cell precursors, migration of immature neurons from the embryo, outgrowth of axonal and dendritic processes; and, ultimately, axonal pruning and cellular apoptosis—all of which are managed by ACh via the nAChRs.

ACh is influential as a trophic factor, promoting mitosis in fetal neurons and later prompting cells to undergo differentiation for specificity (Slotkin et al., 2004). Overall, the cholinergic system plays a major role in corticoceptal cytoarchitectural development: neurite growth, controlling cell number, apoptosis, differentiation, and pruning.

3. Animal models of maternal smoking during gestation

Several decades of clinical research reports provide a rich behavioral composite of the long-term consequences of PCSE. Given the >4000 constituents in tobacco smoke,
animal research provides techniques to isolate the precise behavioral- and-
neuroteratogenic effects of nicotine alone (Slotkin, 1998).

Although not an exact facsimile of the human CNS, there is remarkable
phylogenetic conservation in the rat CNS (Keeler & Robbins, 2011). Animal models of
prenatal drug exposure allow systemic and controlled drug delivery. These models are
based on three routes of administration: subcutaneous, oral, and intravenous injection.
Each has a unique pharmacokinetic profile.

3.1 Subcutaneous route of administration

Historically, the first studies of PN exposure used subcutaneous (SC) injections to
deliver nicotine throughout gestation, and attempted to equate a range of the total amount
of nicotine absorbed by humans during daily smoking (1-5 mg/kg/day), (Becker et al.,
1968; Hammer et al., 1981; Hudson & Timiras, 1972). This method resulted in decreased
maternal weight gain, pup birth weight, maternal food intake, and an increased duration
of gestation (Becker and Martin, 1971; Martin and Becker, 1971). Additionally, this dose
range of SC nicotine reduces uterine blood flow and intrauterine oxygen availability
(Hammer et al., 1981). The reduction of blood flow and oxygen causes transient ischemic
fetal hypoxia following each injection.

The subcutaneously –implanted osmotic minipump (OMP) is the most often cited
route of PN administration. The OMP is placed in a subcutaneous pouch and releases
nicotine into the subcutaneous compartment to be slowly absorbed into the bloodstream
and distributed to the dam and fetuses.
The OMP represents a steady-state model of exposure; nicotine is slowly, continuously released, with most OMPs expiring after 28 days (Slotkin et al., 1987b; Levin et al., 2006). A steady titer of nicotine is released into the dam’s body and fetuses, and early research with this device typically included doses meant to mimic heavy smoking (6-9mg/kg/day), which is higher than corresponding human levels of plasma nicotine produced by smoking. For example, a human female has to smoke 360 cigarettes per day to achieve the blood plasma levels produced by a 6mg/kg/day OMP (Mactutus, 1989), although more contemporary experiments use 2-3mg/kg/day, which represents levels obtained in moderate smokers (Dwyer et al., 2008).

Uterine and placental blood flow are reduced by up to 40% with the OMP (Birnbaum et al., 1994) but the steady-state dosing doesn’t imitate the spike of nicotine bolus in the brain and blood plasma experienced by smokers.

One advantage of the minipump is the elimination of daily stress cause by handling and injections. However, as the devices have standard expiration dates, they must be surgically implanted either immediately prior or following confirmation of pregnancy, which carries the risk of stressing the dam (Mactutus, 1989).

3.2 Oral route of administration

Nicotine dissolves in dams’ drinking water via the oral route of administration. Subjects (1) may have access to only nicotine-dosed drinking water, (Pauly et al., 2004; Peters & Tang, 1982; Schneider et al., 2010), or (2) access to both nicotine water and non-nicotine water during pregnancy (Paz et al., 2007; Thompson et al., 2011). Oral
nicotine is subject to first-pass metabolism, as it slowly passes through hepatic portal circulation, reducing the amount of nicotine absorbed into the blood. Seventy percent of nicotine taken orally is metabolized by the liver before reaching the brain (US DHHS, 1988). These studies typically require a higher dose to reach plasma levels similar to smoking (Pauly et al., 2004; Sparks & Pauly, 1999).

Oral administration has an advantage of being non-stressful to the pregnant dam. Rats are typically neophobic and don’t readily consume nicotine water; introducing as little as 0.2% nicotine into drinking water can reduce overall water consumption by 50% (Murrin et al., 1987). The oral method does not provide ‘spikes’ of nicotine in the blood and brain seen in smokers. This “spike” and concurrent neurotransmission underlies nicotine’s addictive properties (Mactutus et al., 1994; Russell & Feyerabend, 1978). However, as rats drink water periodically throughout the day, the oral method can provide an ongoing dosing regimen, somewhat similar to smoking patterns.

3.3 Intravenous (IV) route of administration

The IV route of nicotine administration closely mimics the pharmacokinetic profile of blood nicotine concentrations via tobacco smoke inhalation (Benowitz et al., 2009; Booze et al., 1999a). The “puff” of inhaled tobacco smoke leads to a rapid peak level of nicotine the brain (Mactutus et al., 1994; Russell & Feyerabend, 1978). Similar to smoke inhalation, an IV injection causes rapid absorption and distribution of nicotine (Booze et al., 1999a).
The IV route offers 100% bioavailability into the arterial circulation (Benowitz et al., 2009; Booze et al., 1999a; Mactutus et al., 1994). In 1993, Henningfield and colleagues captured both arterial and venous plasma nicotine levels from human male habitual smokers, directly after the subjects smoked one cigarette (following several hours’ abstinence). Capillary gas chromatography analysis of frequent samples collected the first few minutes after smoking revealed arterial nicotine in the plasma is dramatically higher than venous levels, with highest levels recorded between 5-6 minutes.

Arterial samples are precise comparisons of drug levels in the brain, because of the rapid temporal equilibration of nicotine concentration between the brain and arterial blood following inhalation (Henningfield et al., 1993). The inhalation and intravenous routes are pharmacokinetically extremely similar. Although the two send drug to the brain via slightly different anatomical paths, the total bioavailability of IV drugs after injection imitates the rapid distribution half-life of inhalation, and effectively removes any variability introduced with the kinetic component of absorption (Booze et al., 1999).

An early preclinical study by Adir et al., (1976) analyzed aortic blood after IV administration of radioligand-labeled nicotine via the penis vein in the rat. Nicotine in blood samples from an indwelling abdominal aortic cannula after injection revealed the distribution half-life (highest drug levels seen 5-6 minutes following injection) of IV nicotine to mirror human levels after tobacco inhalation (Adir et al., 1976; Henningfield et al., 1993).

Booze et al., (1999) demonstrated that using an indwelling jugular catheter first used in Mactutus et al., (1994), to administer IV nicotine in rats, produces an extremely
close match to human arterial nicotine plasma levels following inhalation, with an average 5 minute distribution half-life, and 50 minute elimination half-life.

Furthermore, his technique allows multiple daily injections, capable of modeling the repeated spikes of the nicotine bolus in the brain produced by tobacco smoking (Mactutus, 1989; LeSage et al., 2006). The earliest preclinical studies of IV PN reported use of an indwelling cannula. A tether connected the cannula to an automated pump, to deliver 0.03 mg/kg/injection every 14 minutes, for 16 hours a day, for a total of 2.0 mg/kg/day (Keyler et al., 2005, LeSage et al., 2006).

Using a free-hand injection IV model allows the researcher to administer nicotine concentrations to mimic dosing regimens seen in drug dependence, including nicotine self-administration (Donny et al., 1995; Donny et al., 1998). The IV model in the current set of experiments delivers 0.05 mg/kg/injection of nicotine, three times per day. This dosing regimen maintains nicotine self-administration in rats (e.g., 0.03 – 0.06 mg/kg/infusion; Donny et al., 1995; Donny et al., 1998; Corrigall & Coen, 1989), and produces sensitization of brain regions responsible for reinforcement learning and reward seeking behavior (Booze et al., 1999a; Harrod et al., 2007; Harrod et al., 2004). The elimination half-life ($T_{1/2}\beta$) of IV nicotine via catheter is about 50 minutes (Booze et al., 1999).

In the proposed set of experiments, the dose of 0.05mg/kg were given at 1000, 1300, and 1600. From 1000-1300, and from 1300-1600, 3.5 elimination half-lives will pass, and each dam will have trace amounts of nicotine in their blood plasma. At the time of the second injection, 9.375% of the drug concentration from the first dose (0.0046875 mg/kg) was bioavailable in blood plasma, resulting in an incrementally additive effect of
the first and second injections. The third and final nicotine dose will add 0.05mg/kg to 0.0051269 mg/kg, already present in the dam’s blood plasma. For a 12-hour profile of nicotine elimination in the proposed set of experiments, see Table 1.

Animal models of PN exposure are advantageous for studies of neuropharmacological alterations that may support maladaptive neurobehavioral outcomes following maternal smoking.

4. Neurodevelopmental effects of prenatal nicotine: Preclinical Findings

4.1 The Cholinergic System: changes in brain growth and nAChRs

In PN rats, gross DNA levels are reduced by 10-20% in the brain stem, midbrain, cerebral cortex, and cerebellum. One nucleus is present in each brain cell, so decreased DNA directly reflects fewer numbers of cells in the brain (Slotkin et al., 1987b). Levels of the enzyme ornithine decarboxylase (ODC) are significantly higher among postnatal PN rats; ODC controls cell replication and differentiation. Higher ODC levels reflect a premature switch from replication to differentiation (Slotkin et al, 1987b).

PN interferes with the ability of Ach and nAChRs to properly control neurite growth (Lauder & Schambra, 1999). For example, α7 nAChRs both inhibit and stimulate neurites—and introducing PN wreaks havoc on these tightly orchestrated developmental milestones, and can also highjack nAChRs meant to guide neurites during pathfinding to their proper brain regions.

Significantly lower DNA levels for PN rats two weeks after birth reflects the
interruption of another critical α7 mediated developmental event—apoptotic programmed cell death (Slotkin et al., 1987b; 1999). The α7 nAChR facilitates timed cell death of motoneurons in the spinal cord (Renshaw et al., 1993), and is a known agent of hippocampal and cortical pruning via direct Ca$^{2+}$ influx (Orr-Urteger et al., 2000).

Nicotinic receptors are phasic agents of programmed apoptosis, as a necessary step in healthy brain growth.

However, apoptosis is endogenously accomplished by ACh, and the introduction of exogenous nicotine causes premature cell death, and an increase in apoptosis beyond normal programmed cell death (Slotkin et al., 1987b; Berger et al., 1998). The introduction of a gain-of-function α7 nAChR mutant causes dramatically increased apoptosis and death in the first post-natal day (Orr-Urteger et al., 2000).

PN is also capable of transiently upregulating nAChRs in the developing nervous system (Broide et al., 1995; Duncan et al., 2015; van de Camp & Collins, 1994; Tizabi et al., 1997). Phasic nAChRs express subunits which often change confirmation during critical phases of brain maturation (Abreu-Villaça et al., 2010; Zahalka et al., 1993). Nicotine has different binding affinities for each nAChR confirmation, and because of the (1) variability of receptor confirmations, (2) nicotine’s versatile affinities for nAChRs, and (3) the transient upregulation of receptors during brain development, PN causes complex and far-reaching aberrations of development and neurotoxicity during the earliest stages of growth.

PN perturbs synaptogenesis and synaptic function of the nicotinic receptor; nAChRs experience long-term changes in synaptic morphology when challenged with PN exposure. A seminal set of experiments by Zahalka et al., (1993) showed that PN-
exposed animals had significantly higher numbers of ACh binding sites on nAChRs, and upregulated nAChRs across brain regions.

Zahalka et al., (1993) also reported higher ChAT activity, reflecting prolonged spikes in axonal outgrowth and synaptic proliferation. This indicates the synthesis of both ACh and nAChRs are symbiotically responding to PN exposure, both working to adjust to the unnatural influx of nAChR agonist. PN exposure during vulnerable periods of ontogenesis sets the brain upon a trajectory of structural malformation, and maladaptive functioning for the lifespan.

The arrival of PN during gestation affects the brain during critical periods when the content and connections for areas that drive the behaviors of attention and motivation are developing, giving PN an especially insidious role in abnormal lifespan development (Slotkin 1987a; Navarro et al., 1987).

4.1.1 The Basal Forebrain Cholinergic System:

Neonatal rodents experience the human equivalent of the “third trimester” of gestation during the first week and a half of life—during this stage, burst firing of ACh guides cortical growth. While ACh is prolific in the developing brain, the Basal Forebrain Cholinergic System is the principle source for extrinsic ACh fibers in the neocortex throughout the lifespan. The BFCS is structurally and functionally complex, producing widespread ACh fibers throughout the brain.

Prenatally, the presence of ChAT and AChE indicates that cholinergic projections appear very early on. Enzymatic evidence of ACh first appears in the forebrain by GD 14
BFCS innervation of the neocortex coincides temporally with cortical synapse formation, illustrating the importance of ACh for inducting cortical plasticity (Dwyer, 2009; Hohmann, 2003).

BFCS ACh projections regulate neural activity underlying processes of attention, memory, and motivation, directing a litany of clinically relevant behaviors. There is variability in the neuroanatomical literature defining precise anatomical boundaries of the BFCS. The structures typically named as part of the BFCS include, but are not limited to, the ventral pallidum, globus pallidus, substantia innominata, septum, diagonal band of Broca, and extended amygdala (Mesulam et al., 1983; Zaborsky et al., 2012).

These areas can be further subdivided, to reveal a complex mosaic of ascending acetylcholinergic nuclei. These components project to brain regions important for learning, memory, and motivation. Cholinergic neurons have widespread cerebral cortex projections, and the BFCS provides the main cholinergic drive into the PFC, the hippocampus, and Nacc (Mesulam et al., 1983).

4.1.1.1 The Prefrontal Cortex: attention, and the role of nAChRs

The BFCS is a hub of neural outputs directing attention processes and behaviors. About 19% of cholinergic neurons originating in the BFCS project axons to the PFC. These ‘corticoceptal’ cells project from the substantia innominata/ventral pallidum and nucleus basalis magnocellularis to the PFC (termed ‘Ch4’ cell bodies; Mesulam et al., 1983), where nAChRs mediate attentional processes and executive function by
moderating glutamatergic input into pyramidal cells (McCormick, 1993; Methrate & Ashe, 1993).

ACh acts via the α4β2 nAChR to regulate multiple attentional processes, including cue detection, divided attention, and sustained attention. α4β2 nAChRs manage the responses of pyramidal cells to glutamatergic influence (Dalley et al., 2004; Parikh et al., 2008; 2010; Howe et al., 2010; Sarter and Bruno, 1999). The PFC also regulates behavior related to emotional reactivity—inhbiting inappropriate emotions, impulses, and habits (Miller, 2000).

BFCS lesions show impairment of divided attention behavior and point to BFCS-generated cholinergic corticopetal projections, as responsible for managing attention-processing resources. Turchi & Sarter (1997) report that ACh activity directs behavior to resolve conflicting divided attention demands—in this case, a speed-accuracy tradeoff was directed by ACh, and the task became impossible when cholinergic corticoceptal projections were lesioned.

Both α4 and α7-subunit containing nAChRs are found in the PFC. Kassam et al. (2008), showed that stimulation of the α4α5β2 neuron during the first postnatal month produces pyramidal excitatory currents in the PFC. This effect is paired with evidence that a functional loss of the β2 subunit in the first two postnatal weeks (but not later in life) causes decreases in the ability to learn. BFCS-originating corticothalamic neurons, shown to regulate attentional processing behaviors, are generated by α4α5β2-mediated cholinergic excitatory input during the third trimester (Dwyer et al., 2009).

In vitro animal models of prenatal nicotine (PN) show long-term changes attention-allocating neurochemical brain systems. The five-choice serial reaction task is a
rodent model of attention and cognitive ability.

Adult rats born to pregnant dams who were exposed to a low dose of nicotine (0.06 mg/ml) in drinking water, performed worse on errors of omission, worse on accuracy, and had more variable response times (Schneider et al., 2011).

4.1.1.2 The Hippocampus, Basolateral Amygda and Cingulate Cortex

BFCS projections termed “Ch2” emanate from medial septum and diagonal band of Broca, to provide the main cholinergic innervation of the hippocampus (Mesulam 1983); the hippocampus is characterized as the most vital region for memory functioning (Aggleton & Pearce 2001). Both $\alpha_7$ and $\alpha_4\beta_2$ nAChRs are found in the hippocampus and are active during memory processes. When selective antagonists for both the $\alpha_7$ and $\alpha_4\beta_2$ nAChRs are infused locally into the hippocampus, memory functions and performance on the radial arm maze is compromised (Felix & Levin, 1997; Levin et al., 2002; 2015).

During gestation, transient ACh interneurons act as ontogenetic regulators in the hippocampus. Prenatally and during the early postnatal weeks, the hippocampus needs nAChRs to grow in size, and to change GABA from excitatory to inhibitory before the end of the third trimester (Lauder & Schambra, 1999; Dumas 1995). During this third trimester—in the rat, the first ten postnatal days—hippocampal expression of both $\alpha_7$ and $\alpha_4\beta_2$ nAChRs peaks, followed by a decline to adult levels (Court et al., 1997).

PN exposure is a well-known agent of impaired performance on memory tasks. In a classic study by Sorenson et al. (1991), following high-dose dam prenatal nicotine exposure via drinking water (6.0mg/kg/day), late-adolescent PN subjects were
significantly less capable of learning to navigate an 8-arm radial maze. Li et al., (2014) report that rats exposed to PN (6mg/kg) via OMP had longer escape latency times in a Morris Water Maze experiment, as well as reduced α7 nAChR protein expression levels in the hippocampus in adulthood.

The BLA is functionally connected with the Hippocampus, as a part of neurocircuitry underlying the emotional processing of stimuli, emotional memory and learning, (Pidoplichko et al, 2013; Subramaniyan & Dani, 2015). Acquisition of stimulant-seeking behavior is regulated by efferents sent from the BLA to the NAcc, and lesions of the BLA prevent the acquisition of drug-seeking behavior (Whitelaw et al. 1996). Activation of both α7 and α4β2 nAChRs in the BLA are a function of reward-based learning and seeking behavior.

The Cingulate Cortex has emerged in recent decades as an area integral to the processing of reward-related stimuli. Both α7 and α4β2 nAChRs are located in the CC, and midbrain DA burst firing is activated by stimulation of the CC, and this region is known to be structurally altered by prenatal nicotine exposure (Zhu et al., 2012).

4.1.4 The Pedunculopontine Tegmental Nucleus: attention, drug seeking, and the role of nAChRs

The Pedunculopontine Tegmental Nucleus (also called the Pedunculopontine Nucleus; PPN), is considered the ‘cholinergic arm’ of the reticular activating system (RAS) and is a moderator of arousal and waking states. The PPTg cytoarchitecture is defined by large ‘Ch 5’ cholinergic multipolar neurons. The PPTg receives input from
several brainstem regions relaying autonomic information, with output efferents extending to brain areas key for both attention processes and drug abuse behavior.

The PPTg sends cholinergic projections to the BFCS in response to arousal cues. The PPTg also sends cholinergic axons to the VTA and NAcc, where ACh innervates on DA neurons via nAChRs, moderating DA release (Maskos 2008, 2010).

A set of experiments by Inglis et al., (2001) found that lesioning the PPTg reduces performance on a 5-choice serial reaction time task. Accuracy and speed were both decreased among those with PPTg lesions; the authors conclude that the role of the PPTg in attentional processing is to affect global, rather than specific, attention processes. The PPTg is thus part of an attention-allocating neurocircuitry, likely affecting DA neurons in the PFC responsible for attention processes.

The PPTg is also engaged during actions involved in drug-taking behavior, and PPTg ACh-positive neurons are activate when an animal actively seeks psychostimulants (Urbano et al., 2015). Lesions to the PPTg result in reduced nicotine self-administration (Picciotto & Corrigall 2002; Lanca et al., 2000).

In rats prenatally exposed to tobacco smoke, cholinergic neurons were “rendered more excitable,” according to a study by Good et al., (2006). Following tobacco smoke exposure during gestation, intracellular recording in adult rats showed that changes in membrane potential and action potential threshold made these neurons more unstable. The ACh neurons were more likely to fire, and fired more frequently (Good et al., 2006).
4.2 The Dopaminergic Systems

In the adult rat, DA neurotransmission regulates the neural circuitries underlying cognitive and behavioral processes, including attention and motivation (Nestler & Carlezon, 2006). nAChRs densely innervate neurons in dopaminergic pathways, including those that originate from the A9 (nigrostriatal) and A10 (mesocorticolimbic) dopamine cell bodies (Anden et al., 1964; Chang, 1988). Prenatally, DA neurons of the VTA appear around GD13, and those fibers advance onto neocortical structures by GD15 (Kalsbeek et al., 1988).

nAChRs on DA cell bodies are critical for dopaminergic neurodevelopment. For example, mRNA for DA receptors is expressed prenatally, but doesn’t translate into functional binding sites until nearby differentiated neurons release DA (Jung & Bennett, 1996). Activation of presynaptic nAChRs causes this widespread DA release from DA terminals, allowing DA neurotransmitter to reach receptor mRNA (Leslie et al., 2002; O’Leary & Leslie, 2003). Dopamine is dependent on nAChRs to develop the extensive innervation patterns seen in the adult brain, which allow DA to be one of the most widespread and influential neurotransmitters in the brain.

4.2.1 The Prefrontal Cortex: dopamine, nAChRs, and attention

The PFC has an extensive pattern of connectivity, putting this region in a unique anatomical position to subserve “higher level” executive cognitive functions (Weinberger, 1993). DA is recruited for the cognitive behavior of inhibiting prepotent
responses (von Gaalen et al., 2006), is active during tasks of visual attention and working memory (Chudasama & Robbins, 2004), and fires when an individual is engaged in activities requiring divided, selective, and sustained attention (Granon et al., 2000).

Attentional processes are driven by ACh-positive projections from the BFCS to PFC. In the PFC, ACh fibers innervate GABAergic interneurons and glutamatergic pyramidal neurons (Parikh et al., 2010), influencing DA transmission. Both α7 and α4β2 nAChRs are localized in the PFC, with cortical α4 and β2 mRNA peaking on PND 14 and remaining high throughout adulthood (Aracri et al., 2009; Duffy et al., 2009; Shacka et al, 1997).

4.2.2 Motivational neurocircuitry: role of nAChRs

The neurocircuitry underlying motivated behavior, including drug and reward seeking, is known as the ‘mesocorticolimbic (MCL) dopamine system’. The MCL is primarily composed of the VTA, the NAcc, and the PFC (Evenden & Ryan, 1988; Wise & Rompre, 1989). DA release in this circuit is essential for motivation and reinforcement learning (Beeler et al. 2010; Berridge 2004; Berridge & Robinson, 1998; Everitt et al., 2008; Nicola 2007).

The α7, α4β2 and α6-subunit containing nAChRs are highly expressed in the MCL. Excitatory cholinergic projections from the PPTg innervate DA neurons of the A10 cell group in the VTA (Omelchenko & Sesack 2005), and all three nAChR subunits are localized in the VTA and NAcc, where they modulate DA transmission (Mameli-Engvall et al., 2006; Yang et al., 2009). As part of the MCL, DA projections from the
VTA travel to the NAcc and PFC.

The α6 nAChR subunit only appears in a few midbrain structures, and the main responsibility of this subunit is to regulate DA neurotransmission (De Biasi & Dani, 2011; Exley et al., 2008; 2011; McCallum et al., 2005; Pons et al., 2008). When DA neurons are lesioned or rendered inactive, 87% of the detectable α6 protein disappears in the midbrain (Salminen et al., 2004). Transgenic rats with gain-of-function α6 subunits show enhanced dopamine release and locomotor activity (Drenan 2008; Wang et al., 2014).

The α6 nAChR subunit is expressed in both the VTA and the NAc (Brunzell et al., 2010; Gotti et al., 2010). When an α6 subunit antagonist is applied to the NAc shell, DA release essentially comes to a halt, and α6 nAChRs are thought to control DA release in the NAc (Livingstone & Wannacott, 2009; Yang et al., 2009). These subunits are also the most sensitive to nicotine (Kurytov & Lindstrom, 2011; Salminen et al., 2004).

Prenatal nicotine can extensively affect neurochemical expression in the MCL, as evidenced by changes in motivated behavior. Data from our laboratory shows PN rats are more motivated to self-administer sucrose on a progressive ratio schedule (Lacy et al., 2012), and will also self-administer a lower dose of IV methamphetamine, indicating greater sensitivity to the rewarding effects of methamphetamine (Harrod et al., 2012).

The α4, α6, and β2 subunits are all essential for addiction to nicotine. Pons et al., (2008) used receptor knockout (KO) models to determine the roles of the α4, α6, β2, and α7 subunits in nicotine addiction. KO-wild type pairs were trained to self-administer nicotine, but those subjects who had the α4, α6, or β2 genes knocked out did not self-administer the reinforcing dose of nicotine, compared to WT. When this trio of receptor
subunits were re-expressed in the KO subjects, only those with re-expression in the VTA (compared to the SN) restored responding to nicotine.

The α4, α6, and β2 nAChR subunits are all implicated in cognition, attention, and motivation. Prenatal nicotine exposure is capable of changing expression patterns for nAChRs throughout the CNS, and these changes can last into adulthood.

4.3 Hypotheses

The goal of this experiment is to investigate changes in nAChR expression following prenatal exposure to low-dose IV nicotine or saline. In a semi-quantitative manner, in vitro autoradiography will use radioligands to bind and label nAChRs for density measurements. The autoradiography will label high-affinity α6 and α4β2 heteromeric nAChRs and of low-affinity α7 homomeric nAChRs within the CNS of the rat, for anatomical receptor density comparisons. Previous research by Tizabi et al., (1997; 2000) shows that nAChR expression levels are significantly different between PN and PS rat offspring on postnatal days 35-38.

It is predicted that (1) nAChR expression levels were significantly different between PN and PS rats. Specifically, PN levels of α4β2 nAChR binding are expected to be greater than PS levels in the PFC, NAc, VTA, and PPTg, similar to findings of Tizabi et al., (1997). Expression of the α7-subunit containing nAChRs is expected to be lower in PN animals.

Based on these findings, along with similar results from Tizabi et al., (2000), PN levels of α7 nAChRs are expected to be significantly lower than PS binding levels in the
hippocampus and PFC.

This experiment was the first to investigate expression levels of the α6 nAChR following PN exposure. α6 nAChRs are localized almost exclusively on midbrain DA neurons (Yang et al., 2011), and Richardson & Tizabi (1993) found that PN-exposed offspring express fewer DA neurons in the VTA. It is hypothesized that PN and PS groups will have significantly different α6 expression levels, however, these differences are expected to be a function of age (see below).

(2) nAChR levels across regions are expected to differ as a function of age. This experiment was the first to compare neonatal (PND10) and adolescent (PND35) nAChR expression in PN and PS offspring. Tizabi et al., (1997; 2000), found higher α4β2 levels in the PFC, NAc, and VTA of PN rat at PND 35, but also lower α7 expression in the hippocampus and PFC at PND 36-38.

Considering basal levels, the α6, α7 and α4β2 nAChRs first appear during the second gestational trimester, then climb steadily to peak by PND 21, followed by a decline to levels maintained through adulthood (Azam et al., 2007; Pugh & Berg, 1994; Small et al., 1995; Xiang et al., 1998). It is predicted that there will be significant differences in nAChR expression levels between age groups, as well as an interaction between prenatal nicotine exposure and age.

Lastly, (3) it is hypothesized that nAChR expression will be different between males and females. Tizabi et al., (1997) previously showed that PN males had significantly higher whole-brain levels of α4β2 nAChR expression, compared to PN females. The later Tizabi et al., (2000) report of α7 nAChR levels following PN exposure included only male offspring. No evidence to-date addresses sex differences in α7 or α6
nAChR expression. It is predicted that there will be significant sex differences in nAChR expression, as well as an interaction between prenatal nicotine and sex.
Table 1.1 Nicotine concentration metabolism from blood plasma.

<table>
<thead>
<tr>
<th>Time of day (injection = 0.05mg/kg)</th>
<th>$T_{1/2}\beta$ (elimination half-life)</th>
<th>Percent of nicotine in plasma (since most recent injection)</th>
<th>Mg/kg of drug concentration in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 (1&lt;sup&gt;st&lt;/sup&gt; injection)</td>
<td>0</td>
<td>100%^^</td>
<td>0.05</td>
</tr>
<tr>
<td>1050</td>
<td>1</td>
<td>50%</td>
<td>0.025</td>
</tr>
<tr>
<td>1140</td>
<td>2</td>
<td>25%</td>
<td>0.0125</td>
</tr>
<tr>
<td>1230</td>
<td>3</td>
<td>12.5%</td>
<td>0.0625</td>
</tr>
<tr>
<td>1300 (2&lt;sup&gt;nd&lt;/sup&gt; injection)</td>
<td>3.5</td>
<td>9.375%</td>
<td>0.0046875</td>
</tr>
<tr>
<td></td>
<td>0 (upon injection)</td>
<td>100%^^</td>
<td>0.0046875+0.05 (additive)=0.0546875</td>
</tr>
<tr>
<td>1350</td>
<td>1</td>
<td>50%</td>
<td>0.02734375</td>
</tr>
<tr>
<td>1440</td>
<td>2</td>
<td>25%</td>
<td>0.01367187</td>
</tr>
<tr>
<td>1530</td>
<td>3</td>
<td>12.5%</td>
<td>0.00683593</td>
</tr>
<tr>
<td>1600 (3&lt;sup&gt;rd&lt;/sup&gt; injection)</td>
<td>3.5</td>
<td>9.375%</td>
<td>0.00512695</td>
</tr>
<tr>
<td></td>
<td>0 (upon injection)</td>
<td>100%^^</td>
<td>0.00512695+0.05 (additive)=0.05512695</td>
</tr>
<tr>
<td>1650</td>
<td>1</td>
<td>50%</td>
<td>0.02756347</td>
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<td>1740</td>
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<td>25%</td>
<td>0.01378173</td>
</tr>
<tr>
<td>1830</td>
<td>3</td>
<td>12.5%</td>
<td>0.00689086</td>
</tr>
<tr>
<td>1920</td>
<td>4</td>
<td>6.25%</td>
<td>0.00344543</td>
</tr>
<tr>
<td>2010</td>
<td>5</td>
<td>3.125%</td>
<td>0.00172271</td>
</tr>
<tr>
<td>2100</td>
<td>6</td>
<td>1.5625%</td>
<td>0.00086135</td>
</tr>
<tr>
<td>2150</td>
<td>7</td>
<td>0.78125%</td>
<td>0.00043067</td>
</tr>
</tbody>
</table>

^Percent of bioavailable drug returns to 100 with each injection.
CHAPTER II

METHODS

5.1 Animals

A total of 60 female and 30 male, adult nulliparous Sprague-Dawley rats, were acquired from Harlan Industries, Inc. (Indianapolis, IN). All rats were transported to the animal care facilities in the psychology department at the University of South Carolina and rodent food (ProLab Rat/Mouse/Hamster Chow 3000) and water were provided ad libitum throughout the course of the experiments. The animal colony was maintained at 21 ± 2°C, 50% ± 10% relative humidity and a 12L:12D cycle with lights on at 0700 h.

All animal cages were provided with Nylabones (Nylabone, Inc.; Neptune, NJ) and Nestlets (NestletsT; Ancare, Bellmore, NY) for environmental enrichment. A Nylabone was replaced if it is thoroughly chewed, and one Nestlet nesting product was placed in each cage when the cage is changed for sanitation, which occurred 2×/week. This experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

5.1.1 Internalized jugular catheter surgeries

The internalized jugular catheters that were implanted in the dams are commercially available from Harlan Industries (Indianapolis, IN), where the
catheterization procedure was performed according to the methods of Mactutus et al. (1994), prior to arrival in the colony at the University of South Carolina. In summary, animals were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg/ml) and xylazine (3.3 mg/kg/ml).

Following anesthesia, a sterile Intracath IV catheter (Becton, Dickinson and Co., Franklin Lakes, NJ) with a Luer-Lok injection cap (Medex, Inc., Carlsbad, CA) was implanted dorsally in a subcutaneous pouch. The distal end of the catheter was inserted into the left jugular vein, advanced toward the heart, and bound with a sterile suture. Animals were kept under post-operative observation and returned to the colony upon recovery. The day after surgery, catheters were flushed with 0.2 ml of heparinized saline.

5.1.2 Breeding

Upon arrival, all animals were habituated to the colony room for 7 days. Following the habituation period, female rats were housed three per cage and one male were placed in the cage, overnight, from approximately 1700 to 0900 for the purposes of breeding.

Lavage samples were acquired from each female every morning and vaginal cytology recorded using a low-magnification objective on a standard Nikon microscope. On the day sperm was detected, the dams were single-caged. Day of sperm-positive lavage sample was gestational day (GD)0.
5.2 Drugs

Nicotine hydrogen tartrate (Sigma-Aldrich Pharmaceuticals; St. Louis, MO) was weighed as base and dissolved in physiological saline (0.9%; Hospira, Inc. Lake Forest, IL).

The pH of the nicotine solution was neutralized to ~ 7.0. Heparin (APP Pharmaceuticals, Schaumburg, IL) was added to saline and the heparinized saline solution (2.5%) was used to flush the IV catheters.

5.2.1 Prenatal Nicotine Treatment

Pregnant dams were randomly assigned to either the PN (0.05 mg/kg/injection) or PS groups. Nicotine or saline was administered 3×/day from gestational days (GD) 8-21. Following the first two daily injections, catheters were flushed with 0.2 ml of 0.9% physiological saline, and heparinized saline (2.5%) were used to flush the catheter after the final daily NIC or SAL administration. Injections will occur at 1000, 1300, and 1600 daily.

5.3 Surrogate Fostering, Litter Composition, and Postnatal Testing

Post-natal day (PND) 0 was recorded when pups were found in the cage. On PND 1, Litters were culled 10 pups with 5 males and 5 females whenever possible. All pups
were surrogate-fostered to timed-pregnant, drug naïve dams to prevent poor maternal care on PND 1 (Vorhees, 1986).

Offspring were weaned and pair housed, same sex, on PND 21. Behavioral developmental milestones were assessed as follows: righting reflex (PND 3-5), negative geotaxis (PND 8-10) eye opening (PND 13-17), (Heyser, 2004). Subjects were sacrificed either on PND 10 or PND 35; data provided for developmental milestones reflect assessments for righting reflex and negative geotaxis for the first group; the second (PND 35) group has representative data for all three developmental milestone behavioral assessments.

The righting reflex assessment was conducted in blocks of 3 trials on 3 consecutive days. Briefly, pups were placed on their backs, and the length of time it will take the animals to right themselves onto their stomachs was recorded, with a maximum latency of 25 seconds per trial. Negative geotaxis was measured in blocks of 3 trials across 3 consecutive days. For this task, pups were placed with their heads towards the ground on a wire mesh grid positioned on a 25° downward angle. The latency (30 second maximum) for the animals to turn 180° to face up the slope was measured.

Eyes were checked for degree of openness across 5 consecutive days. The degree of openness was rated on a scale of 0-3: 0 = completely closed; 1 = any opening exposing the cornea; 2 = cornea and pupil exposed but eye lids are not fully open; 3 = fully open. All animals’ weights were recorded on PND 1, 7, 14, and 21. Rats were weaned and pair housed, same sex, on PND 21.
5.4 Nicotinic Receptor Autoradiography

10 males (5 PS, 5PN) and 10 females (5PS, 5PN) were sacrificed via rapid decapitation on postnatal day 10. A second group of 10 males (5 PS, 5 PN) and 10 females (5PS, 5PN) was sacrificed via rapid decapitation on postnatal day 35 (see Table 2, Figure 2). Radioligand binding produced expression densities for the PFC, Hippocampus, NAc, VTA, and PPTg.

5.4.1 Nicotinic Receptor Autoradiography: α4β2 nAChR

Tissue was prepared prior to ligand application: first, a Krebs-Ringer HEPES (KRH, pH 7.5) buffer solution of 4.8mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 118mM NaCl, 20mM hepes and 10mM NAOH was used to incubate slides for 40 min. at room temperature.

Following this, a separate KRH solution served as incubation for 90 minutes at room temperature to facilitate ligand binding: [¹²⁵I]-epibatidine (100 nM 2200 Ci/mmol). The slides were rinsed in chilled KRH buffer (4°C, 2x). RayMax Beta High Performance Autoradiography Film (ICN Biomedicals, Aurora, Ohio) was used to visualize ligand binding. Exposure time was 3 days for [¹²⁵I]-epibatidine (Table 3).

5.4.2 Nicotinic Receptor Autoradiography: α7 nAChR

Whole brains were flash frozen in isopentane (−35°C). Samples were stored at −70°C for at least 24h. On a cryostat, brains were sectioned (~16μm) and mounted on
gelatin-and chrom alum coated slides. For $[^{125}]$-bungarotoxin autoradiography, a Krebs-Ringer HEPES (KRH, pH 7.5) buffer solution of 4.8mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 118mM NaCl, 20mM hepes and 10mM NAOH was used to incubate slides for 30 min. at room temperature. Following this, a separate KRH solution will serve as incubation for two hours at room temperature to facilitate ligand binding: 2.5nM α-$[^{125}]$-Tyr54-BTX (specific activity:102.9 Ci/nmol on day of binding) under equilibrium binding conditions.

The slides were rinsed in chilled KRH buffer (4°C, 2x), diluted KRH buffer 1x, and deionized H$_2$O 1x, respectively. Nonspecific blanks were assayed with 10 mM L-Nicotine, without exceeding film background (Pauly & Collins, 1993). Samples were kept at room temperature in a desiccator overnight, exposed to RayMax Beta high-performance autoradiography film (ICN Biomedicals Inc., Aurora, OH) for 14 days (Table 3).

5.4.3 Nicotinic Receptor Autoradiography: α6 nAChR

Before incubation with $[^{125}]$α-CtxMII, sections were incubated in binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgSO$_4$, 20 mM HEPES, 0.1% BSA (w/v), pH 7.5) phenylmethylsulfonyl fluoride (1 mM, to inactivate endogenous serine proteases) at 22°C for 15 min. For all $[^{125}]$α-CtxMII binding reactions, the standard binding buffer was supplemented with BSA [0.1% (w/v)], 5 mM EDTA, 5 mM EGTA, and 10 g/ml each of aprotinin, leupeptin trifluoroacetate, and pepstatin A to protect the ligand from endogenous proteases. The sections were then incubated with 0.5 nM $[^{125}$
I\textsuperscript{125}I\alpha-CtxMII for 2h at 22°C. After incubation with I\textsuperscript{125}I\alpha-CtxMII, the slides were washed as follows: 30 sec in binding buffer 0.1% (w/v) BSA (22°C), 30 sec in binding buffer 0.1% (w/v) BSA (0°C), 5 sec in 0.1 binding buffer 0.01% (w/v) BSA (twice at 0°C), and twice at 0°C for 5 sec in 5 mM HEPES (pH 7.5) (Table 3).

5.5 Collecting Luminance Values

All autoradiography slides were digitally analyzed using the software program Densita (MicroBrightField, 2014; MBF), to outline brain regions of interest and labeled radioligand density. The MBF Densita platform was used to calculate ‘luminance’ values (as the dependent variable), with ‘luminance’ indicating density of receptors.

Because the autoradiograms do not have traditional standards, each image was loaded into the Densita program as total binding (for each section), and a gray scale image with values from 0 (darkest, almost black) to 250 (lightest, almost white) will provide a linear black-to-white gradient for comparison (see Figure 1). Luminance values are based on comparing the shade, or gradient, of the region of interest to the gray scale spectrum. This is the “Step Wedge” method in the MBF Densita program.

Using Step Wedge, high luminance values indicate lower density, and low luminance values indicate a greater density of radioligand binding for the labeled receptor (Figure 2). All cortical tissues analyzed for luminance include information about which laminar layer density measures were taken from. Each report of outcome data for a given brain area also includes information about the location of the slices, relative to Bregma.
5.6 Data Analysis

5.6.1 Litter Parameters

Litter parameters were analyzed with the following factors: treatment (2) as the between-subjects factor; within-subjects factor of gestational day (4) – dam weight gain for gestational days: 0, 7, 14, and 21; for pup weight gain, within-subjects factor of postnatal day (4) pup weight gain neonatal days: 0, 7, 4, 21. A 2 × 4 Treatment × Gestational Day mixed ANOVA was used to ascertain any differences between PN and PS dams on the measure of dam weight gain, and a 2 × 4 Treatment × Postnatal Day mixed ANOVA was used to analyze offspring weight gain.

One-way ANOVAs with Treatment (2) as the between-subjects factor analyzed number of male offspring born, as well as number of female offspring born, followed by a Chi-Square test to compare the ratio of males to females born to PN and PS dams. Sex × Treatment × Postnatal Day mixed-factorial ANOVAs were performed for pup weight gain, righting reflex, negative geotaxis and eye opening data. A Kruskall-Wallis test analyzed each day of eye-opening, with Treatment as grouping factor.

5.6.2 Preliminary One-way ANOVA: Litter as a Random Subject effect

For this experiment, four rats were used per dam (Litter), for a total of 40 subjects from 10 dams. The random Litter variable can confound data interpretation, and threaten the statistical assumption of independence (Aart et al., 2014) (Figure 2).

Litter of origin is a random subjects effect, because each dam has an equal
likelihood of being selected at random from the population of possible dams (Gamst, Meyers & Guarino, 2008). Each Litter is ‘nested’ within each Treatment group, and each of the 40 subjects is then ‘nested’ within its Litter of origin (Figure 2).

To determine the relative contribution of Litter to the variability of distribution of Luminance values, multilevel modeling (also called nested modeling, hierarchical linear modeling, mixed linear modeling, and random-effects modeling) techniques can determine an estimate of dependence (Aart et al., Moen et al., SPSS, 2014). In SPSS, the procedure for multilevel modeling with random effects, is the Mixed Models Linear (MML) procedure. With MML, Litter is entered into the model as a random “subjects” factor, indicating the random effect is a sampling effect.

To determine if data should be modeled with Litter as a random subject effect, a preliminary one-way ANOVA with a conservative alpha level of $p< 0.25$ was ran for each dataset; ($F=\frac{MS_{\text{Litters}}}{MS_{\text{Subjects}}}$; Denenberg, 1976). For each preliminary one-way ANOVA, the $H_0$: no Litter effect. If $H_0$ was rejected, then data analysis proceeded with an MML analysis in SPSS. If the $H_0$ was not rejected, data analysis proceeded with Univariate ANOVA in SPSS (see sections 6.2 and 6.3 below).

According to Tabachnick & Fidell, (2013), as well as Denenberg (1976), the one-way ANOVA test is an appropriate first step of data analysis with Litter effects. If the $H_0$ was rejected, variance results produced by the MML analysis allowed calculation of the IntraCluster Correlation (ICC); which is labeled in the results section with the Latin symbol for rho, “$\rho$”. In multilevel modeling, the ICC is a measure of random (Litter) effect.

The MML procedure in SPSS produces variance estimates between groups, as
well as variance estimates within Litter groups, and this data was used to calculate an estimate of the ICC. ICC was calculated to estimate how much of the variance is associated with the Litter Variable, as a next step in MML analysis. The ICC is calculated using the Estimates of Covariance Parameters from the linear mixed models analysis:

\[
\text{Variability between groups} = \frac{\text{ICC}}{\text{Variability between groups} + \text{Variability within (Litter) groups}} \times 100
\]

Litter was entered as the random subject variable for the mixed models MML, and the fixed models in MML analysis were as follows: Treatment, Sex, and Age, and interactions of those variables, (when available. Some datasets only contained images available for densitometry from the adolescent subjects. In those cases, Age was not a factor). The MML used Sum of Squares III. Both fixed and random effects models included the intercept, and used the Variance Components covariance structure.

\[5.6.3\] Mixed Models Linear Analysis: Luminance

Because of the nested experimental design, if the \(H_o\) from the initial analysis is rejected, Luminance values were analyzed in a Mixed Models design, with Litter as random effect. For data collected on each brain region expressing the nAChR of interest, a \(2 \times 2 \times 2 \times 10\) MML (SPSS 23) was done, with Treatment (2), Sex(2), and Age(2) as fixed factors, and Litter(10) as random factor.
MML is a variation of regression analysis, but MML allows for random intercepts and slopes (Tabachnick & Fidell, 2013), adjusted so that fixed effects contribute to the primary sources of variability of the DV, but random effects influence the covariance structure. In MML, the responses (of the rat) are the sum (i.e., linear) of the fixed and random effects models’ entered into the analysis.

MML analysis considers the variances that are unequal, and weights the variance contributed by effects to the linear model. In the SPSS (IBM) statistical software, the MML procedure treats Litter as a random “subjects” variable. The adjustment of covariance in a standard general linear model or univariate ANOVA isn’t sufficient, because structurally, both procedures still assume the independence of data (SPSS 2002).

Additionally, MML in SPSS is a more appropriate statistic when the Litter has significant influence on the variability of the DV, because of the estimation procedure. In the Univariate ANOVA, the outcome is predicated on a balanced design, and the estimation produced is based only on a minimum variance model.

In SPSS, MML variance estimations are based on either maximum, or restricted maximum likelihood (ML, REML)—estimations modeled on asymptotic normality, and the estimates of covariance parameters predict the DV score for each individual (rat) by a random intercept that varies across groups (Litters), (Gamst et al, 2008; SPSS 2002; Tabachnick & Fidell 2013). For the sake of uniformity, the results section (Chapter III) will report MML analysis with each significant fixed effect model ($p$-values were obtained by likelihood ratio tests of the full model with the effect in question, against the model without the effect in question) using the same basic structure as the ANOVA results—that is, [Fixed effect ($df_{\text{between}}$, $df_{\text{error}}$) = ____ , $p=_____$], $\eta^2$, post-hoc power = .
5.6.3.1 MML Effect Size Calculation

Lastly, the estimate of effect size is typically a challenging statistic to procure when using multilevel modeling. The ANOVA produces partial $\eta^2$, but MML analysis typically is more complex and the effect size estimation is not generated by SPSS. The effect size for the MML results (Chapter III) are calculated here using the error variance, which can increase as fixed and random variables are added to the multilevel model (Kreft & DeLeeuw, 1998).

For the brain regions and nAChR subtypes using MML analysis, the effect size is calculated as follows (from Kreft & DeLeeuw, 1998; and Tabachnick & Fidell, 2013):

\[
\eta^2 = \frac{S_1^2 - S_2^2}{S_1^2}
\]

In this calculation, the effect size is calculated using portions of the mixed model analysis. This is conceptually similar, but calculated differently, from the ICC and $\eta^2$ produced from the Univariate ANOVA; those measures of effect size evaluate the difference in variances between- and within-groups without consideration of the random-effects.

For confirmation of the validity of the $\eta^2$ calculated in the Results section for
MML analysis (Chapter III), each time a $\eta^2$ was calculated, the entire dataset was analyzed a second time using standard Univariate ANOVA. The partial $\eta^2$ produced by the factorial ANOVA was compared to the $\eta^2$ calculated from the MML analysis variance estimates, to make certain the calculations were within a hundredth of a point of each other.

5.6.4 Univariate ANOVA: Luminance & Relative Optical Density

If $H_o$ from the preliminary one-way ANOVA is not rejected, datasets for each brain region expressing an nAChR of interest were analyzed with a $2 \times 2 \times 2$ factorial ANOVA with Treatment, Sex, and Age as between-subjects factors for the Luminance variable. Interactions were also entered into the ANOVA analysis, comparing Treatment $\times$ Sex, Treatment $\times$ Age, Sex $\times$ Age, and Treatment $\times$ Sex $\times$ Age.

Each ANOVA also produced means and SEMs for each main effect, across brain region and nAChR type (Tables X-X), $F$-ratios, significance values and partial $\eta^2$. (Data analyses for MML, one-way ANOVA, and Univariate ANOVA, were produced using SPSS 23 (IBM Software), and graphs were generated with Graphpad Prism 6 (Graphpad Software, Inc.).

5.6.5 Power Analysis

The power (1-\(\beta\)) for each significant main or interaction effect from the MML or factorial ANOVA was calculated using G*Power 3.1.9.2 (Heinrich Heine, Universität
Düsseldorf). With the G*Power software, the power statistic for each significant effect was determined post-hoc; $1-\beta$ was computed as a function of the ANOVA or MML $\alpha$, effect size, and sample size.

The G*Power software is also chosen because it allows effect size to be included either either by direct entry of partial $\eta^2$, (as is obtained from the factorial ANOVA), or by inputting the ‘variance explained by special effect’ and ‘error variance,’ (as is obtained from the MML analysis) into the power calculator. Using either of these effect size estimates enables G*Power to produce the actual power of the statistical model.

Table 2.1 Age, sex and treatment group assignment.

<table>
<thead>
<tr>
<th>Post Natal Day</th>
<th>Group</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>PS n =10</td>
<td>Male =5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female = 5</td>
</tr>
<tr>
<td>10</td>
<td>PN n =10</td>
<td>Male = 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female = 5</td>
</tr>
<tr>
<td>35</td>
<td>PS n =10</td>
<td>Male =5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female = 5</td>
</tr>
<tr>
<td>35</td>
<td>PN n =10</td>
<td>Male =5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female = 5</td>
</tr>
</tbody>
</table>
Table 2.2. Three radioligands used to detect putative nAChRs.

Radioligands used to assess nicotinic acetylcholinergic receptors (nAChRs) markers in rat brains of offspring exposed to prenatal nicotine or saline.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Concentration</th>
<th>Native venom origin of synthetic ligand</th>
<th>Detects</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{125}I]$-epibatidine</td>
<td>100 nM</td>
<td><em>Epipedobates anthonyi</em> – Ecuadorian frog</td>
<td>α4β2 nAChRs</td>
</tr>
<tr>
<td>$[^{125}I]$-bungarotoxin</td>
<td>2.5nM</td>
<td><em>Bungarus multicinctus</em>-Taiwanese krait snake</td>
<td>α7 –containing nAChRs</td>
</tr>
<tr>
<td>$[^{125}I]$-conotoxin MII</td>
<td>0.5 nM</td>
<td><em>Conus Magus</em>- Cone sea snail</td>
<td>α6 –containing nAChRs</td>
</tr>
</tbody>
</table>

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<td>α6 –containing nAChRs</td>
</tr>
</tbody>
</table>
Figure 2.1 Gray Scale binding reference. Gray scale from Step Wedge procedure in MicroBrightField Densa. “Zero” indicates the darkest color, with 11 gradients increasing in numerical units of 25 (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250), and ranging from dark-to-light (0=black, center gray=125, lightest=250). Each pixel within each of the 11 boxes on the gray scale is mapped to a specific color, with a luminance value associated with that pixel. The generated autoradiograph images of brain sections are loaded into Densa as .jpg pictomicrographs. Pixels of the selected brain area are compared to the standardized gray scale. Luminance data is generated when a brain area is selected, contoured for accurate shaping, and finalized for analysis.
Figure 2.2 Graphical representation of research objects and observations in dataset. N=10 dams/litters (N=5 prenatal nicotine litters, N=5 prenatal saline litters) each litter contributed one male and one female (2 subjects) on postnatal day 10, and again (2 subjects) on postnatal day 35.
CHAPTER III

RESULTS

6.1 Litter Parameters

Analysis of the maternal weight gain revealed a significant main effect of gestational day \([F(3, 81) = 754.36, p = 0.000]\) partial \(\eta^2 = 0.98\); with all dams increasing weight throughout gestation. The ANOVA revealed no significant main effect of maternal treatment, and no interaction between maternal treatment and gestational day, as illustrated in Figure 3a.

There was no Treatment effect for number of female offspring produced, \([F(1, 8) = 0.259, p = 0.171]\) (Figure 4), or male offspring produced, \([F(1, 8) = 0.031, p = 0.864]\) (Figure 4) nor difference in the ratio of males to females, \(\chi^2 = 15.69, p = 0.403\), or total number of pups born between prenatal treatments, \([F(1, 8) = 0.459, p = 0.517]\), (Figure 4).

A significant effect of postnatal day, \([F(3, 81) = 654.86, p = 0.000]\) partial \(\eta^2 = 0.996\) indicated all pups gained weight consistently across the three postnatal weeks (Figure 3b). No Treatment effect was found for neonatal weight gain, \([F(3, 36) = 1.89, p = 0.119]\) (Figure 3b). The main effect of Sex was not significant for postnatal weight gain \([F(3, 36) = 1.02, p = 0.388]\).

The analysis of Righting Reflex latency showed a significant main effect of postnatal day, \([F(2, 72) = 41.87, p = 0.000]\), as all offspring reduced the latency to
complete the reflex behavior across three consecutive days of testing. There were no significant main effects of Treatment \( [F(2, 72) = 0.070, p = 0.993] \) (Figure 5a), or Sex, \( [F(2, 72) = 2.22, p = 0.114] \), and no interaction, for latency to perform the Righting Reflex.

A significant effect of postnatal day was found for negative geotaxis \( [F(2, 72) = 7.00, p = 0.002] \) partial \( \eta^2 = 0.209 \), post hoc power=1.00, indicating all offspring improved latency to perform the Negative Geotaxis task, across the consecutive days of testing with increasingly shorter latencies to face up the inclined apparatus: Day 8 \( (M= 8.88 \pm 0.517) \), Day 9 \( (M= 7.19 \pm 0.347) \), Day 10 \( (M= 6.23 \pm 0.501) \) (Figure 5b). Neither Treatment nor Sex had a significant main effect on Negative Geotaxis latency, \( [F(2, 72) = 1.71, p = 0.196]; \) \[F(1, 72) = 0.101, p = 0.752]\], respectively; nor was the interaction significant.

Note: statistical analyses of Eye Opening data only included \( N= 20 \) offspring. The total \( N = 40 \) offspring in these experiments, but \( n = 20 \) were sacrificed at postnatal day 10 (representing the neonatal Age group), and Eye Opening is assessed on postnatal days 13-17. The ANOVA analysis of Eye-Opening data revealed a significant main effect of Day, \( [F(2, 108) = 172.22, p = 0.000] \), but no significant main effects of Treatment \( [F(1, 108) = .665, p = 0.420] \), or Sex \( [F(1, 108) = .079, p = 0.780] \), or interaction, across days of eye opening.

The Kruskall-Wallis test revealed no significant differences between Treatment groups for eye opening as follows: Day 13: \[H(2) p= 0.317], Day 14, \[H(2) p= 1.00], Day 15 \[H(2) p= 0.418], Day 16, \[H(2) p= 0.278], Day 17 \[H(2) p= 0.139], indicating that there were no significant differences in eye opening between groups.

** For the remainder of the results section: significant findings, which are underpowered, are reported (with power estimate) and double asterisk **. However, significant but
underpowered interactions did not undergo further probative statistical investigation.

The Results Section also includes data analysis for those brain regions which did not undergo hypothesis testing: Substantia Nigra, Dorsal Striatum, Basolateral Amygdala, Cingulate Cortex (Anterior), Medial Habenula, Interpeduncular Nucleus, Raphe Nucleus, Insular (agranular) Cortex, Auditory Cortex, Motor Cortex, Parietal Cortex, and Somatosensory Cortex.

6.2 Mesocorticolimbic Dopamine Circuit: VTA, NAc, PFC

6.2.1 $[^{125}\text{I}]$-epibatidine (Table 3)

VTA: The preliminary between-subjects one-way ANOVA revealed no significant effect of Litter [$F(9, 29)=1.221, p=.321$], so data analysis proceeded with the between-subjects factorial ANOVA. The analysis revealed a significant effect of Treatment [$F(1, 31)=8.40, p=0.007$, partial $\eta^2=0.213$, post-hoc power=0.89 (Table 4a; Figure 6)]. Neither Sex nor Age were significant main effects (Tables 4b&c) and no significant interactions were found.

NAc: The preliminary between-subjects one-way ANOVA revealed a significant effect of Litter [$F(9, 31) = 1.43, p = 0.220$], so a MML analysis followed. Results of the MML analysis provided variance information to compute the ICC (by hand, using formula in section 4.3.2); $\rho = 0.316$; indicating Litter constitutes 31.6% of the variability across subjects and fixed effects.

The MML analysis revealed a significant fixed effect of Treatment, [Fixed Effect $(1, 32) = 12.71, p = 0.001$, $\eta^2=0.194$, power = 0.85 (Table 4a; Figure 7)]. No significant main effects or interactions were found for Sex or Age (Tables 4b&c).
PFC: The prefrontal cortex is labeled “Frontal Cortex,” in the 6th ed. Rat Brain Atlas (Paxinos & Watson, 2006). A preliminary between-subjects one-way ANOVA revealed no significant effect of Litter, \( F(9, 30) = 0.354, p = 0.947 \), so the analysis proceeded with a between-subjects factorial ANOVA.

There was no significant main effect of Treatment (Table 4a), but significant main effects of Sex \( F(1, 31) = 9.40, p = 0.004 \), partial \( \eta^2 = 0.233 \), post-hoc power = 0.92 (Table 4b), and Age \( F(1, 31) = 50.93, p = 0.000 \), partial \( \eta^2 = 0.622 \), post-hoc power = 1.00 (Table 4c) and a significant but unpowered, Sex \( \times \) Age interaction \( F(2, 31) = 7.74, p = 0.009 \) \( \eta^2 = 0.200 \), post-hoc power = 0.69** was found. For this ligand, the Frontal cortex data was collected using the most rostral slices available, where the labeling appeared in the deepest laminar layer (V/VI) for both neonatal rats. In adolescents, layers III and VI were labeled and data collected from the Frontal cortex.

6.2.2 \([^{125}\text{I}]\-\alpha\ bungarotoxin\) (Table 3)

VTA: Densitometry measurements were not possible for \([^{125}\text{I}]\-\alpha\)-bungarotoxin binding in the VTA.

NAc: The preliminary one-way ANOVA revealed no significant effect of Litter, \( F(9, 31) = 0.853, p = 0.575 \), so the analyses continued with a between-subjects factorial ANOVA, revealing a significant main effect of Treatment \( F(1, 32) = 7.59, p = 0.010 \) partial \( \eta^2 = 0.192 \), post-hoc power = 0.83 (Table 5a; Figure 13), but no significant effects of Sex or Age (Tables 5a&b), or interactions of Sex or Age with Treatment.

However, a significant but unpowered Sex \( \times \) Age interaction was revealed \( F(3, 32) = 8.17, p = 0.007 \) partial \( \eta^2 = 0.203 \), post-hoc power = 0.70**.
PFC: Densitometry measurements were not possible for $[^{125}\text{I}]$ α-bungarotoxin binding in the Frontal Cortex.

6.2.3 $[^{125}\text{I}]\alpha$-CtxMII (Table 3)

VTA: $[^{125}\text{I}]\alpha$-CtxMII binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed no significant effect of Litter, $[F(9, 11)= 1.26, p=0.379]$, so the analysis proceeded with between-subjects factorial ANOVA. The ANOVA revealed a significant but unpowered main effect of Treatment, $[F(1, 16)=6.84, p=0.020]$ partial $\eta^2=0.328$, post-hoc power=0.78** (Table 6a), no significant main effect of Sex (Table 6b); but a significant, unpowered, interaction of Treatment $\times$ Sex, $[F(2, 16)= 5.93, p=.029]$ partial $\eta^2= 0.297$, post-hoc power= 0.50**.

NAc: $[^{125}\text{I}]\alpha$-CtxMII binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed no significant Litter effect, $[F(9, 11)= 1.53, p=0.332]$, and analyses proceed with between-subjects factorial ANOVA. However, no significant main effects or interactions of Treatment and/or Sex were revealed (Tables 6a&b).

PFC: $[^{125}\text{I}]\alpha$-CtxMII binding did not occur in the Frontal Cortex.

6.3 Nigrostriatal Dopamine Circuit: Substantia Nigra and Dorsal Striatum

6.3.1 $[^{125}\text{I}]$-epibatidine (Table 3)

Substantia Nigra: The preliminary one-way ANOVA revealed no significant effect of Litter, $[F(9, 29)= 0.637, p= 0.756]$, so analyses proceeded with between-
subjects factorial ANOVA. However, the analyses revealed no significant effects or interactions of Treatment, Sex, or Age (Table 4a, b&c).

**Dorsal Striatum:** The preliminary one-way ANOVA revealed no significant effects of Litter, \(F(9, 29)= 0.187, p=0.994\), so the analyses continued with between-subjects factorial ANOVA. A significant main effect of Treatment \(F(1, 30)= 8.41, p=0.007\) partial \(\eta^2= 0.213\), post-hoc power=0.88 (Table 4a; Figure 8); as well as a significant main effect of Age \(F(1, 30)= 202.97, p=0.000\) partial \(\eta^2= 0.868\), post-hoc power= 1.00 (Table 4c), was revealed, but no interaction between the two factors, and Sex was not a significant factor (Table 4b).

**6.3.2 \[^{125}\text{I}]\alpha\text{-bungarotoxin (Table 3)}**

**Substantia Nigra:** \[^{125}\text{I}]\alpha\text{-bungarotoxin binding only visible in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed a significant effect of Litter \(F(9, 11)= 1.62, p=0.243\). Luminance values were then analyzed with an MML analysis; the ICC generated was \(\rho= 0.1539\), or 15.39\% of the variance in the model was explained by Litter. Fixed effects of Treatment and Sex were not statistically significant, (Tables 5a&b), and analyses were concluded.

**Dorsal Striatum:** visible \[^{125}\text{I}]\alpha\text{-bungarotoxin binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed no significant effect of Litter \(F(9, 11)= 0.856, p=0.587\), so the analyses proceeded with between-subjects ANOVA, revealing no significant main effects of Treatment or Sex (Tables 5a&b), and no significant interaction.
6.3.3 $[^{125}\text{I}]\alpha$-CtxMII (Table 3)

*Substantia Nigra:* The preliminary one-way ANOVA revealed no significant effect of Litter, $[F(9, 31)= 0.326, p = 0.957]$, so the analyses proceeded with between-subjects factorial ANOVA. Treatment (Table 6a) was not a significant effect, nor was Sex (Table 6b). The ANOVA did reveal a significant main effect of Age, $[F(1, 32)= 108.43, p=0.00] \text{ partial } \eta^2 = 0.819, \text{ post-hoc power} = 1.00$ (Table 6c). No interactions were significant.

*Dorsal Striatum:* $[^{125}\text{I}]\alpha$-CtxMII binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed no significant effect of Litter $[F(9, 11)= 0.790, p=0.634]$, so the analyses proceeded with between-subjects factorial ANOVA, revealing no significant effect of Treatment (Table 6a), but an unpowered significant main effect of Sex, $[F(1, 16)= 6.94, p=0.019] \text{ partial } \eta^2=0.316, \text{ post-hoc power} = 0.79**$ (Table 6b). No significant effect of the treatment by sex interaction.

6.4 Pedunculopontine Tegmental Nucleus

6.4.1 $[^{125}\text{I}]]\text{-epibatidine}$ (Table 3)

*PPTg:* The preliminary one-way ANOVA revealed no significant effect of Litter, $[F(9, 30)= 0.326, p=0.959]$, so the analyses proceeded with between-subjects factorial ANOVA, revealing no significant effects of Treatment or Sex (Tables 4a&b), but a
significant main effect of Age \( [F(1, 31)= 38.71, p=0.000] \) partial \( \eta^2 = 0.563 \), post-hoc power= 0.99 (Table 4c) is evident. None of the interactions were statistically significant.

6.4.2 \( [^{125}\text{I}] \alpha\text{-bungarotoxin} \) (Table 3)

\( PPTg: \) \( [^{125}\text{I}] \alpha\text{-bungarotoxin} \) binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed a significant effect of Litter \( [F(9, 11)= 4.69, p=0.015] \), and further main effects and interactions were investigated with MML analysis. The ICC was calculated, \( \rho = 0.6590; \) indicating 65.90% of the variability of Luminance values is attributed to Litter. There were no main effects of Treatment or Sex (Tables 5a&amp;b), and no interaction of the two factors.

6.4.3 \( [^{125}\text{I}]\alpha\text{-CtxMII} \) (Table 3)

\( PPTg: \) No \( [^{125}\text{I}]\alpha\text{-CtxMII} \) binding was visible in the PPTg.

6.5 Extended Reward Neurocircuitry: Hippocampus, BLA, Cingulate Cortex

6.5.1 \( [^{125}\text{I}]\text{-epibatidine} \) (Table 3)

\( \text{Hippocampus:} \) No visible \( [^{125}\text{I}]\text{-epibatidine} \) binding was evident in the hippocampus.

\( \text{BLA:} \) The preliminary one-way ANOVA revealed a significant effect of Litter, \( [F(9, 31) = 1.64, p = 0.149] \) and was followed up with MML analysis. From this MML
analysis, the ICC was computed; \( \rho = 0.637 \), indicating Litter is responsible for 63.7% of the variability in the distribution of luminance values.

MML analysis revealed a significant unpowered effect of Treatment, \([F(1, 32) = 6.48, p=0.016 ] \) partial \( \eta^2 = 0.142 \), post-hoc power = 0.70** (Table 4a) Age and Sex did not have significant effects (Tables 4b&c), and no interaction was significant.

**Cingulate Cortex:** The preliminary one-way ANOVA revealed no significant effect of Litter, \([F(9, 31) = 0.398, p = 0.926] \), so analysis continued with a between-subjects ANOVA. The ANOVA revealed a significant effect of Treatment \([F(1, 32) = 7.68, p =0.009] \) partial \( \eta^2 = 0.194 \), post-hoc power = 0.85 (Table 4a; Figure 9); a significant but unpowered effect of Sex \([F(1, 32) = 4.51, p =0.042] \) partial \( \eta^2 = 0.123 \), post-hoc power = 0.63** (Table 4b); a significant effect of Age \([F(1, 32) = 42.32, p =0.000] \) partial \( \eta^2 = 0.569 \), post-hoc power = 0.99 (Table 4c); significant but unpowered interactions between Sex \( \times \) Age \([F(2, 32) = 4.13, p =0.050] \) partial \( \eta^2 = 0.114 \), post-hoc power = 0.40**, as well as an unpowered three-way interaction of Treatment \( \times \) Sex \( \times \) Age \([F(3, 32) = 5.85, p =0.021] \) partial \( \eta^2 = 0.154 \), post-hoc power = 0.32**. For the Cingulate cortex, this ligand was labeled mostly in the deepest cortical layer for both age groups (laminar layer V/VI).

6.5.2 \([^{125}I]\) \( \alpha \)-bungarotoxin (Table 3)

**Hippocampus:** The preliminary one-way ANOVA revealed a significant effect of Litter, \([F(9, 31)= 2.46, p=0.031] \), so MML analysis was used for main effects and interactions. The MML analysis provided information to generate the ICC; \( \rho = 0.126, \)
indicating the Litter variable accounted for 12.6% of variance in the distribution of the Luminance DV.

MML analysis revealed a significant main effect of Treatment, \( [F(1, 32)= 17.89, p=0.000] \) partial \( \eta^2= 0.229 \), post-hoc power= 0.91 (Table 5a; Figure 14). Sex did not have a significant main effect (Table 5b), but Age was a significant, unpowered main effect \( [F(1, 32)= 11.54 p=0.002] \) partial \( \eta^2= 0.101 \), post-hoc power=0.54** (Table 5c). No significant interactions were revealed.

**BLA:** The preliminary one-way ANOVA revealed no significant effect of Litter, \( [F(9, 31)= 0.663, p=0.735] \), and the analysis continued with a between-subjects factorial ANOVA. The ANOVA revealed no significant effect of Treatment (Table 5a), but Sex had a significant main effect, \( [F(1, 32) = 8.31, p =0.007] \) partial \( \eta^2=.206 \), post-hoc power = 0.87 (Table 5b). No significant effect of Age was found (Table 5c), but a significant unpowered interaction of Treatment and Age \( [F(2, 32) = 4.19, p =0.049] \) partial \( \eta^2=.116 \), post-hoc power = 0.41**.

**Cingulate Cortex:** The preliminary one-way ANOVA revealed significant effect of Litter, \( [F(9, 31) = 1.44, p =0.314] \), so the factorial ANOVA was performed; the ANOVA revealed a significant Treatment effect, \( [F(1, 32)= 7.27, p =0.011] \) partial \( \eta^2= .185 \), post-hoc power = 0.81 (Table 5a; Figure 15), but Sex was not a significant main effect (Table 5b). Age was a significant main effect, \( [F(1, 32) = 11.78, p= 0.002] \) partial \( \eta^2= .269 \), post-hoc power = 0.96 (Table 5c). No interactions reached statistical significance. For this ligand, the Cingulate Cortex was labeled most intensely in layers III and IV; density measurements reflect labeling in layers III and IV.
6.5.3 $^{[125]}I\alpha$CtxMII (Table 3)

**Hippocampus:** No $^{[125]}I\alpha$CtxMII binding was evident in the hippocampus.

**BLA:** No visible $^{[125]}I\alpha$CtxMII binding was capable of densitometry analysis.

**Cingulate Cortex:** No $^{[125]}I\alpha$CtxMII binding was evident in the Cingulate Cortex.

6.6 Cholinergic pathway: Medial Habenula, Interpeduncular Nucleus, Raphe

**Nucleus**

6.6.1 $^{[125]}I$-epibatidine (Table 3)

**Medial Habenula:** The preliminary one-way ANOVA revealed no significant effect of Litter, $F(9, 31)$= 1.13, $p=0.374$, so the analyses proceeded with a between-subjects ANOVA: Treatment was revealed as a significant, but unpowered main effect, $F(1, 32)$= 4.80, $p=0.036$ partial $\eta^2= 0.130$, post-hoc power=0.660** (Table 4a).

Sex was not a significant main effect (Table 4b); Age emerged as significant unpowered effect, $F(1, 32)= 4.17$, $p= 0.050$ partial $\eta^2= 0.115$, post-hoc power= 0.599** (Table 4c). A significant, unpowered interaction of Sex $\times$ Age $F(2, 32)$= 7.63, $p=0.009$ partial $\eta^2=0.192$, post-hoc power= 0.753**.

**Interpeduncular Nucleus:** The preliminary one-way ANOVA revealed no significant effects of Litter $F(9, 31)$= 0.591, $p=0.794$, so the analysis proceeded with a between subjects ANOVA, which revealed no significant effects of Treatment (Table 4a), Sex (Table 4b), or Age Table 4c), but a singular significant unpowered interaction
between Treatment × Age, \(F(1, 32) = 4.08, p =0.052\) partial \(\eta^2= 0.113\), post-hoc power =0.39**.

**Raphe Nucleus:** \(^{125}\)I-epibatidine binding was was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed a significant effect of Litter, \([F(9, 30) = 2.49, p =0.085]\), so the data underwent MML analysis, and from the MML analysis, ICC was generated; \(\rho=0.453\). So 45.3% of the variability of distribution of data was attributed to the Litter variable. No significant effect of Treatment was revealed (Table 4a) but a significant main, unpowered effect of Sex emerged, \([F(1, 13) =6.30, p =0.036]\) partial \(\eta^2= 0.283\), post-hoc power =0.73** (Table 4b). The interaction of Treatment and Sex was not significant.

6.6.2 \(^{125}\)I-\(\alpha\)-bungarotoxin (Table 3)

**Medial Habenula:** \(^{125}\)I-\(\alpha\)-bungarotoxin binding was not evident in the Medial Habenula.

**Interpeduncular Nucleus:** \(^{125}\)I-\(\alpha\)-bungarotoxin binding was not evident in the Interpeduncular Nucleus.

**Raphe Nucleus:** \(^{125}\)I-\(\alpha\)-bungarotoxin binding was was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed \([F(9, 11)=2.08, p=0.135]\), so a MML analysis proceeded, and results of the MML were used to calculate the ICC; \(\rho=0.214\), or, Litter contributed 21.5% of the variance associated with the distribution of the Luminance values. No significant effects of Treatment (Figure 5a) or Sex (Figure 5b) or the interaction of Treatment and Sex were revealed, and analysis ended.
6.6.3 $[^{125}\text{I}]\alpha$-CtxMII (Table 3)

Medial Habenula: $[^{125}\text{I}]\alpha$-CtxMII binding was visible only in the adolescent pictomicrographs (postnatal day 35). The preliminary one-way ANOVA revealed a significant effect of Litter, $[F(9, 10) = 4.19, p = .018]$; MML analysis proceeded. Variance data from the MML analysis were used to compute the ICC; $\rho = 0.445$. This indicates that an estimated 44.5% of the variability of the Litter DV is attributed to the Litter variable. No significant effects of Treatment or Sex (Tables 6a&b), or the interaction of Treatment and Sex were significant, and analysis ended.

Interpeduncular Nucleus: $[^{125}\text{I}]\alpha$-CtxMII binding was not evident in the Interpeduncular Nucleus.

Raphe Nucleus: $[^{125}\text{I}]\alpha$-CtxMII binding was not evident in the Raphe Nucleus.

6.7 Cortices involved in motivated behavior or attention: Agranular Insular Cortex, Auditory Cortex, Motor Cortex, Parietal Cortex, Somatosensory Cortex

6.7.1 $[^{125}\text{I}]$-epibatidine (Table 3)

Agranular Insular Cortex: The preliminary one-way ANOVA revealed no significant effect of Litter, $[F(9, 30) = 0.092, p = 1.00]$; this was followed by a between-subjects factorial ANOVA. The ANOVA revealed no significant effects of Treatment (Table 4a) or Sex (Table 4b), but Age was significant, $[F(1, 31) =100.73, p =0.000]$ partial $\eta^2 = 0.759$, post-hoc power =1.00 (Table 4c). Significant, but unpowered interactions between Treatment $\times$ Sex $[F(2, 31) =4.47, p =0.042]$ partial $\eta^2 = 0.123$, post-
hoc power =0.501**; as well as between Sex × Age \([F(2, 31) =4.49, p=0.042]\) partial \(\eta^2= 0.123\), post-hoc power =.501**. For this ligand, the neonates had greatest density labeling in the deepest cortical layer. In the adolescent brain tissue, cortical layer III had the most prominent epibatidine labeling.

**Auditory Cortex:** The preliminary one-way ANOVA revealed no significant effect of Litter, \([F(9, 30) =0.521, p =0.848]\), so the analysis proceeded with a between-subjects factorial ANOVA. The ANOVA revealed a significant main effect of Treatment, \([F(1, 31) = 7.144, p=0.012]\) partial \(\eta^2= 0.183\), post-hoc power =.82 (Table 4a; Figure 10), no significant effect of Sex (Table b), but a significant main effect of Age, \([F(1, 31) =55.62, p=0.000]\) partial \(\eta^2= 0.635\), post-hoc power =1.00 (Table 4c). No interactions were significant. For the neonates, density measurements were collected from a thin, but intense, labeling in the deepest cortical layer. In the adolescent rats, the Auditory cortex had the most intense binding apparent in cortical layers III and IV.

**Motor Cortex:** Densitometry measurements were not possible for \([^{125}I]\)-epibatidine binding in the Motor Cortex.

**Parietal Cortex:** The preliminary one-way ANOVA revealed no significant effect of Litter, \(F(9, 31)= 1.19, p=0.331\), so the analysis continued with a between-subjects factorial ANOVA. A significant Treatment effect was revealed, \([F(1, 32) = 8.61, p=0.006]\) partial \(\eta^2= 0.212\), post-hoc power =.88 (Table 4a; Figure 11). There were no significant main effects of Sex (Table 4b) or Age (Table 4c), and no interactions. In the neonatal rats, density measurements for this ligand were collected from the deepest cortical layer of the Parietal cortex. In the adolescents, the labeling was intense for layers I, III, V, and VI, with measurements taken from layers V and VI.
**Somatosensory Cortex:** The preliminary one-way ANOVA revealed no significant effects of Litter, $F(9, 31) = 0.436$, $p=0.904$, so the analysis proceeded with a between-subjects factorial ANOVA. The ANOVA revealed a significant effect of Treatment, $[F(1, 32) = 8.22, p=0.007]$ partial $\eta^2 = 0.204$, post-hoc power =.865 (Table 4a; Figure 12a), and a significant but unpowered main effect of Sex, $[F(1, 32) = 5.39, p=0.027]$ partial $\eta^2 = 0.144$, post-hoc power =.699** (Table 4b), as well as a significant main effect of Age $[F(1, 32) = 91.88, p=0.000]$ partial $\eta^2 = 0.742$, post-hoc power = 1.00 (Table 4c).

Treatment $\times$ Age was significant, $[F(2, 32) = 11.54, p=0.002]$ partial $\eta^2 = 0.215$, post-hoc power =.813 (Figure 12b). For the both neonatal and adolescent rats, cortical layers III, IV and V were clearly labeled, with densitometry data collected from layer III.

6.7.2 $[^{125}\text{I}] \alpha$-bungarotoxin (Table 3)

**Agranular Insular Cortex:** The preliminary one-way ANOVA revealed no significant effects of Litter, $[F(9, 31) = 0.837, p = 0.589]$, so a between-subjects factorial ANOVA revealed a significant Treatment effect, $[F(1, 32) = 7.20, p = 0.011]$ partial $\eta^2 = 0.184$, post-hoc power = 0.82 (Table 5a; Figure 16), a significant, but unpowered, effect of Sex $[F(1, 32)=4.88, p=0.034]$ partial $\eta^2 = 0.132$, post-hoc power = 0.67** (Table 5b), as well as a significant effect of Age $[F(1, 32) = 52.85, p = 0.000]$ partial $\eta^2 = 0.623$, post-hoc power = 1.00 (Tables 5c). No significant interactions were revealed. For this ligand, the most rostral brain areas were used in the neonates, with data collected from intensely labeled cortical layer III. For the adolescent group, layer III was also used, but occasionally the best images were slightly more caudal; for those, IV and V were labeled most intensly.
**Auditory Cortex:** The preliminary one-way ANOVA revealed no significant effect of Litter, \(F(9, 29) = 0.480, p = 0.876\), so the analyses proceeded with a between-subjects factorial ANOVA. Significant effect of Treatment was found \(F(1, 31) = 7.48, p = 0.10\) partial \(\eta^2 = .194\), post-hoc power = 0.833 (Table 5a; Figure 17), and no effect of Sex (Table 5b), but a significant main effect of Age \(F(1, 31) = 80.84, p = .000\) partial \(\eta^2 = 0.723\), post-hoc power = 1.00 (Table 5c). No significant interactions were revealed. For both the neonatal and adolescent groups, cortical layer III was labeled most intensely and used for densitometry data collection.

**Motor Cortex:** The preliminary one-way ANOVA revealed a significant effect of Litter, \(F(9, 30) = 1.87, p = 0.097\), so a MML analysis was done; results of the MML output were used to generate the ICC; \(\rho = 0.317\). So, 31.7% of the variability in the model can be explained by the random Litter variable. Treatment and Sex did not have significant main effects (Tables 5a&b) or interactions, but Age did have a significant, but unpowered, main effect \(F(1, 30) = 12.59, p = 0.002\) partial \(\eta^2 = 0.172\), post-hoc power = 0.776** (Table 5c). In the motor cortex, layer II was labeled most intensely and was the darkest layer available for data collection.

**Parietal Cortex:** No visible \(^{125}\text{I}\) α-bungarotoxin binding was capable of densitometry analysis.

**Somatosensory Cortex:** The preliminary one-way ANOVA revealed a significant effect of Litter, \(F(9, 30) = 2.13, p = 0.059\), so the analysis proceeded using MML. The results of the MML analysis were used to calculate ICC; \(\rho = 0.413\), meaning the Litter variable contributes 41.3% of the variance in the Luminance DV. The MML analysis revealed no significant main effects of Treatment or Sex (Tables 5a&b), but a main effect
of Age, $[F(1, 31) = 14.36, p=0.001]$ partial $\eta^2 = 0.188$, post-hoc power =0.83 (Table 5c).

No interactions achieved statistical significance. For the neonatal group, cortical layers I, III and IV were labeled clearly; for both the neonatal and adolescent group, cortical layer V was selected for data collection, because of the intensity of ligand binding.
Table 3.1a | [T]epibatidine binding. Luminance values and relative optical density (means/±S.E.M.) in offspring exposed to prenatal nicotine (0.05 mg/kg/day) or saline. Results are collapsed across age and sex. Significant p-values reflect between-subjections comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

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<th>LUMINANCE</th>
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<th>Sig.</th>
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<td>PN</td>
<td>PS</td>
<td>PN</td>
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<td>Auditory Cortex</td>
<td>191.95±4.37</td>
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<td>BLA</td>
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Table 3.1b Leptibatidine binding. Luminance values and relative optical density (means/±S.E.M.) in male and female brain regions. Results are collapsed across age, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

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<tr>
<td>Cingulate Cortex</td>
<td>170.22±2.27</td>
<td>175.83±3.5</td>
<td>0.1799±0.0059</td>
<td>0.1619±0.0085</td>
<td>0.042</td>
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<tr>
<td>Dorsal Striatum</td>
<td>183.69±2.55</td>
<td>186.12±2.45</td>
<td>0.1436±0.0067</td>
<td>0.1375±0.0061</td>
<td>0.659</td>
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<tr>
<td>Frontal Cortex</td>
<td>177.05±2.14</td>
<td>186.33±3.85</td>
<td>0.1608±0.0040</td>
<td>0.1379±0.0084</td>
<td>0.004*</td>
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<tr>
<td>Insular Cortex</td>
<td>182.69±3.22</td>
<td>186.70±4.41</td>
<td>0.1456±0.0057</td>
<td>0.1380±0.102</td>
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<tr>
<td>IPN</td>
<td>86.03±2.05</td>
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<td>0.4752±0.0053</td>
<td>0.4865±0.0048</td>
<td>0.635</td>
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<tr>
<td>MHb</td>
<td>78.59±1.76</td>
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<tr>
<td>NAcc</td>
<td>182.40±2.32</td>
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<tr>
<td>Parietal Cortex</td>
<td>206.30±2.01</td>
<td>203.76±1.48</td>
<td>0.0930±0.0036</td>
<td>0.0982±0.0037</td>
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<tr>
<td>PPTg</td>
<td>175.82±3.00</td>
<td>173.92±5.92</td>
<td>0.1620±0.0069</td>
<td>0.1676±0.0110</td>
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<tr>
<td>Raphe Nucleus</td>
<td>187.11±6.93</td>
<td>172.13±5.92</td>
<td>0.1354±0.0120</td>
<td>0.1722±0.0118</td>
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<tr>
<td>Somatosensory Cortex</td>
<td>196.9±3.95</td>
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<td>0.1265±0.0089</td>
<td>0.1281±0.0084</td>
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<tr>
<td>Substantia Nigra</td>
<td>125.81±3.05</td>
<td>123.39±1.80</td>
<td>0.3128±0.0072</td>
<td>0.3231±0.0050</td>
<td>0.511</td>
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<tr>
<td>VTA</td>
<td>126.96±2.30</td>
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<td>0.3313±0.0043</td>
<td>0.176</td>
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Table 3.1c 

**Epibatidine binding.** Luminance and relative optical density (means/±S.E.M.) in neonatal and adolescent rat brain regions. Results are collapsed across sex, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjections comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
<thead>
<tr>
<th>Region</th>
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<th>Neonate</th>
<th>Adol.</th>
<th>Sig.</th>
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<tbody>
<tr>
<td>Auditory Cortex</td>
<td>199.95±3.06</td>
<td>175.00±2.08</td>
<td>0.1064±0.0070</td>
<td>0.1639±0.0054</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>BLA</td>
<td>187.94±2.24</td>
<td>185.04±2.69</td>
<td>0.1332±0.0057</td>
<td>0.1401±.0065</td>
<td>p=0.398</td>
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<tr>
<td>Cingulate Cortex</td>
<td>181.62±2.74</td>
<td>164.43±1.71</td>
<td>0.1485±0.0064</td>
<td>0.1912±0.0042</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>Dorsal Striatum</td>
<td>200.60±1.89</td>
<td>163.35±1.97</td>
<td>0.1056±0.0040</td>
<td>0.1945±0.0046</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>191.16±2.72</td>
<td>171.48±1.75</td>
<td>0.1260±0.0067</td>
<td>0.1732±0.0044</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>Insular Cortex</td>
<td>198.67±2.57</td>
<td>170.71±1.76</td>
<td>0.1092±0.0056</td>
<td>0.1754±0.0045</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>IPN</td>
<td>85.36±1.79</td>
<td>85.54±1.75</td>
<td>0.4910±0.0046</td>
<td>0.4881±0.0044</td>
<td>p=0.914</td>
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<tr>
<td>MHB</td>
<td>77.56±1.70</td>
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<td>0.5186±0.0040</td>
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<tr>
<td>NAcc</td>
<td>182.10±2.15</td>
<td>178.17±3.08</td>
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<td>0.1569±0.0071</td>
<td>p=0.271</td>
</tr>
<tr>
<td>Parietal Cortex</td>
<td>205.78±1.72</td>
<td>204.28±1.84</td>
<td>0.0938±0.0044</td>
<td>0.0963±0.0047</td>
<td>p=0.519</td>
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<tr>
<td>PPTg</td>
<td>166.05±1.95</td>
<td>182.81±1.79</td>
<td>0.1878±0.0045</td>
<td>0.1471±0.0046</td>
<td>p=0.000*</td>
</tr>
<tr>
<td>Raphe Nucleus</td>
<td>187.11±6.93</td>
<td>172.13±5.92</td>
<td>0.1363±0.0102</td>
<td>0.1718±0.0105</td>
<td>p=0.036</td>
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<tr>
<td>SSC</td>
<td>206.38±2.95</td>
<td>180.66±1.89</td>
<td>0.0926±0.0068</td>
<td>0.1505±0.0049</td>
<td>p=0.000*</td>
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<tr>
<td>Substantia Nigra</td>
<td>122.99±2.95</td>
<td>126.19±2.74</td>
<td>0.3200±0.0068</td>
<td>0.3084±0.0064</td>
<td>p=0.382</td>
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<tr>
<td>VTA</td>
<td>123.99±1.89</td>
<td>126.06±2.21</td>
<td>0.3147±0.0049</td>
<td>0.3081±0.0057</td>
<td>p=0.949</td>
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</table>
Table 3.2a | 1bungarotoxin binding. Luminance and relative optical density (means/±S.E.M.) in brain regions of offspring exposed to nicotine (0.05 mg/kg/day) or saline during gestational days 8-21. Results are collapsed across sex and age. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
<thead>
<tr>
<th>Region</th>
<th>LUMINANCE</th>
<th>ROD</th>
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<td></td>
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<td>PS</td>
<td>PN</td>
<td>PS</td>
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<tr>
<td>Auditory Cortex</td>
<td>175.61±2.66</td>
<td>182.09±2.99</td>
<td>0.1632±0.0069</td>
<td>0.1472±0.0070</td>
<td>p=0.010*</td>
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<tr>
<td>BLA</td>
<td>142.09±1.47</td>
<td>142.61±1.33</td>
<td>0.2584±0.0049</td>
<td>0.2633±0.0038</td>
<td>p=0.773</td>
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<tr>
<td>Cingulate Cortex</td>
<td>167.89±1.61</td>
<td>174.19±2.08</td>
<td>0.1827±0.0042</td>
<td>0.1671±0.0054</td>
<td>p=0.011*</td>
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<tr>
<td>Dorsal Striatum</td>
<td>231.00±4.31</td>
<td>225.74±4.85</td>
<td>0.0421±0.0101</td>
<td>0.0534±0.0105</td>
<td>p=0.447</td>
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<tr>
<td>Insular Cortex</td>
<td>175.09±2.46</td>
<td>181.31±2.69</td>
<td>0.1660±0.0062</td>
<td>0.1511±0.0064</td>
<td>p=0.011*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>100.74±1.94</td>
<td>112.86±2.75</td>
<td>0.4107±0.0045</td>
<td>0.3577±0.0066</td>
<td>p=0.004*</td>
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<tr>
<td>Motor Cortex</td>
<td>179.89±1.79</td>
<td>178.51±1.74</td>
<td>0.1525±0.0047</td>
<td>0.1556±0.0045</td>
<td>p=0.680</td>
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<tr>
<td>NAcc</td>
<td>169.95±1.85</td>
<td>162.20±1.87</td>
<td>0.1772±0.0047</td>
<td>0.1992±0.0047</td>
<td>p=0.010*</td>
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</tr>
<tr>
<td>PPTg</td>
<td>153.91±2.10</td>
<td>155.12±2.79</td>
<td>0.2211±0.0055</td>
<td>0.2194±0.0067</td>
<td>p=0.720</td>
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<tr>
<td>Raphe Nucleus</td>
<td>150.47±1.39</td>
<td>146.74±3.31</td>
<td>0.2364±0.0040</td>
<td>0.2404±0.0077</td>
<td>p=0.408</td>
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<tr>
<td>Somatosensory Cortex</td>
<td>170.96±1.88</td>
<td>175.73±2.49</td>
<td>0.1745±0.0048</td>
<td>0.1624±0.0061</td>
<td>p=0.271</td>
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<tr>
<td>Substantia Nigra</td>
<td>118.23±3.27</td>
<td>123.66±3.44</td>
<td>0.3365±0.0058</td>
<td>0.3215±0.0065</td>
<td>p=0.290</td>
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</table>
**Table 3.2b** bungarotoxin binding. Luminance and relative optical density (means/±S.E.M.) in male and female brain regions. Results are collapsed across age, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjactions comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
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<th>Region</th>
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<th>LUMINANCE Female</th>
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<th>ROD Female</th>
<th>Sig.</th>
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<tbody>
<tr>
<td>Auditory Cortex</td>
<td>178.04±3.02</td>
<td>179.53±2.79</td>
<td>0.1575±0.0071</td>
<td>0.1531±0.0067</td>
<td>p=0.571</td>
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<tr>
<td>BLA</td>
<td>139.8±0.98</td>
<td>144.91±1.51</td>
<td>0.2742±0.0020</td>
<td>0.2474±0.0038</td>
<td>p=0.007*</td>
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<tr>
<td>Cingulate Cortex</td>
<td>175.64±2.38</td>
<td>172.37±2.18</td>
<td>0.1641±0.0044</td>
<td>0.1711±0.0057</td>
<td>p=0.244</td>
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<tr>
<td>Dorsal Striatum</td>
<td>226.46±5.25</td>
<td>230.24±3.91</td>
<td>0.0518±0.0150</td>
<td>0.0456±0.0088</td>
<td>p=0.378</td>
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<tr>
<td>Insular Cortex</td>
<td>175.64±2.38</td>
<td>180.76±2.82</td>
<td>0.1649±0.0058</td>
<td>0.1522±0.0068</td>
<td>p=0.034</td>
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<tr>
<td>Hippocampus</td>
<td>108.88±3.22</td>
<td>104.74±2.08</td>
<td>0.3736±0.0076</td>
<td>0.3945±0.0054</td>
<td>p=0.143</td>
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<tr>
<td>Motor Cortex</td>
<td>177.67±1.65</td>
<td>180.73±1.82</td>
<td>0.1588±0.0045</td>
<td>0.1504±0.0046</td>
<td>p=0.123</td>
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<tr>
<td>NAc</td>
<td>164.13±1.66</td>
<td>168.02±2.18</td>
<td>0.1920±0.0046</td>
<td>0.1817±0.0057</td>
<td>p=0.097</td>
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<tr>
<td>PPTg</td>
<td>154.10±2.47</td>
<td>154.83±2.42</td>
<td>0.2194±0.0062</td>
<td>0.2173±0.0061</td>
<td>p=0.950</td>
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<tr>
<td>Raphe Nucleus</td>
<td>150.76±1.56</td>
<td>146.44±3.19</td>
<td>0.2309±0.0045</td>
<td>0.2421±0.0075</td>
<td>p=0.147</td>
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<tr>
<td>Somatosensory Cortex</td>
<td>174.12±2.38</td>
<td>172.58±2.16</td>
<td>0.1675±0.0044</td>
<td>0.1706±0.0041</td>
<td>p=0.525</td>
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<tr>
<td>Substantia Nigra</td>
<td>121.21±3.47</td>
<td>120.44±3.47</td>
<td>0.3395±0.0081</td>
<td>0.3292±0.0081</td>
<td>p=0.651</td>
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Table 3.2c | [I]bungarotoxin binding. Luminance and relative optical density (means/±S.E.M.) in neonatal and adolescent rat brain regions. Results are collapsed across sex, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
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<td>Auditory Cortex</td>
<td>168.21±1.78</td>
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<tr>
<td>BLA</td>
<td>141.65±1.45</td>
<td>143.06±1.33</td>
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<tr>
<td>Cingulate Cortex</td>
<td>186.63±2.16</td>
<td>169.78±1.47</td>
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<tr>
<td>Insular Cortex</td>
<td>186.63±2.16</td>
<td>169.78±1.47</td>
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<tr>
<td>Hippocampus</td>
<td>111.68±1.67</td>
<td>101.94±3.15</td>
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<tr>
<td>Motor Cortex</td>
<td>175.80±1.73</td>
<td>182.60±1.44</td>
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<td>NAc</td>
<td>167.81±2.08</td>
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<tr>
<td>Somatosensory</td>
<td>168.82±1.83</td>
<td>1177.88±2.21</td>
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Table 3.3a | Iconotoxin MII binding. Luminance and relative optical density (means/±S.E.M.) in brain regions of offspring exposed to nicotine (0.05 mg/kg/day) or saline during gestational days 8-21. Results are collapsed across sex and age. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
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<th>Region</th>
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<td>PN</td>
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<tr>
<td>Dorsal Striatum</td>
<td>220.71±1.40</td>
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<td>0.0645±0.0047</td>
<td>0.0629±0.0020</td>
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<tr>
<td>MHb</td>
<td>177.66±3.76</td>
<td>191.24±3.32</td>
<td>0.1580±0.0082</td>
<td>0.1254±0.0077</td>
<td>p=0.064</td>
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<tr>
<td>NAc</td>
<td>217.37±2.88</td>
<td>216.52±1.70</td>
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<td>0.0709±0.0043</td>
<td>p=0.656</td>
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<tr>
<td>Substantia Nigra</td>
<td>184.64±3.70</td>
<td>181.78±4.53</td>
<td>0.1415±0.0084</td>
<td>0.1483±0.0099</td>
<td>p=0.925</td>
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<tr>
<td>VTA</td>
<td>204.50±1.87</td>
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<td>0.1000±0.0049</td>
<td>0.0920±0.0082</td>
<td>p=0.020</td>
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</table>
Table 3.3b [125]conotoxin MII binding. Luminance and relative optical density (means/±S.E.M.) in male and female brain regions. Results are collapsed across age, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
<thead>
<tr>
<th>Region</th>
<th>LUMINANCE</th>
<th>ROD</th>
<th>Sig.</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Dorsal Striatum</td>
<td>218.94±1.12</td>
<td>222.91±0.84</td>
<td>0.0676±0.0039</td>
</tr>
<tr>
<td>MHB</td>
<td>184.24±2.78</td>
<td>184.65±5.25</td>
<td>0.1420±0.0065</td>
</tr>
<tr>
<td>NAcc</td>
<td>215.05±2.45</td>
<td>219.34±0.68</td>
<td>0.0745±0.0062</td>
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<tr>
<td>Substantia Nigra</td>
<td>184.64±3.70</td>
<td>181.78±4.53</td>
<td>0.1418±0.0084</td>
</tr>
<tr>
<td>VTA</td>
<td>204.50±1.87</td>
<td>206.30±2.88</td>
<td>0.0967±0.0045</td>
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</tbody>
</table>

Table 3.3c [125]conotoxin MII binding. Luminance values and relative optical density (means/±S.E.M.) in neonatal and adolescent rat brain regions. Results are collapsed across sex, and prenatal nicotine (0.05 mg/kg/day) or saline exposure during gestational days 8-21. Significant p-values reflect between-subjects comparisons; an asterisk * indicates a significant group difference with statistical power ≥ 0.80.

<table>
<thead>
<tr>
<th>Region</th>
<th>LUMINANCE</th>
<th>ROD</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neonate</td>
<td>Adol.</td>
<td>Neonate</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>199.46±1.76</td>
<td>171.04±1.82</td>
<td>0.1069±0.0045</td>
</tr>
</tbody>
</table>
Figure 3a. Dam Weight Gain

Figure 3b. Pup Weight Gain
**Figure 3.1 Dam and offspring weight gain.** (A) Mean weight gain (±SEM) of dams (in grams), presented by maternal treatment group. Daily weights were recorded for pregnant dams, gestational days 0-21; graphs represent grams of gained weight on gestational days 0, 7, 14 and 21. A Mixed Models ANOVA with Repeated Measures revealed that gestational treatment had no significant effect on weight gain. (B) Mean weight gain (± SEM) of offspring (in grams), presented by prenatal treatment. Weight gain for postnatal days 1, 7, 14, and 21 are represented. A Mixed Models ANOVA with Repeated measures found no significant main effect of prenatal treatment on weight gain.

**Number of Pups**

![Graph showing number of pups born by males, females, and total.]

**Figure 3.2 Number of male and female offspring produced.** The mean number (±SEM) of male (left), female (middle), and total (right) offspring delivered by dams in each Treatment group. ANOVAs revealed no differences between number of pups born to each group, and Chi-Square test showed no difference in the ratio of males to females born.
Righting Reflex

![Graph showing the mean righting time (seconds) for different gestational days across two groups, PN and PS.]

Negative Geotaxis

![Graph showing the mean time (seconds) for different gestational days across two groups, PN and PS.]

Figure 5a.

Figure 5b.
Figure 3.3 Latency for righting reflex and negative geotaxis. (A) Mean latency (±SEM) (in seconds) for PN and PS offspring on Righting Reflex task, as measured across three consecutive days (postnatal days 3-5) during the neonatal period. A Mixed Models ANOVA with Repeated Measures revealed that prenatal treatment had no significant effect on Righting Reflex latency. (B) Mean latency (±SEM) (in seconds) for PN and PS offspring on Negative Geotaxis task, as measured across three consecutive days (postnatal days 8-10) during the neonatal period. A Mixed Models ANOVA with Repeated Measures revealed that prenatal treatment had no significant effect on Negative Geotaxis latency.

Ventral Tegmental Area~ α4β2 nAChRs

![Graph showing Luminance values for PN and PS treatments]

Prenatal Treatment

\[ F(1,32) = 8.40, \ p = .007, \ \eta^2_p = .213 \]

Figure 3.4 Treatment effect: α4β2 nAChRs in VTA. Mean Luminance (±SEM) value for [\(^{125}\)I]-epibatidine binding in the Ventral Tegmental Area. A 2 × 2 ×2 Univariate ANOVA revealed a significant main effect of Treatment [\(F(1,31) = 8.40, \ p = 0.007\), partial \(\eta^2 = 0.213\), post-hoc power=0.89. PN \(M=121.13±1.98;\) PS \(M=129.17±1.72.\)
Nucleus Accumbens ~ α4β2 nAChRs

Prenatal Treatment

$F(1,32) = 12.71, p = .001, \eta^2 = .194$

Dorsal Striatum ~ α4β2 nAChRs

Prenatal Treatment

$F(1,31) = 8.41, p = .007, \eta_p^2 = .213$
Figure 3.5 Treatment effect: α4β2 nAChRs in NAcc. Mean Luminance (±SEM) values for [125I]-epibatidine binding in the NAcc. MML analysis found a significant fixed effect of Treatment, [Fixed Effect (1, 32) = 12.71, \(p = 0.001\), \(\eta^2 = 0.194\), power = 0.85. PN \(M = 175.01 \pm 2.61\); PS \(M = 185.79 \pm 2.15\).

Figure 3.6 Treatment effect: α4β2 nAChRs in Dorsal Striatum. Mean Luminance (±SEM) values for [125I]-epibatidine binding in the Dorsal Striatum. A 2 × 2 × 2 Univariate ANOVA revealed a significant main effect of Treatment \([F(1, 31) = 8.41, \ p = 0.007\), partial \(\eta^2 = 0.213\), post-hoc power=0.88. PN \(M = 185.68 \pm 1.83\); PS \(M = 179.06 \pm 1.89\).
Cingulate Cortex ~ α4β2 nAChRs

Prenatal Treatment

$F(1,32)=7.68, p=.009, \eta_p^2=.194$

Auditory Cortex ~ α4β2 nAChRs

Prenatal Treatment

$F(1,32)=7.14, p=.012, \eta_p^2=.183$
Figure 3.7 Treatment effect: α4β2 nAChRs in Cingulate Cortex. Mean Luminance (±SEM) values for \(^{125}\text{I}\)-epibatidine binding in the Cingulate Cortex. A \(2 \times 2 \times 2\) Univariate ANOVA revealed a significant main effect of Treatment \([F(1, 32)=7.68, p=0.009, \text{partial } \eta^2=0.194, \text{post-hoc power}=0.85\). PN \(M=169.36±2.66\); PS \(M=176.69±3.12\).

Figure 3.8 Treatment effect: α4β2 nAChRs in Auditory Cortex. Mean Luminance (±SEM) values for \(^{125}\text{I}\)-epibatidine binding in the Auditory Cortex. A \(2 \times 2 \times 2\) Univariate ANOVA revealed a significant main effect of Treatment \([F(1, 32)=7.14, p=0.012, \text{partial } \eta^2=0.183, \text{post-hoc power}=0.82\). PN \(M=191.95±4.37\); PS \(M=183.00±3.12\).
Parietal Cortex $\alpha4\beta2$ nAChRs

Prenatal Treatment

$F(1,32)=8.61, p=.006, \eta_p^2=.212$

Somatosensory Cortex $\sim \alpha4\beta2$ nAChRs

Prenatal Treatment

$F(1,32)=11.19, p=.002, \eta_p^2=.259$
Figure 3.9 Treatment effect: α4β2 nAChRs in Parietal Cortex. Luminance (±SEM) values for [125I]-epibatidine binding in the Parietal Cortex. A 2 × 2 ×2 Univariate ANOVA revealed a significant main effect of Treatment [F(1, 32)=8.61, p=0.006, partial η²=0.212, post-hoc power=0.88. PN M=208.41±1.62; PS M=201.66±1.61.

Figure 3.10 Treatment effect: α4β2 nAChRs in Somatosensory Cortex. Luminance (±SEM) values for [125I]-epibatidine binding in the Somatosensory Cortex. A 2 × 2 ×2 Univariate ANOVA revealed a significant main effect of Treatment [F(1, 32)=11.19, p=0.002, partial η²=0.259, post-hoc power=0.95. PN M=198.07±4.5; PS M=188.97±2.68.

Figure 3.11 Treatment X Age interaction: Somatosensory Cortex. Luminance (±SEM) values for [125I]-epibatidine binding in the Somatosensory Cortex. The ANOVA revealed a significant interaction between Treatment × Age, [F(1, 32)=8.37, p=0.007, partial η²=0.206, post-hoc power=0.95. PN neonates: M=213.84±2.68; PS neonates: M=197.13±2.77; PN adolescents: M=180.50±2.18, PS adolescents: M=181.81±2.65. The PN neonates had fewer receptors early in life, compared to the PS group; both PN and PS subjects significantly increased receptor density to similar levels by adolescence.
Nucleus Accumbens ~ $\alpha_7$ nAChRs

Prenatal Treatment

$F(1,32)=7.59, p=.010, \eta^2=.192$

Hippocampus ~ $\alpha_7$ nAChRs

Prenatal Treatment

$F(1,32)=17.89, p=.000, \eta^2=.229$
Figure 3.12 Treatment Effect: α7 nAChRs in Nucleus Accumbens Luminance (±SEM) values for [125I]α-bungarotoxin binding in the NAcc. MML analysis found a significant fixed effect of Treatment, [Fixed Effect (1, 32) = 7.59, \(p = 0.010\), \(\eta^2 = 0.192\), power = 0.83. PN \(M = 162.95 \pm 1.85\); PS \(M = 169.20 \pm 1.89\).

Figure 3.13 Treatment Effect: α7 nAChRs in Hippocampus. Luminance (±SEM) values for [125I]α-bungarotoxin binding in the Hippocampus. MML Analysis revealed a significant main effect of Treatment, [Fixed Effect (1, 32) = 17.89, \(p = 0.000\), \(\eta^2 = 0.229\), power = 0.91. PN \(M = 100.74 \pm 1.94\); PS \(M = 112.86 \pm 2.75\).
Cingulate Cortex ~ α7 nAChRs

Prenatal Treatment

$F(1,32)=7.27, p=.011, \eta^2_p=.185$

Agranular Insular Cortex ~ α7 nAChRs

Prenatal Treatment

$F(1,32)=7.20, p=.011, \eta^2=.184$
Figure 3.14 Treatment Effect: α7 nAChRs in Cingulate Cortex. Luminance (±SEM) values for $[^{125}\text{I}]\alpha$-bungarotoxin binding in the Cingulate Cortex. A $2 \times 2 \times 2$ Univariate ANOVA revealed a significant main effect of Treatment, $[F(1, 32) = 7.27, p = 0.011]$ partial $\eta^2 = 185$, post-hoc power = 0.82. PN $M=167.88\pm1.61$; PS $M=174.19\pm2.08$.

Figure 3.15 Treatment Effect: α7 nAChRs in Insular Cortex. Luminance (±SEM) values for $[^{125}\text{I}]\alpha$-bungarotoxin binding in the Agranular Insular Cortex. A $2 \times 2 \times 2$ Univariate ANOVA revealed a significant main effect of Treatment, $[F(1, 32) = 7.19, p = 0.011]$ partial $\eta^2 = 184$, post-hoc power = 0.82. PN $M=175.09\pm2.46$; PS $M=181.31\pm2.69$.

Figure 17. Luminance (±SEM) values for $[^{125}\text{I}]\alpha$-bungarotoxin binding in the Auditory Cortex. A $2 \times 2 \times 2$ Univariate ANOVA revealed a significant main effect of Treatment, $[F(1, 31) = 7.48, p = 0.010]$ partial $\eta^2 = 194$, post-hoc power = 0.83. PN $M=175.61\pm2.66$; PS $M=182.09\pm2.99$.

Auditory Cortex ~ α7 nAChRs

Prenatal Treatment

$F(1,31)=7.48, p=.010$, $\eta_p^2=.193$
Figure 3.16 Treatment Effect: α7 nAChRs in Auditory Cortex. Luminance (±SEM) values for $[^{125}\text{I}]\alpha$-bungarotoxin binding in the Auditory Cortex. A $2 \times 2 \times 2$ Univariate ANOVA revealed a significant main effect of Treatment, [$F(1, 31) = 7.48, p = 0.010$] partial $\eta^2 = 194$, post-hoc power = 0.83. PN $M=175.61\pm2.66$; PS $M=182.09\pm2.99$. 
CHAPTER IV

CONCLUSIONS

Low-dose IV prenatal nicotine administration during gestational days 8-21 resulted in quantifiable changes in nicotinic receptor expression in brain regions underlying motivated behavior and attention processing. Autoradiography and densitometry revealed significant, sufficiently powered differences between PN and PS treatment groups in α4β2 nAChR expression levels in the VTA and NAc.

As hypothesized (Hypothesis 1, section 4.3), PN subjects had greater α4β2 nAChR density in the VTA and NAc. No differences in α4β2 nAChR receptor density were found in the PFC as a function of Treatment.

As hypothesized (1) PN subjects had fewer α7-containing nAChRs in the NAc. However, the opposite was found in the Hippocampus; a greater density of α7-containing nAChRs found in the Hippocampus for PN animals (no differences were found for the VTA or PFC). As hypothesized, nAChR expression levels following prenatal nicotine were also altered as a function of age (2).

nAChR expression density increased between postnatal day 10 and postnatal day 35, as hypothesized (2), in the PFC (α4β2) and Hippocampus (α7). However, in the PPTg, α4β2 expression decreased with age. No age differences were found in the hypothesized brain regions of VTA or NAc. Sex differences in nAChR expression levels were found in the PFC; males had greater density of α4β2 nAChRs, as hypothesized (3),
but no other differences between males and females were found, within any hypothesized brain regions.

Collectively, these results support the hypothesis that prenatal nicotine exposure changes nicotinic receptor expression in brain areas engaged during reward-seeking behavior and attention processing.

More research is needed to validate and expand upon these findings. The current set of experiments had methodological and design limitations, discussed below. Several other brain areas implicated in reward-seeking behavior, known to be altered by prenatal nicotine, and expressing α4β2, α7-containing, and α6-containing nAChRs, were analyzed for receptor density—those findings are discussed later in the conclusions section.

7.1 Litter Parameters

The use of IV PN resulted in a lack of growth related deficits, as reported in previous published reports from this laboratory (Harrod et al., 2011; Lacy et al., 2011; Harrod et al., 2012; Lacy et al., 2012; Morgan et al., 2013; Lacy et al., 2014; Lacy et al., 2016). Clinical research has consistently reported low birth weight following gestational tobacco exposure (via maternal smoking), and this reduced weight has historically confounded clinical findings of altered neurodevelopmental capacity throughout the lifespan (Comstock et al., 1971; Fried & O’Connell, 1987; Meyer & Comstock, 1972; Simpson 1957).

Preclinical research is rife with similar outcomes; reduced birth weight is often reported across several gestational nicotine administration routes (Becker & Martin,
1971; Slotkin 2004; Schneider 2010); however, birth weight differences between prenatal treatment groups become nonsignificant about a month after parturition (Levin et al., 2006).

The findings reported here cannot be attributed to any nutritional deficits; during gestation, no differences in dam weight gain as a function of gestational treatment. After parturition, no significant differences in weight gain between PN and PS offspring appeared through the first three weeks of life.

In addition to lower birth weight, fetal ischemia/hypoxia is a cited source of abnormal developmental trajectories. For example, the pharmacokinetic distribution of nicotine administered through the OMP method results in much less nicotine entering the brain, compared to the amount released from the OMP continuously.

Because much of the drug is metabolized before reaching target sites, many published experiments use a daily dose of nicotine around 2.4 mg/kg/day (Birnbaum et al., 1994). This dose is about 15x higher than the dose used for this experiment (0.05mg/kg/injection, which is 100% bioavailable upon injection). The higher doses of nicotine prenatally administered (via OMP) result in a 40% reduction of uterine and placental blood flow.

Low-dose IV prenatal nicotine administration does not produce confounds of restricted intrauterine growth or low birth weight, and the ontogenetic measurements of postnatal development (litter size/composition, reflexes, and eye opening) were not disrupted by low-dose IV nicotine exposure on GD 8-21.

*The remainder of the Conclusions section is organized to review results specific to the hypotheses (Section 4.3.1), followed by information*
about experimental limitations. A brief summary of non-hypothesized experimental results follows the limitations section.

7.2 Mesocorticolimbic DA pathway: VTA, NAc, PFC

A hypothesized increase in α4β2 nAChR expression levels in the MCL DA reward neurocircuitry was partially confirmed by statistically significant increases in receptor density in the VTA and NAc as a function of Treatment (hypothesis 1). This is supported by similar research by Tizabi et al., (1997) using the OMP route of administration for nicotine.

This finding is also supported by evidence for the capacity of nicotinic receptors to upregulate. Although the fetal compartment is unique, chronic adult nicotine exposure during tobacco dependence causes prolonged nAChR desensitization, this deep desensitization causes an upregulation in nAChR receptor number (Dani & Heinemann, 1996; Fenster et al., 1999). This upregulation is the result of homeostatic regulation. Because offspring were exposed to chronic nicotine during gestational days 8-21, the NAc and VTA may have upregulated α4β2 nAChRs in these regions.

The α4β2 nAChR in particular is critical for addiction to nicotine, and induces firing of dopamine neurons in the VTA following stimulation (Pons et al., 2008). An upregulation of expression for this receptor subtype in the VTA and NAc fits well with the clinical literature, which shows higher rates of nicotine dependence and early initiation of tobacco smoking behaviors following PCSE (Buka et al., 2003; Kandel et al., 1994; Brennan et al., 2002; O’Callagahan et al., 2006; Goldschmidt et al., 2012).
The NAc had fewer \( \alpha 7 \) nAChRs expressed as a function of Treatment (hypothesis 1). This reduced expression was hypothesized (1) to extend to VTA, PFC, Hippocamous and PPTg. Tizabi et al., (2000) reported reduced expression of the \( \alpha 7 \) nAChR following prenatal nicotine exposure.

The \( \alpha 7 \) nAChR subtype is critical during gestation, and is responsible for the development of the DA reward neurocircuitry. During GD13, the \( \alpha 7 \) nAChR guides the newly-generated DA neuron to the DA receptor for the first time during gestation, establishing the earliest structure of the DA neurocircuitry (Dwyer et al., 2008).

The reduced \( \alpha 7 \) nAChR expression in the NAc, which projects to the PFC, could underlie reduced dopamine firing in this region following PN exposure. PN exposure changes the development of catecholamine neurons in the VTA and substantia nigra (Slotkin et al., 1987a; Navarro et al., 1988; Ribary & Lichtensteiger, 1989; Kane et al., 2004; Chen et al., 2005; Franke et al., 2008); and results in decreased DA content and D\( _2 \) receptors in the NAc and striatum on postnatal day 22 (Richardson & Tizabi, 1994). \( \alpha 7 \) nAChRs are located on and act on DA neurons in the NAc and Striatum, so the reduced DA content in these regions may be reflected by lower \( \alpha 7 \) nAChR levels.

No other hypothesized brain regions showed reduced \( \alpha 7 \) nAChR expression levels, and the hypothesis was only confirmed for one brain area (see Limitations section).

Males had significantly greater \( \alpha 4\beta 2 \) nAChR expression in the PFC than females, which is consistent with published literature (Tizabi et al., 1997) and confirms the hypothesis (3) of higher nAChR levels in Males following PN exposure. Males are also
more likely to experience problems with conduct disorder and ADHD, following prenatal tobacco smoke exposure.

The dysregulation of attention processing and impulse regulation in this population may be a function of cholinergic receptor changes, as the α4β2 nAChR to regulates multiple attentional processes (cue detection, divided attention, and sustained attention; Turchi & Sarter, 1997). nAChRs in the PFC also regulate behavior related to emotional reactivity—inhibiting inappropriate emotions, impulses, and habits (Miller, 2000).

Receptor expression levels were not a function of Sex for any other hypothesized brain regions (see Limitations, below), and the hypothesis was not confirmed.

Age was a significant main effect for α4β2 nAChR expression in the PFC, with higher expression levels found in the adolescent group. This is consistent with the hypothesis (2) predicting greater density of all five hypothesized brain regions. Tizabi et al., (1997; 2000) reported significantly higher nAChR levels in brains of adolescent rats (PND36-38) following OMP prenatal exposure. A significant effect of Age was not revealed in VTA or NAc.

A lack of significant treatment, sex, or age effects are possibly attributable to the experimental Limitations (see below).

### 7.3 Hippocampus and PPTg

A significant Treatment effect was found for α7 nAChR expression levels in the Hippocampus, with PN-exposed offspring expressing higher levels of nAChRs in the
Hippocampus. This finding is the opposite of the hypothesized results (hypothesis 1, reduced \( \alpha_7 \) nAChR expression). However, upregulation of the \( \alpha_7 \) nAChR as a function of Treatment may contribute to impaired memory function in prenatal-nicotine exposed offspring.

Hippocampal \( \alpha_7 \) nAChRs are active during memory processes. Several studies report reduced acquisition of memory tasks following PN exposure (Sorenson et al. 1991; Li et al., 2014).

In the PPTg, the only main effect revealed was Age, for \( \alpha_4\beta_2 \) nAChR expression. Higher nAChR levels were found in neonates, with lower levels in adolescents. This could be attributed to the widely-reported expression pattern typical of nAChRs; \( \alpha_6, \alpha_7 \) and \( \alpha_4\beta_2 \) nAChRs first appear during the second gestational trimester, then expression levels climb steadily, to peak by PND 21, followed by a decline to levels maintained through adulthood (Azam et al., 2007; Pugh & Berg, 1994; Small et al., 1995; Xiang et al., 1998). Treatment and Sex did not emerge as significant main effects in the PPTg.

### 7.4 Limitations

Limitations of the experiments reported here include 3 possible sources of variability in data analysis. (1) Experimental design: with 10 dams contributing 4 offspring each, the statistical power was not present to conclude multiple significant group differences were not spurious.

The MML analysis, used when Litter was shown to have an undue effect on the variability of data analysis, often violated the multilevel modeling assumption of
convergence. This was likely because the sample size requirement wasn’t met for robust multilevel modeling. Future research with higher sample size would undoubtedly find statistically significant, sufficiently powered differences in receptor expression for many brain regions, as a function of Treatment.

(2) Tissue slicing/preparation: a proportion of tissue, approximately a third of tissue images submitted for densitometry, were torn, with the ventral half of the slice missing, or with holes in the slice. Perhaps uneven tissue slicing resulted in the loss of tissue. Within the \[^{[125]}I\]-bungarotoxin dataset, for example, the tissue was too damaged (in both age groups), that there were not enough intact Frontal Cortex images intact for densitometry.

Damage of tissue negatively affected densitometry analysis in many brain areas, identified in the results section with the statement “binding was not capable of densitometry analysis.” For sections in which the nAChR was not expressed, the statement “binding was not evident” is used.

(3) Autoradiography: The images obtained following autoradiography and ligand binding were damaged, in many cases, beyond what could be expected from tissue slicing errors. One culprit may be the age of the tissue at the time of radioligand binding, with neonatal tissue being prone to damage which can occur with application of harsh radioactive isotopes. Many slices appeared as though a third or more of the slice had been disintegrated.

Additionally, the tissue images retained for densitometry following \[^{[125]}I\] Conotoxin MII (binding α6-containing nAChRs) binding were limited to the areas of α6-
containing nAChR expression (namely, midbrain dopamine neurons); Beyond this limitation, there were very few slice images available for the neonatal age group.

Of the \( n=17 \) neonatal subjects with images of bound tissue, an average of 13 images of single slices were available (per subject) for densitometry analysis. The adolescent group had twice as many slice images available for densitometry analysis to collect luminance values, but this still resulted in a limited number of options to obtain the 4 images with the brain region of interest available and intact for densitometry.

7.5 Summary of Brain Regions Analyzed

7.5.1 Substantia Nigra and Dorsal Striatum

The Substantia Nigra (SN) and Dorsal Striatum (DS) were selected for Densitometry analysis shortly after formal hypothesis testing began. The SN is another hub (along with the VTA) of dopamine cell bodies; SN dopamine neurons project to the DS. The only significant differences in Substantia Nigra expression found was a function of Age; Adolescent rat brains expressed a greater density of \( \alpha 6 \) nAChRs, compared to neonates (across Treatment and Sex).

Density of \( \alpha 4\beta 2 \) nAChRs in DS (which receives projections from SN) were significantly higher for the PS Treatment group, compared to the PN-exposed offspring. This region also had increased \( \alpha 4\beta 2 \) nAChR density in adolescence, compared to the neonatal group.
7.5.2 BLA & Cingulate Cortex

The Anterior Cingulate Cortex (CC) is recognized in clinical literature, characterized by reduced activation during cognitive processing for drug-addicted subjects (Goldstein et al., 2009). Because of its involvement in attention processing and drug addiction, the CC was analyzed for nAChR receptor densitometry.

α4β2 nAChR expression pattern variability was significantly different as a function of Treatment, with PN offspring expressing significantly greater density of α4β2 nAChRs in the CC, compared to PS offspring. For both α4β2 and α7 nAChRs, the CC offspring had greater receptor density binding in adolescence, compared to the neonatal group.

The BLA is functionally connected with the Hippocampus, as a part of neurocircuitry underlying the emotional processing of stimuli, emotional memory, and learning, (Pidoplichko et al, 2013; Subramaniyan & Dani, 2015). Acquisition of stimulant-seeking behavior is regulated by efferents sent from the BLA to the NAc, and lesions of the BLA prevent the acquisition of drug-seeking behavior (Whitelaw et al. 1996). Activation of both α7 and α4β2 nAChRs in the BLA are a function of reward-based learning and seeking behavior.

This study found one significant difference in the BLA; Males expressed greater density of the α7 nAChR, compared to females.
7.5.3 Medial Habenula, Interpeduncular Nucleus, Raphe Nucleus

The Medial Habenula (MHb) was examined for nAChR densitometry, as it is often-cited for its involvement in tobacco smoking dependence (Kenny et al., 2012); although the MHb is commonly associated with the α5 containing nAChR subunits, both α4β2 and α6 nAChR subunits are expressed in the MHb (Shih et al., 2014).

However, no statistically significant, sufficiently powered main effects were found for the MHb in this sample.

The Interpeduncular Nucleus (IPN) and Raphe Nucleus (RN) were selected for analysis; both of these regions are involved in directing and maintaining sleep-wake cycles. The clinical literature indicates that sleep problems are common, especially early in life, for offspring exposed to maternal tobacco (Blood-Siegried et al., 2010); Boychuck et al., (2011) found that newborns experience withdrawals following parturition, and nicotine withdrawal during the early days of birth, shifts sleep-wake patterns, potentially making offspring more vulnerable to cardiac complications.

No significant, sufficiently powered differences were found for any receptor subtype in either the IPN or the RN.

7.5.4 Cortices: Insular (Agranular) Cortex, Auditory Cortex, Motor Cortex, Parietal Cortex, Somatosensory Cortex

The Insular Cortex was selected for densitometry because of this region’s involvement in smoking behavior. The insular cortex is important for tobacco addiction
in human smokers, and it’s ablation can dramatically decrease, and even eliminate smoking behaviors (Hollander et al., 2008). In the Insular Cortex, receptor density was altered as a function of Treatment; the PN group had greater levels of α7 nAChR expression density, and both α4β2 and α7 levels were significantly different between neonatal and adolescent groups; adolescents expressed greater density for both types of nAChR.

The Auditory Cortex function is appreciably damaged by nicotine perinatal nicotine exposure (Armamakis et al., 2000). Moreover, the Auditory Cortex is part of the circuitry involved in attention and sensory processing (along with the Parietal and Somatosensory Cortices), and nicotine exposure during prenatal and perinatal periods underlies several attention and sensory processing cognitive deficits among offspring (Heath & Piccioto, 2008).

Densitometry results in the Auditory Cortex were mixed, but nAChR expression was changed for both α4β2 and α7 nAChRs; PN animals had lower levels of α4β2 nAChRs compared to PS animals, but the opposite was found for density of α7 nAChR expression; PN animals had significantly higher levels of nAChR expression.

The Motor Cortex was included as a control region; as expected, the only significant difference for this region was a function of Age, with adolescents expressing higher nAChR levels. The Parietal Cortex densitometry revealed fewer α4β2 nAChRs expressed in the PN group. In the Somatosensory Cortex, nAChR expression varied as a function of Treatment and Age, with a significant interaction revealed.

PN animals expressed fewer α4β2 nAChRs, but adolescents expressed higher levels of α4β2 nAChRs. The graphic in this report shows that PN animals, as neonates,
have significantly higher α4β2 levels, but by adolescence, both PN and PS groups appear to approach similar means.

7.6 Summary

The results of this study indicate low-dose IV prenatal nicotine has complex effects on nAChR expression levels in the neonatal and adolescent rat brain. The upregulation of nAChRs in brain regions underlying motivated behavior, point to a potential mechanism for the long-term changes in motivated, drug-seeking behavior seen in the population of maternal-tobacco smoke exposed offspring.
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