UNIVERSITY OF CALIFORNIA,
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Sex and Genotype Effects of the *CHRNA*6 3'-UTR Single Nucleotide Polymorphism on Nicotine-Seeking Behavior and Neurotransmitter Profiles in Adolescent Rats.

DISSERTATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacological Sciences

by

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
α6*	α6 subunit containing
α-CTX	Alpha conotoxin
Ach	Acetylcholine
ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of Variance
AUD	Alcohol use disorder
β2* nAChRs	Beta 2 subunits containing nAChRs
β2KO	Beta 2 null mutant
β3KO	Beta 3 null mutant
β4KO	Beta 4 null mutant
β4* nAChRs	Beta 4 subunit containing nAChRs
BLA	Basolateral amygdala
*CHRNA*3	Gene encoding the alpha3 nicotinic subunit
*CHRNA*4	Gene encoding the alpha4 nicotinic subunit
*CHRNA*5	Gene encoding the alpha5 nicotinic subunit
*CHRNA*6	Gene encoding the alpha6 nicotinic subunit
*CHRNB*2	Gene encoding the beta2 nicotinic subunit
*CHRNB*3	Gene encoding the beta3 nicotinic subunit
*CHRNB*4	Gene encoding the beta3 nicotinic subunit
CNS	Central nervous system
CPD	Cigarettes per day
CPP	Conditioned place preference
COMT	Cathecol-O methyl transferase
DA	Dopamine
DAT	Dopamine transporter
DHβE	Dihydro-β-erythroidine
DOPAC	3,4-Dihydroxyphenylacetic acid
ECD	Electrochemical detection
e-cigarettes	Electronic cigarettes
EXT	Extinction
FR	Fixed ratio schedule of reinforcement
GABA	γ-amino butyric acid
GWAS	Genome wide association study
GLU	Glutamate
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IL	Infralimbic
i.p.	Intraperitoneal
IPN	Interpeduncular nucleus

i.v.	Intravenous
IVSA	intravenous self-administration
KOR	Kappa opioid receptor
LC	Locus coeruleus
LDTn	Lateraldorsal tegmental nuclei
mg/kg	Milligram/kilogram
MAO	Monoamine Oxidase
NAc	Nucleus Accumbens
NE	Norepinephrine
NIC	Nicotine
NorBNI	Norbinaltorphimine
PFC	Prefrontal cortex
PL	Prelimbic
PPn	Pedunculopontine nucleus
PPTg	Pedunculopontine tegmental nucleus
PR	Progressive Ratio
SAL	Saline
s.c.	Subcutaneous
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SNg	Substantia Nigra
VTA	Ventral tegmental area
WT	Wild type

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CONFERENCE ABSTRACTS (~talk, †poster presentation)

† Carreño, D., , Lotfipour, S. (November, 2022) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297). Society for Neuroscience (SfN), San Diego, CA.

† Carreño, D., , Lotfipour, S. (September 2022) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297). National Hispanic Science Network Conference, Grand Rapids, MI.

†Carreño, D., , Lotfipour, S. (June 2022) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297). Associated Graduate Students (AGS) Symposium, Irvine, CA.

†Carreño, D., Lotfipour, S. (March 2022) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297). SRNT 2022, Baltimore, MD.

†Carreño, D., Lotfipour, S. (March 2021) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297) in the alpha(α)6 nicotinic acetylcholine receptor subunit. UCI AGS 2021, Virtual.

†Carreño, D., Cano M., Lotfipour, S. (March 2021) Nicotine plus cue-induced reinstatement is enhanced in adolescent Sprague-Dawley rats containing the Human 3'-UTR polymorphism (rs2304297) in the alpha(α)6 nicotinic acetylcholine receptor subunit. NIDA 2021, Virtual.

~Carreño, D. and Lotfipour, S. (Feb 2021) Human 3'-UTR polymorphism (rs2304297) in the alpha(α)6 nicotinic acetylcholine receptor subunit enhances nicotine plus cue induced reinstatement in adolescent Sprague-Dawley rats. SRNT 202, Virtual

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ABSTRACT OF THE DISSERTATION

Nicotine, the main psychoactive constituent in tobacco, is highly addictive and poses a particular vulnerability to adolescents. Adolescence is a critical period of neurodevelopment when nicotine use is initiated. Nicotine is the exogenous ligand for nicotinic acetylcholine receptors (nAChRs). The alpha(α)6 nAChR subunit (encoded by the *CHRNA*6 gene) reaches peak expression in dopaminergic neurons of the midbrain during adolescence. A 'C' to 'G' single nucleotide polymorphism (SNP) in the 3'-untranslated region (UTR) of the *CHRNA6* gene (rs2304297) has been associated with nicotine addiction in adolescent humans. Discerning the genetic and neurobiological mechanisms impacting adolescent nicotine seeking could help with improved prevention and intervention strategies in humans. My findings illustrate that nicotine-seeking behavior could be assessed in adolescent wild type male and female Sprague Dawley rats using a self-administration and reinstatement paradigm, a model of relapse behavior in humans. Further, I show that the *CHRNA*6 3'-UTR SNP elicits sex- and genotype-dependent nicotine seeking behavior, with $\alpha6^{GG}$ male adolescents most vulnerable. Mechanistically, I illustrate that dopaminergic mechanisms underlie deficits in nicotine seeking behavior, Taken together, the results of the present study will add to a growing body of literature that the *CHRNA*6 3'-UTR SNP sex- and genotype-dependently contribute to adolescent nicotine addiction with dopaminergic circuits involved.

Chapter 1:

Introduction

Significance and Background

The prevalence of tobacco addiction and dependence is higher than any other drug of abuse (Centers for Disease Control and Prevention, 2012; Centers for Disease Control and Prevention (US), 2014), with tobacco use being the leading preventable cause of death and disease in the world (Samet, 2013). Smoking disproportionately affects those in low-socioeconomic status, women, the homeless, racial minorities, LGBTQ+ individuals, and those suffering from mental health and substance use disorders (Baskerville et al., 2017; Upson, 2015; Y, 2017). Adolescents are a vulnerable population, given that smoking initiation and maintenance begins during this developmental period. Everyday approximately 4000 adolescents try a cigarette, of which 1/3 become habitual daily smokers (Song et al., 2009). Of those smokers who try to quit, less than 5 percent are successful at any one time (Bancej et al., 2007; Diemert et al., 2013; Gray et al., 2019). E-cigarettes have been the most commonly used tobacco product among adolescents in the US since 2014 (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health., 2016). A study evaluating adolescents in 2016 found that the likelihood of ever smoking a conventional cigarette increased significantly with lifetime e-cigarette use by 2.5 times (Kim and Selya, 2020). In addition, the delivery of nicotine, the primary reinforcing component in tobacco products, is enhanced in e-cigarettes (Vollstädt-Klein et al., 2021). Furthermore, e-cigarettes may be more appealing to new smokers as an alternative to traditional smoking and encouraging non-smokers to start smoking (DiFranza et al., 2007). In 2022, more than 2.5 million U.S. middle and high school students currently used e-cigarettes (Park-Lee et al., 2022). The use of e-cigarettes has been associated with a reduced odds of smoking cessation from

either e-cigarettes or conventional tobacco products among adolescents (Glantz, 2023; Park et al., 2016). Reducing tobacco/nicotine use among adolescence and other vulnerable populations is particularly challenging, requiring a comprehensive understanding of biological, environmental, and social factors contributing to its practice.

Nicotine and the Adolescent Brain

Adolescence

Adolescence is a developmental period characterized by significant hormonal, psychosocial, and neural changes (Spear, 2000). In humans, adolescence is defined from the age of 12-18 years of age, whereas rodents, from postnatal day (P) 28-42 (Spear, 2000). Human and rodent adolescents may undergo transitions beyond this conservative age range however, the hallmark of biological, physiological and behavioral changes are conserved across mammalian species (Spear, 2016, 2013, 2000). During this developmental stage the adolescent brain undergoes structural and neurochemical maturation. For instance, synaptic connections of neural cells are overproduced, receptor levels increased, increased myelination of axons, and the crosstalk between neural circuits is immature (Spear, 2013; Yuan et al., 2015). In particular, during adolescence, an increase in modulatory neurotransmitters and glutamatergic projections to the prefrontal cortex (PFC) are observed (Del et al., 2009). Dopamine (DA) neurons from the Ventral Tegmental Area (VTA) innervate the PFC throughout adolescent development period as observed in rats and macaque brains (Kalsbeek et al., 1988; Rosenberg and Lewis, 1995). Furthermore, the maturation of the DA system is accelerated in the mesolimbic and nigrostriatal pathways, while in the mesocortical pathway is delayed in adolescence (Naneix et al., 2012). Thus, brain development during this developmental period emerges from interactions of genetic and environmental factors.

Hormonal changes are also a hallmark of the adolescence developmental period (Cross et al., 2017; Sisk and Zehr, 2005). Whereas puberty is a discrete period of sexual maturation resulting from the activation of hypothalamic-pituitary-gonadal axis, as characterized by gonadotropin-releasing hormone (GnRH) (Avendaño et al., 2017; Yuan et al., 2015), adolescence extends beyond the physical features of puberty (Yuan et al., 2015). Estradiol, progesterone and its metabolites can influence nAChRs often in opposing effects (Cross et al., 2017; Ke and Lukas, 1996; Paradiso et al., 2001, 2000; Valera et al., 1992).

Nicotinic Acetylcholine Receptors Underlie Nicotine Addiction in Adolescence.

Nicotine binds to neuronal nicotinic acetylcholine receptors (nAChRs) in the periphery and central nervous system (CNS). These receptors belong to the superfamily of cys-loop ligand-gated ion channels which includes γ-amino butyric acid (GABA), 5-hydroxytriptamine (5-HT) and glycine receptors (Changeux et al., 1998; Gotti et al., 2009; Thompson et al., 2010). nAChRs are pentameric structure composed of various subunits including alpha 2 – alpha 10 (α2-α10) and beta 2 – beta 4 (β2-β4) clustered around a central ion pore. Homomeric neuronal nAChRs are composed of α7 subunits. Heteromeric neuronal nAChRs are composed of a combination of α and β subunits i.e., α2-6, 9-10 and β2-4 . Ligands bind to the interface between α subunits in homomeric receptors, whereas they bind to the α and β interface of heteromeric receptors. Heteromeric receptor subunit composition and assembly vary, with subunit stoichiometry conferring sensitivity to ligand-initiated depolarization events, agonist and antagonist sensitivity and desensitization. The most common functional subunit combination found in the CNS with the highest affinity for nicotine is α4β2. α5 and β3 are accessory subunits as they do not participate in ligand binding, but rather they contribute to receptor binding affinity, permeability, sensitivity to allosteric modulators, upregulation sensitivity, and desensitization (Changeux et al., 1998; Engle et al., 2013;

Gotti et al., 2009; Salminen et al., 2007; Zoli et al., 2002). Of particular interest is the α6 subunit which, as expression studies have demonstrated, assembles into functional triplet receptors, including α6β3β4, α3α6β4, and α3α6β2 (Gotti et al., 2009). Other conformations have also been observed including α4α6β2β3 and α6β2β3 receptors (Exley et al., 2008).

Upon stimulation from either its endogenous ligand, acetylcholine (ACh), or exogenous nicotinic agonists and antagonists, the five subunits undergo a conformational change causing the central pore to open allowing extracellular ions to enter the cell. nAChRs can exist in three conformational states: resting, open, or desensitized. During the resting state, in the absence of a ligand, the channel is closed, blocking the movement of cations. In the presence of an agonist, such as ACh, it binds to the receptor, undergoing a conformational change and transitions to the open

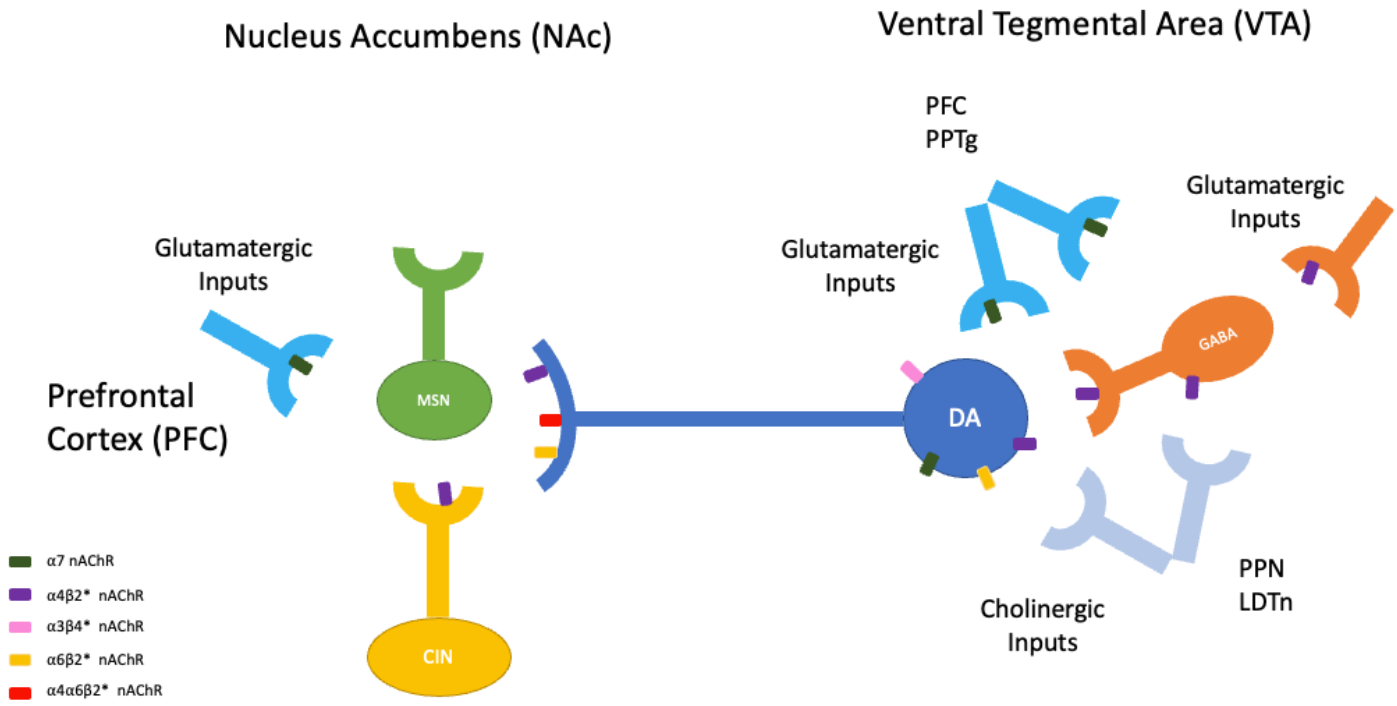

Figure 1.1 Diagram of the mesolimbic dopamine (DA) pathway and its expressions of acetylcholine receptors (nAChRs). The mesolimbic DA pathway contains neurons that originate from the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc). These neurons synapse with medium spiny neurons (MSN) in the NAc. The MSN receive glutamatergic input from the prefrontal cortex (PFC) and cholinergic input from interneurons (CIN). DA neurons in the VTA receive glutamatergic input from the PFC and pedunculopontine tegmental nucleus (PPTg), GABAergic input from GABA interneurons, and cholinergic input from the pedunculopontine (PPn) and lateraldorsal tegmental nuclei (LDTn).

state, allowing the flow of calcium (Ca^{2+}), sodium (Na^{+}) down their electrochemical gradient into the cell. The cell quickly undergoes depolarization of neurons, neurotransmitter release, or activation of downstream signaling cascades. Further, desensitization of nAChRs by ACh is thought to be inhibited by acetylcholinesterase, breaking down Ach into acetic acid and choline (Katz and Thesleff, 1957). Nicotine exerts its effects via nAChRs, with longer duration than ACh as there is no enzymatic breakdown of nicotine.

Nicotine increases the firing rate of DA neurons and increases DA release from synaptic terminals (Grenhoff et al., 1986; Pidoplichko et al., 1997; Rowell et al., 1987). The α4α6β2* (* denotes other subunits) have the highest sensitivity to nicotine and are activated in VTA (Exley et al., 2008). Studies with α-conotoxin have identified α-conotoxin MII (α-Ctx MII)-insensitive and α-Ctx MII-sensitive nAChRs. α-Ctx MII-sensitive are selectively expressed in catecholaminergic nuclei. α6β2* are located in the mesolimbic dopamine pathway on DA cell bodies in the VTA and DA projection terminals in the nucleus accumbens (NAc). The α6 and β3 subunits have been shown to combine with β2 with and without the α4 subunit suggesting these subunits contribute to nicotine-sensitive currents in DA neurons. Figure 1.2 Illustrates mesolimbic dopamine pathways and their expression of nAChRs.

Neurocircuitry of Reward

Drug addiction, including nicotine addiction, has been characterized as a chronically relapsing disorder of compulsive drug-seeking and taking which progresses through three stages: (a) binge/ intoxication, (b) withdrawal/negative affect, and (c) preoccupation/anticipation (Figure 1.2) (Koob and Volkow, 2010; Volkow et al., 2012; Volkow and Morales, 2015). One of the biggest obstacles in the treatment of drug addiction is high rates of relapse, however, much less is known about relapse among adolescents. It is important to highlight the neurocircuitry underlying

the primary reinforcing properties of nicotine and mechanisms known for nicotine-seeking behavior. Nicotine exerts its action, i.e., neurotransmitter release, by binding to nAChRs expressed throughout the CNS and the periphery. Administration of nAChR antagonists has been shown to block nicotine self-administration in rodent models (Corrigall et al., 1994, 1992). The neurocircuitry involved in mediating effects of nicotine and other drugs of abuse is a complex integration of multiple neurochemical systems. The mesolimbic DA system is considered the central system implicated in mediating the reinforcing and rewarding effects of nicotine and drugs of abuse. As mentioned previously, nAChRs are densely expressed in the VTA. (Azam and McIntosh, 2005; Brunzell et al., 2010; Cartier et al., 1996; Cui et al., 2003; Dowell et al., 2003; Kulak et al., 1997; Lebbe et al., 2014; Salminen et al., 2007; Whiteaker et al., 2000)The VTA is known to be a heterogeneous structure, containing different types of neurons, including glutamate and GABAergic neurons (Cai and Tong, 2022). Administration of nicotine increases the firing of dopamine neurons in the VTA inducing DA release in terminal regions including the NAc, basolateral amygdala (BLA), and PFC. DA release has been shown to be antagonized by concomitant intra-VTA, but not intra-NAc, infusions of the nAChR antagonist, mecamylamine (Nisell et al., 1994). Nicotine enhances DA neurotransmission more potently in the NAc shell as compared to the core (Lecca et al., 2006; Nisell et al., 1997).

Other neurochemical systems in the brain also play a role in modulating the effects of nicotine and other drugs of abuse. Such systems include the cholinergic (Lança et al., 2000), glutamatergic (Gipson et al., 2013; McGehee et al., 1995), GABAergic (Kalivas et al., 1993), serotonergic (Dao et al., 2011; Ribeiro et al., 1993), cannabinoid (Cohen et al., 2005; Dukes et al., 2020) and opioid systems (Houdi et al., 1991). Any disruption in these circuits can have lasting effects in the onset, development and maintenance of drug abuse. Disruptions in these regions

especially for the adolescent brain, can heighten the sensitivity of drug-associated cues to trigger drug-seeking (i.e., increasing incentive salience), reducing the function of the executive control system, and reducing sensitivity of brain systems in the experience of pleasure and reward. An illustration of the cycle of addiction cycle and regions implicated is in Figure 1.2.

Neurocircuitry of Cue-Induced Drug-Seeking

Human studies have revealed an increase in DA receptor occupancy in the dorsal striatum of frequent cocaine users; this increase was directly proportional to the intensity of craving experienced by the subjects as a result of cue-exposure (Volkow et al., 2008; Wong et al., 2006). In addition, fMRI data have revealed that smoking cues abnormally stimulate brain regions including anterior cingulate cortex (ACC), orbitoprefrontal cortex, amygdala and the striatum

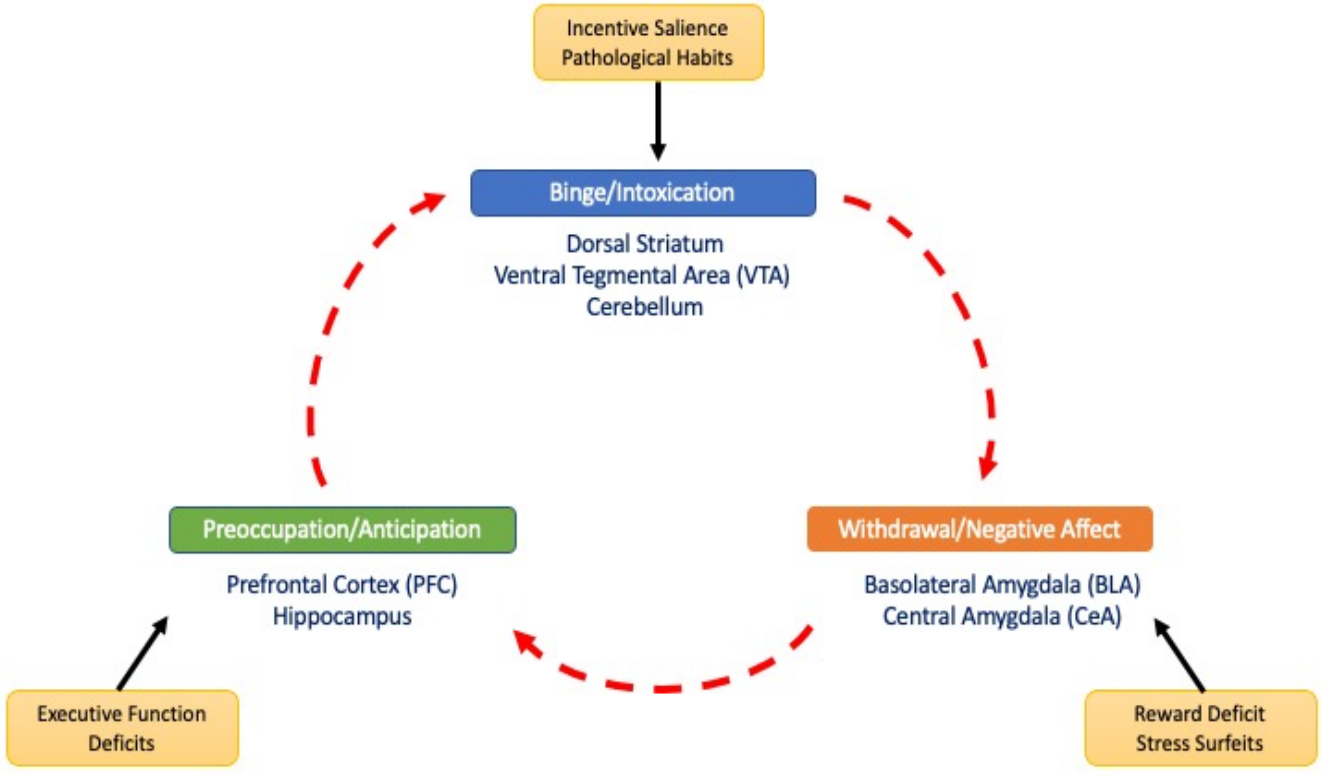

Figure 1.2 The three stages of addiction cycle and the main regions brain regions implicated. Modified from US Department of Health and Human Services General's Report on Alcohol, Drugs and Health, 2016

(Brody et al., 2007; Due et al., 2002; Franklin et al., 2011; Zhang et al., 2011). In animal studies key structures underlying conditioned reinforcement and cue-induced drug-seeking are the amygdala and the NAc and their dopaminergic innervations. The BLA and the central nucleus (Ce) of the amygdala have been shown to be involved in reward-related processes and conditioned reinforcement. Excitotoxic lesions of the insula and orbitofrontal cortex in C57BL/6J mice selectively disrupted nicotine-induced cue response to nicotine (Scott and Hiroi, 2011).

Mammalian Implication of α6 nAChR in Nicotine Addiction

Function and Distribution of α6 nAChR in the Human Brain

The *CHRNA*6 gene in humans encodes the human α6 nAChR subunit. This gene is located on human chromosome 8 (or rat chromosome 16) alongside the *CHRNB*3 gene cluster which is transcribed in opposite direction and encodes β3 nAChR subunit (Zeiger et al., 2008). Functional human α6 nAChR subunit is believed to play a role in smoking initiation, initial sensitivity to nicotine, and positive subjective effects that predict vulnerability to smoking (Culverhouse et al., 2014; Nicole R. Hoft et al., 2009; Saccone et al., 2010, 2009; Thorgeirsson et al., 2010; Zeiger et al., 2008). Genome wide association studies (GWAS) have identified a single nucleotide polymorphism in the 3'-untranslated (UTR) region of the *CHRNA*6 gene to influence adolescent substance use (Cannon et al., 2014; Nicole R. Hoft et al., 2009; Lee et al., 2012; Lotfipour et al., 2010; Pugach et al., 2017; Rigbi et al., 2008; Zeiger et al., 2008). The 'C' to 'G' single nucleotide change in the *CHRNA*6 3'-UTR SNP is located in the 123 position, a genomic region considered a molecular hotspot for pathology (Conne et al., 2000; Egervari et al., 2016). The majority of studies have associated the GG-allele to be the genotype primarily associated with enhanced tobacco/nicotine use and related problems. However, this is not always the case, suggesting that GG genotype being a risk allele may be an oversimplification. The *CHRNA*6 3'-UTR SNP has

been associated with increased likelihood and severity of prenatal tobacco exposure with the potential to alter the dopaminergic system and prime the developing brain for nicotine dependence (Lotfipour et al., 2010b; Selya et al., 2018). Furthermore, a clinical study revealed a sex-treatment interaction for the *CHRNA*6 3'-UTR genetic variant (rs230497), with a two-fold greater abstinence in the bupropion arm in males versus females by end of treatment, although the effects did not reach Bonferroni corrected significance (Lee et al., 2012). In conclusion, clinical findings provide evidence for the *CHRNA*6 3'-UTR SNP contributing to the susceptibility of tobacco/nicotine addiction and dependence.

Function and Distribution of α6 nAChR in Rodent Models.

In the mammalian brain the most prevalent nAChRs implicated in addictive properties of nicotine are those containing α4 and β2 subunits (Hendrickson et al., 2013; Picciotto et al., 1998; Tapper et al., 2004). These subunits can independently partner with α6* nAChRs subunits to make up α6β2β3 (non-α4) and α6α4β2β3 nAChRs (Changeux et al., 1998; Gotti et al., 2010; le Novère et al., 2002; Picciotto et al., 1998). α6*-containing nAChRs are localized on catecholaminergic cell body or terminal regions of catecholaminergic projections areas (Changeux et al., 1998; Gotti et al., 2007; le Novère et al., 2002; Salminen et al., 2007). α6 nAChRs subunit reaches highest mRNA expression in the VTA and substantia nigra (SNg) during adolescence (Azam et al., 2007).

Pharmacological and genetic tools have aided in understanding the role of α6 nAChR subunits in substance use disorders. For instance, α6 knockout (KO) mice exhibit no apparent neurological, or developmental alterations in the mouse visual and dopaminergic system (Pons et al., 2008). In contrast, α6 KO mice as compared with wild type mice do not self-administer nicotine even with a range of nicotine doses (Pons et al., 2008). This phenotype can be rescued using a lentiviral re-expression of α6 nAChRs directly into the VTA (Pons et al., 2008).

Furthermore, α6 KO mice have decreased nicotine-stimulated DA release (Champtiaux et al., 2003). In adult rats administration with an α6β2β3 antagonist, alpha(α)-conotoxin MII, in the NAc shell attenuated nicotine self-administration (Brunzell et al., 2010). A single point mutation in the pore-forming region of the α6 nAChRs subunit, α6*L9S, renders α6β2* nAChRs hypersensitive to nicotine, led to nicotine-associated stimulation of VTA DA neurons and substantially increased locomotor activation (Drenan et al., 2008). Recent publication of a novel *CHRNA*6 3'-UTR single nucleotide polymorphism (SNP) mutant rat line evaluated the role of nicotine induced mRNA expression, locomotor activity, anxiety-like behavior, and methamphetamine self-administration (Cardenas et al., 2022). The humanized *CHRNA*6 3'-UTR SNP (rs2304297) were generated via CRISPR/Cas9 gene editing methods in our lab as described in Cardenas et al., 2022. The 3'-UTR of the rat was replaced with the human *CHRNA*6 3'-UTR containing SNP, rs2304297. The rs2304297 is a C to G polymorphism located at nucleotide position 123 of the *CHRNA*6 3'-UTR (Cardenas et al., 2022). In brief, donor vectors and sgRNA were designed to target the 3'-UTR (95 nucleotides) of the rat *CHRNA*6 gene (GenBank accession number: NM_057184.1; Ensembl: ENSRNOG00000012283). Double stranded DNA breaks were repaired via homologous recombination. Donor vectors contained the human *CHRNA*6 3'-UTR (559 nucleotides) with either the minor SNP rs2304297 allele, C, or major SNP rs2304297allele, G, at nucleotide position 123 and replaced the rodent 3'-UTR. In addition to the human *CHRNA*63'-UTR, the vector contained left (766 bp) and right (913 bp) homology arms. The *CHRNA*6 3'-UTR knock-in does not alter baseline behaviors that have not been shown to be impacted by the hα6* nAChR subunit; however, bidirectional sex-dependent nicotine-induced behavioral effects were found (Cardenas et al., 2022). Nicotine-induced behavioral effects in humanized *CHRNA*6 3'-UTR SNP, male and female, $\alpha6^{GG}$ and $\alpha6^{CC}$, adolescents were assessed in acute (1-day) and sub-chronic (4-day)

nicotine exposure paradigm modeling nicotine initiation (Cardenas et al., 2022; McQuown et al., 2009). Sub-chronic nicotine exposure revealed an increased in locomotion in females $\alpha6^{CC}$ as compared to $\alpha6^{GG}$ females, whereas in males, nicotine increased locomotion in $\alpha6^{GG}$ rats as compared to saline-treated $\alpha6^{GG}$ rats. These results illustrate that sub-chronic, but not acute, nicotine exposure leads to sex- and genotype-dependent enhancement of locomotion in *CHRNA*6 3'-UTR SNP rats (Cardenas et al., 2022). Taken together, these studies highlight the possible involvement of α6 nAChRs reward and reinforcement of drugs of abuse.

Experimental Aims

This dissertation evaluates the role of *CHRNA*6 3'-UTR SNP in nicotine-self-administration, reward, extinction, and reinstatement behavior, sex and the impact of sex effects. Whereas numerous studies have evaluated the role of nicotine in adult male rats, much less is known about the role in female rodents or adolescents, two vulnerable at-risk populations. I evaluated male and female adolescent wild type Sprague Dawley rats in preclinical model of drug-seeking behavior (Chapter 2), subsequently evaluating nicotine-seeking behavior in our novel *CHRNA*6 3'-UTR SNP knock-in rat line. I assessed nicotine-seeking behavior in mutant male and female adolescents (Chapter 3). To understand the mechanisms involved, I assessed the neurochemical profiles in brain reward circuitry in naïve and nicotine-seeking male and female *CHRNA*6 3'-UTR SNP adolescent rats. (Chapter 4). Finally, to assess the functional impact of the *CHRNA*6 3'-UTR SNP, I tested nicotine-induced DA release in our humanized *CHRNA*6 3'-UTR SNP male and female adolescents rats, within the primary drug reward region of the brain, i.e., NAc shell (Chapter 5).

Chapter 2:

Male and Female Sprague Dawley Rats Exhibit Equivalent Natural Reward, Nicotine Self-Administration, Extinction, and Reinstatement During Adolescent-Initiated Behaviors

Introduction

Approximately 4.7 million middle and high school students use at least one tobacco product daily (Park-Lee et al., 2021; Wang et al., 2020). The primary constituent in tobacco leading to addiction is nicotine. While the majority of studies illustrate poly-tobacco use is greater among adolescent males when compared to female (Berry et al., 2019; Duan et al., 2021; Kong et al., 2017; Kowitt et al., 2013; Veliz et al., 2020), there is evidence to support equivalent rates of nicotine use in males and females (Barnett et al., 2015; Barrington-Trimis et al., 2016, 2015; Westling et al., 2017). Factors impacting these differing results may be multifold, including biological underpinnings, such as age, sex, and nicotine dose.

Preclinical studies support the role of age, sex, and nicotine dose in self-administration and reinstatement behavior (Cardenas et al., 2022; Cross et al., 2017; Leslie, 2020; Leslie et al., 2013; Ren et al., 2022; Ren and Lotfipour, 2019; Yuan et al., 2015). For example, during late adolescence (postnatal day (PN) 43-45), females exhibit enhanced nicotine self-administration at 0.005 and 0.010 mg/kg/infusion doses when compared with males (Lynch, 2009). Similarly, during adulthood, females exhibit enhanced reinforcement behavior at a 0.020 mg/kg/infusion dose (Donny et al., 2000). However, these effects do not persist at higher doses or during reinstatement behavior during late adolescence and adulthood (PN 57-74) (Donny et al., 2000; Feltenstein et al., 2012; Lynch, 2009). Further, while females in estrus have enhanced nicotine reinforcement behavior during late adolescence (PN 43-45) (Lynch, 2009), estrous cycle does not impact nicotine self-administration and/or reinstatement during late adolescence and adulthood (PN 57-74)

(Donny et al., 2000; Feltenstein et al., 2012). Whereas prior studies have examined the sex effects on nicotine acquisition, extinction, and reinstatement of nicotine self-administration behavior in older male and female adolescents and adults, assessment of nicotine-seeking in younger adolescents has only been investigated in male adolescents (Shram et al., 2008).

Rodent studies have provided substantial evidence for how adolescent as compared to adult nicotine exposure can impact drug self-administration, addiction, and drug-seeking(Cardenas and Lotfipour, 2021; Leslie, 2020; Linker et al., 2020; Mojica et al., 2014; Ren and Lotfipour, 2019). Nicotine mediates its effects via nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated ion channels widely distributed throughout the brain (Cardenas et al., 2022; Changeux et al., 1998; Gotti et al., 2009; Zoli et al., 2015). nAChRs are widely expressed during brain development and play essential maturational roles during development, including adolescence (Dwyer, J.B., McQuown, Leslie, 2009; Leslie et al., 2006). Exposure to nicotine during adolescence affects reward-related behaviors and drug-seeking learning (Mojica et al., 2014).

In the present study, sex differences were assessed between male and female adolescent rats under natural reward, nicotine self-administration under fixed ratio (FR)-5 schedule of reinforcement, progressive ratio (PR) schedule, days to extinction, and reinstatement of nicotine-seeking induced by nicotine-, cue-, and a combination of nicotine and cue (Figure 1). It has been previously shown that male adolescents and adults will reinstate nicotine-seeking behavior with drug-priming and a combination of drug and cue (Cross et al., 2020; Shaham et al., 2003). Further, no sex effects have been observed in drug-seeking behavior between adult males and females for multiple behaviors including reinstatement induced by nicotine-priming and nicotine associated cue (Feltenstein et al., 2012). Thus, based on these studies, we hypothesize no sex effects will be

observed in our adolescent rats when tested on natural food-reward, acquisition, and progressive ratio schedules of self-administration, days of extinction, and reinstatement condition. Our findings add to the literature by providing a feasible approach for assessing nicotine seeking in male and female Sprague Dawley rats when behavior is initiated during adolescence.

Methods

Animals

Male and female Sprague Dawley rats (Charles Rivers) were bred in-house. Animals were aged matched based on Spear (2000). Upon weaning, PN 21, animals were transferred out of the mother's cage and separated by sex and group-housed throughout the experiment. All rats were maintained on a 12-hr light/dark cycle (lights on at 07:00 am). All experimental procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Animals were handled for 2 minutes daily before testing began. No more than one animal per litter per experimental group was used to avoid potential confounds. Rats were minimally food-restricted beginning two days prior to operant conditioning to promote exploration of the operant chamber. Adolescent rats were fed 15-25 g of food to maintain normal growth during self-administration testing (Mojica et al., 2014). Animal

weights based on postnatal day are represented in Figure 2.1. Attrition rates for animals in our current study are shown in Table 2.1.

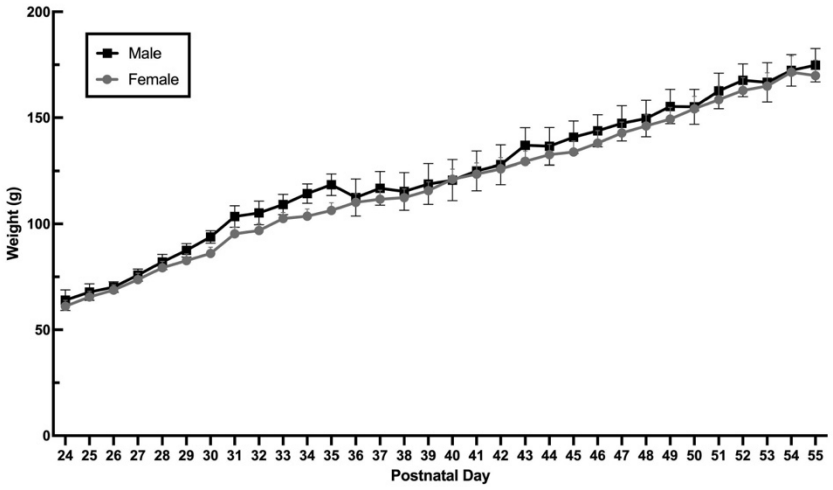

Figure 2.1 Male and Female adolescent Sprague Dawley weights across postnatal ages. No sex differences for body weight across postnatal day. Mean ± SEM body weight from PN 24 to PN 55.

Table 2-1 Attrition rates for rats included in this study.

Condition	No. Animals
Total No. of Animals	70 (34F; 36M)
Failed Food Self-Administration	18 (6F; 12M)
Surgery Complications	6 (2F; 4M)
Failed NIC IVSA	24 (14F; 10M)
Reinstatement Outliers (Box and Whisker Plot)	5 (1F; 4M)
Completed Study	17 (11F; 6M)

Estrous Cycle Assessment

Given that estrous cycle has limited impacts on reinforcement during late adolescence (PN 43-45), a lack of effect on reinstatement during adulthood, and can be a stressor, we chose not to administer vaginal swabs in the current study to assess estrous cycle (Donny et al., 2000; Feltenstein et al., 2012; Lynch, 2009).

Drugs

Nicotine tartrate (Glentham Life Sciences, Corsham, Wiltshire, UK) was calculated as a base, dissolved in saline, with a final pH 7.2-7.4. Pentobarbital (Sigma Aldrich, St. Louis, MO, USA) was dissolved in saline, propylene glycol, and ethanol to make Nembutal. Nembutal was further diluted to make Equithesin. Carprofen (Zoetis, Parsippany, NJ, USA) was diluted in saline. Nicotine, Equithesin, and carprofen were filtered via 0.22 μm sterile filters (VWR, Radnor, PA, USA). Propofol (Medvet, Mettawa, IL, USA) in a 5 mg/kg, intravenous (i.v.) was administered to test for catheter patency.

Behavioral studies

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers (Figure 1). The required number of responses at the reinforced (Reinf) lever turned on a cue light over the lever, turned off the house light, and activated an externally mounted syringe pump that infused drug. During the infusion (5.6 s yielding 100 μl of solution) and timeout period (20 s) the cue light remained illuminated, and the house light remained off. After the timeout period, the house light turned on and signaled the availability of a reinforcer. Responses on the non-reinforced (NonReinf) lever were recorded but had no consequences. Procedures modeled previous work (Cross et al., 2020; Gellner et al., 2016).

Food self-administration

To facilitate learning, male, and female adolescents (PN 24) were trained twice per day in a 30 min session to lever press for food pellets (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) in a lever pressing operant testing chambers (Med Associates, St. Albans, VT) based on previous studies (Costello et al., 2014; Cross et al., 2020). One wall of the chamber contained two levers, a cue light over each, and a house light. The right lever was assigned as the active (Reinf) lever, each response at which was rewarded with delivery of food. The left lever was inactive (NonReinf) had no consequences but was recorded as a measure of nonspecific activity. The animals started at an FR1TO1 (fixed-ratio 1, 1s timeout) schedule of reinforcement, followed by FR1TO10, FR2TO20 and finally FR5TO20, progressing upon earning 35 reinforcers.

Surgery

Following successful acquisition of food training, rats were anesthetized with Equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al., 2005; Cardenas et al., 2021; Cardenas and Lotfipour, 2021). After surgery, rats were given the analgesic carprofen (5 mg/kg, subcutaneous). During the 2-3-day recovery period, catheters were flushed daily with heparinized saline solution (1 ml of 1000 units/ml heparin into 30 ml of bacteriostatic saline) to maintain patency. Catheter patency was tested for rapid (5-10 s) anesthesia by infusing propofol (5 mg/kg, i.v.) before and after the completion of self-administration experiments. Only animals showing rapid anesthesia were included in analyses.

Nicotine self-administration and extinction

Animals (PN 34) initiated intravenous self-administered (IVSA) of nicotine (0.015 mg/kg/infusion) at an FR5 schedule for 1-h daily session based on a prior study (Cross et al., 2020). Nicotine IVSA was for a minimum of 5 days until animals reached stable responding or day 10

and were patent. Stable responding is defined as having reinforced responses be within ±20% of the mean of the last 3-days of nicotine IVSA, with reinforcers needing to be a minimum of 5 and two-times greater or equal to non-reinforcers. Using this paradigm, all animals met criteria for nicotine IVSA. A dose of 0.015 mg/kg/infusion was chosen based on previous adult and adolescent studies (Cross et al., 2020; Gellner et al., 2016). Baseline responding was defined as the average reinforced responses over the last three days of self-administration. Rats were then allowed to respond to at the dose of 0.015 mg/kg/infusion on Progressive Ratio (PR) schedule. The PR schedule of reinforcement is a measure of motivation to obtain the drug (Richardson and Roberts, 1996). The sequence was determined using the exponential formula (5 exp (0.2 x infusion number)-5) such that the required responses per infusion were as followed: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492 (Richardson and Roberts, 1996). PR conditions were the same for FR sessions, with the exception that the sessions were 4-h duration. Breakpoint was achieved when >20 min of inactivity on the active lever elapsed. After reaching stable responding and two-day of PR schedule (~PN 39), extinction-reinstatement testing began.

During extinction (~PN 41), animals were placed in the same operant testing chambers; the animals were not connected to the infusion tubing, the house light remained on and responses on the levers were counted but had no consequences. Extinction sessions were 1-h per day for a minimum of 5 days, or until responding was reduced to 25% of baseline.

Cue and nicotine-induced reinstatement

After meeting extinction criteria, reinstatement testing began (~PN 47). Nicotine-seeking was reinstated using three reinstatement conditions given in a within-subjects counterbalanced design: cue-, nicotine-primed alone, and nicotine-primed paired with cue. Presentation of cue

consisted of cue light illumination and sound in the testing chamber. Nicotine-prime injections contained 0.15 mg/kg nicotine and was administered intraperitoneally immediately before the reinstatement test. The nicotine-prime dose was chosen based on previous work (Cross et al., 2020). Between reinstatement tests, animals were returned to extinction condition for a minimum of two days, or until extinction criteria were met. Reinstatement was defined as a significant increase in responding from the last day of extinction.

Data analysis

Data were analyzed using JMP (SAS Institute) software. Food acquisition was analyzed by a compound 3-way multivariate ANOVA for lever presses x sex (male and female) x FR schedule (FR1TO1, FR1TO10, FR2TO20, and FR5TO20) with repeated measures on lever presses and FR schedule, with Bonferroni corrected t-test post hoc comparisons. Nicotine self-administration data were analyzed by a compound 3-way multivariate ANOVA for Reinf/NonReinf Responses x ex (male and female) x day (day 1-5) with repeated measures on Reinf/NonReinf Responses and day. Reinstatement data were analyzed as reinforced responding. Mean responses for reinstatement condition were analyzed by a 2-way multivariate ANOVA for sex x reinstatement condition (cue only, nicotine only, and nicotine plus cue), with repeated measure on reinstatement condition. Significant main effects were further analyzed with Bonferroni-corrected paired or unpaired.

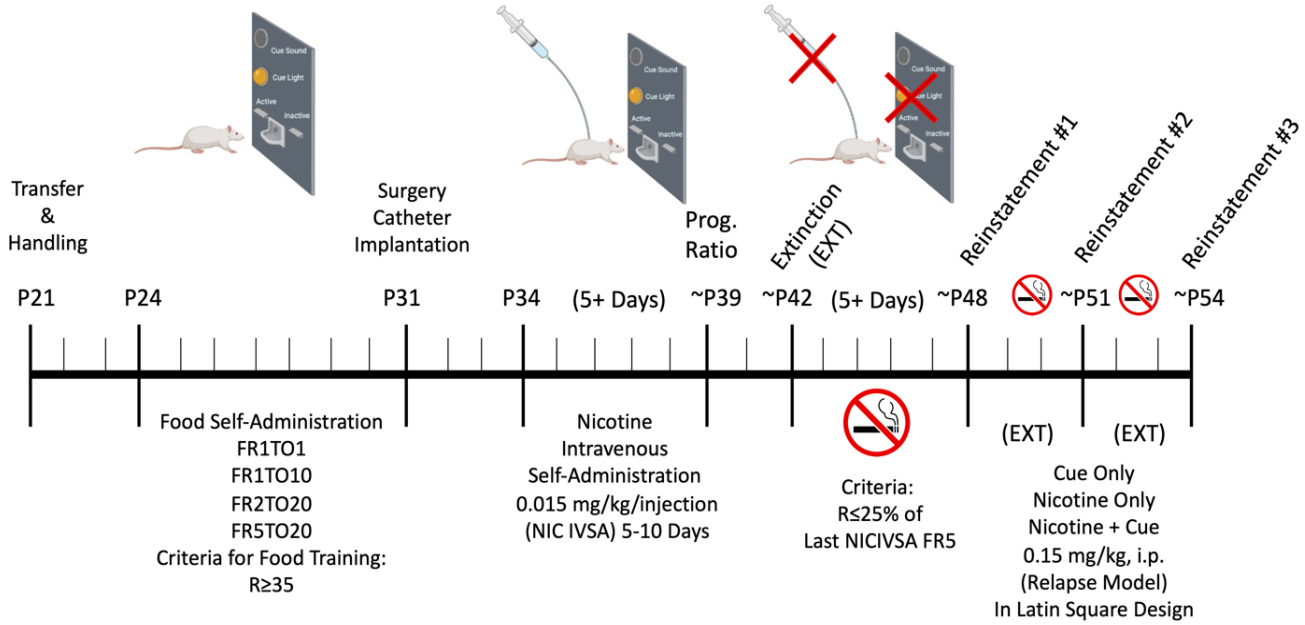

Figure 2.2 Timeline of behavioral experiment. Adolescent male and female underwent food training, catheter implantation, nicotine self-administration, progressive ratio, extinction, and cue-, nicotine-, and nicotine + cue-induced reinstatement of nicotine.

Results

Food self-administration

Male and female adolescent rats were trained to perform an instrumental lever-press response for food reward. Main effects were found for Reinf/NonReinf Response [$F(1,15)=347.73$, $p<0.0001$] and FR Schedule of Reinforcement [$F(3,45)=26.9258$, $p<0.0001$], with interaction effects of Reinf/NonReinf Responses x FR Schedule of Reinforcement [$F(3,45)=26.2187$, $p<0.0001$]. As we observed no sex-dependent effects, results were combined across sex. Figure 2.3 shows the mean lever-pressing for food reinforcement food reinforced versus nonreinforcement as a function of schedule of reinforcement, separated by sex for clarity. Thus, our results illustrate that males and females equally learned to distinguish between Reinf and

NonReinf lever responding across escalating fixed ratio schedules of reinforcement for natural food rewards ($p<0.0001$).

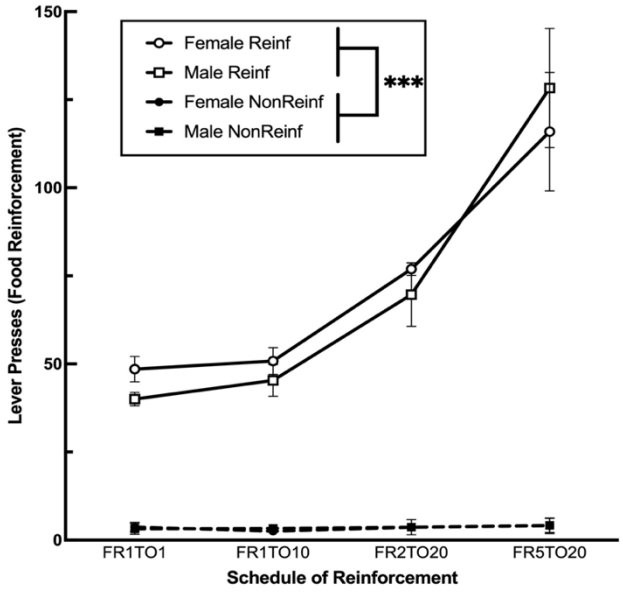

Figure 2.3 No sex differences for food acquisition. Mean ±SEM number of lever presses on the reinforced (Reinf) and nonreinforced (NonReinf) earned to meet criteria during FR1TO1, FR1TO10, FR2TO20, and FR5TO20. ***$p<0.001$ Reinf vs NonReinf lever. N=17.

Nicotine self-administration, progressive ratio, and extinction

Both male and female rats achieved stable nicotine self-administration. Main effects for Reinf/NonReinf Response [$F(1,15)=50.918$, $p<0.0001$], with an interaction effects of Reinf/NonReinf Responses x Days [$F(4,12)=4.9924$, $p<0.0133$] were found. Since no sex differences were found, male and female data were combined for analysis (Figure 2.4a). No sex effects were observed for days to stabilize for nicotine self-administration (i.e., ~6 days, data not shown), total nicotine intake (Figure 2.4b) or breakpoint values (Figure 2.4c). Following stable self-administration, drug-seeking behavior was extinguished by removal of drug and associated

cue. All animals significantly reduced their responding on the reinforced lever beginning on Day 1 ($p<0.05$) and continued throughout extinction. No sex differences were observed for days to meet extinction criteria as shown in Figure 2.4d, males and females showing equivalent 25% or less last day of extinction responding with an average of 6 days (Figure 2.4d).

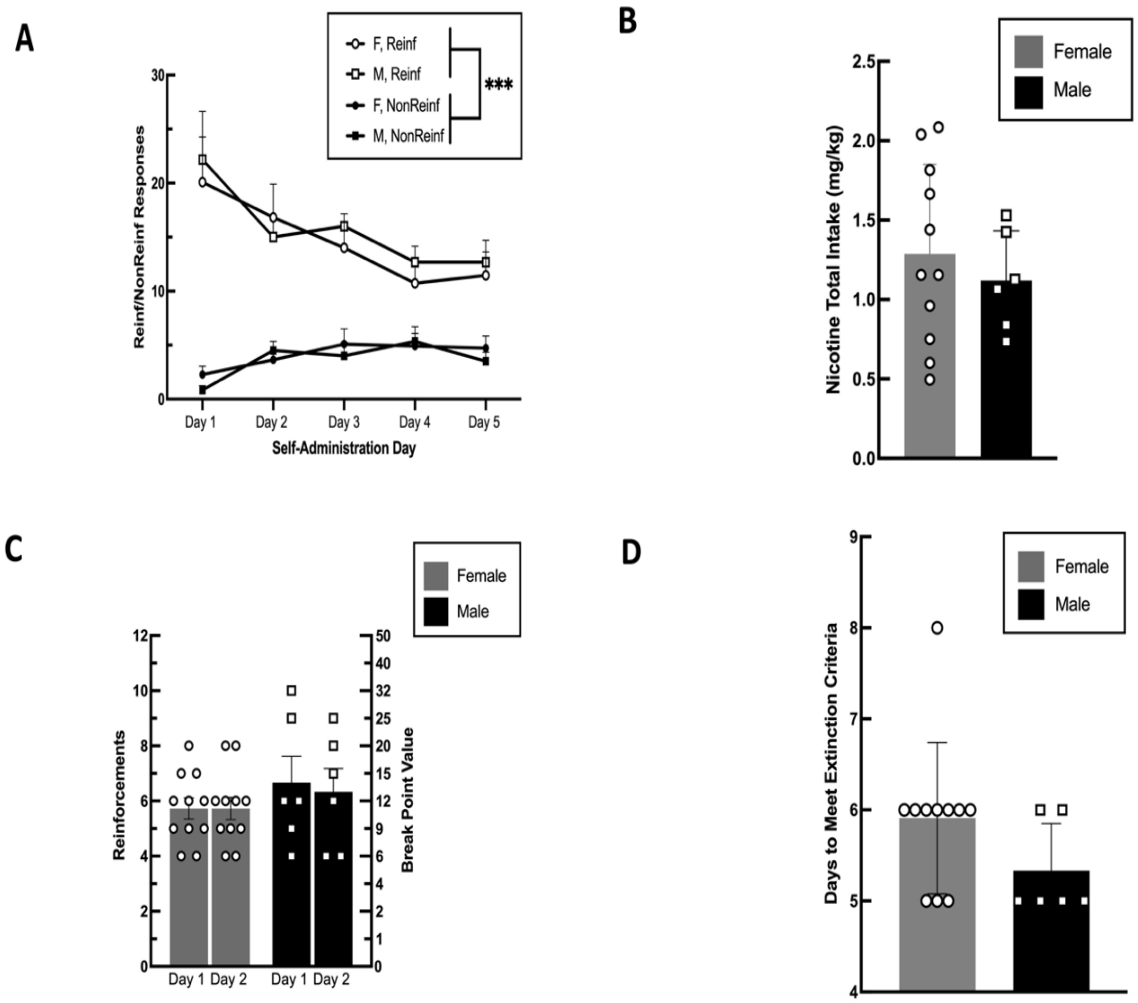

Figure 2.4 No sex difference in nicotine self-administration, nicotine intake, breakpoint for progressive ratio, or extinction. A. The lines represent the mean ± SEM responses leading to an infusion (Reinf) of nicotine (open circles) and nonreinforced (NonReinf) closed circles. Data are separated by sex to illustrate the lack of sex effect for nicotine IVSA. ***p<0.001 Reinf vs NonReinf responses. N=17. B. No sex effects observed for total nicotine intake. The bar graph represents the total number ± SEM of nicotine intake in mg/kg separated by sex for clarity. N=17. C. No sex effects for progressive ratio schedules of reinforcement for nicotine-self-administration break point values. The bar graph represents the number of responses and the equivalent ratio for breakpoint for day 1 and day 2 of testing. N=17. D. No sex effects on the number of days to reach extinction criteria. The bar graph represents the days to meet extinction criteria ± SEM; separated by sex for clarity. N=17.

Reinstatement

Following extinction, animals were triggered to reinstate drug-seeking behavior with cue, nicotine-priming, and the combination of nicotine and cue. Main effects of reinstatement stimuli

were found [F(3,13)=8.4854, p=0.0022]. Since no sex differences were found, data were collapsed by sex. All three-reinstatement stimuli reinstated drug-seeking behavior in male and female adolescent rats. Nicotine + cue induced significantly greater responding than either stimulus alone (Figure 2.5).

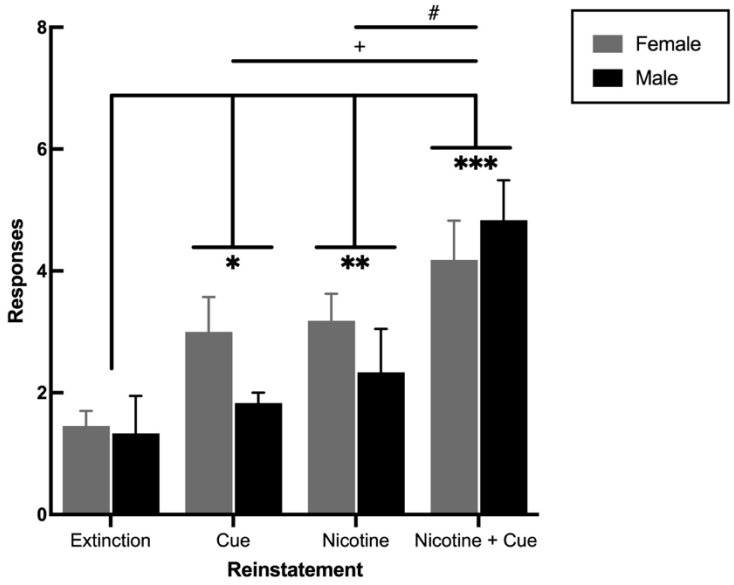

Figure 2.5 No sex effects observed for cue-, nicotine-, and nicotine plus cue-primed reinstatement. Nicotine plus cue-primed reinstatement is greater than nicotine, cue and extinction behavior. Extinction behavior is lower than all other groups. The bar graph represents the mean ± SEM responding that would have led to an infusion if drug was present for adolescent male and females Sprague Dawley rats. ***p<0.001 vs Extinction, **p<0.01 vs Extinction, *p< 0.05 vs Extinction, #p<0.05 vs Nicotine-primed, +p<0.05 vs Cue-primed; data collapsed by sex. N=17.

Discussion

We adapted a preclinical paradigm to assess natural and drug reward self-administration, extinction, and reinstatement in male and female rats that initiated behavior during adolescence. Whereas prior studies illustrated adult male rats that self-administered nicotine required the presentation of cue to reinstate after nicotine-priming (Cross et al., 2020), we now provide

evidence that younger male and female rats will reinstate nicotine-seeking behavior with cue-, nicotine-, and combination of nicotine-primed and cue. Our results illustrate that male and female Sprague Dawley rats exhibit similar natural and drug self-administration, extinction, and reinstatement of drug-seeking behavior when behaviors were initiated during adolescence. These effects were not impacted by the age of males and females across nicotine self-administration, progressive ratio, extinction, and reinstatement (Figure 2.6).

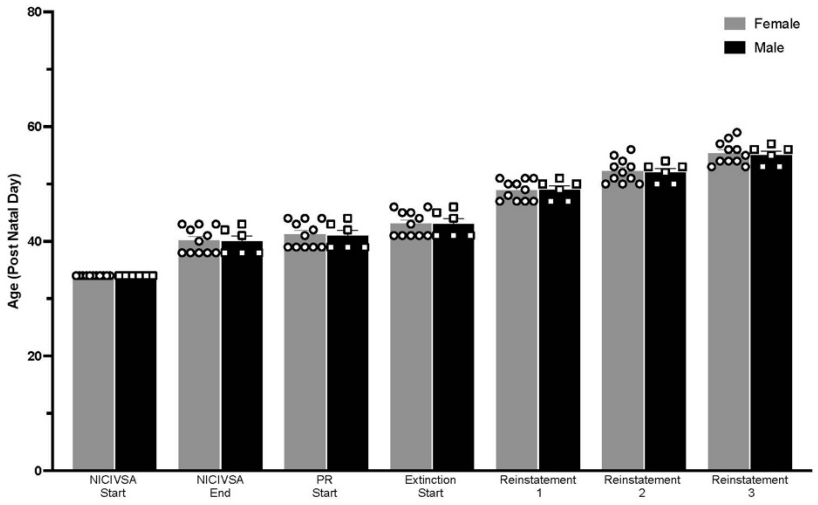

Figure 2.6 Postnatal day across nicotine self-administration, extinction, and reinstatement. No sex differences for the age of initiation of behavior for nicotine self-administration, progressive ratio, extinction, and reinstatement. Mean ± SEM postnatal across study conditions.

Food self-administration

In our studies, male and female adolescents (PN 24-30) equally learned to lever press for a natural reward independent of sex. These results are equivalent to our recent manuscript that illustrate similar food reinforcement in male and female Sprague Dawley rats that have a functional human 3'-UTR genetic polymorphism (Cardenas et al., 2022). The majority of the prior studies evaluating food reinforcement during different time points during adolescence have only used males (Gellner et al., 2016; Levin et al., 2007; Mojica et al., 2014; Shram et al., 2008) or females (Levin et al., 2003). The studies are unique as adolescence represents a developmental window

vulnerable to a number of psychiatric disorders including avoidant/restrictive food intake disorders and substance use. The lack of sex dependent effects on a natural food reinforcement in our studies highlights that males and females are equally able to learn natural rewards and find food equally reinforcing during this age group.

Nicotine self-administration and progressive ratio schedules

Our current findings demonstrate that both, male and female, adolescents (PN 34) self-administer nicotine at a dose of 0.015 mg/kg on a fixed and progressive ratio schedule of reinforcement in a similar manner. In support of our findings, previous studies have shown no sex differences for nicotine IVSA in male and female adolescent rats (PN 43-55) for 23-hr under fixed-ratio 1 for 10-days (Chen et al., 2007). Similarly, no sex differences were observed for adolescents (PN 30) allowed to nose-poke to receive nicotine IVSA of varying doses (0.003 – 0.1 mg/kg) during 1-hr sessions for 16-days (Schassburger et al., 2016). Using different methodologies to our current paradigm or those described above, sex effects for adolescent nicotine self-administration have been observed. For example, male and female adolescents beginning at PN 30-45 tested on an extended 23-h paradigm where nicotine infusions were available under fixed-ratio 1 and progressive ratio schedules of reinforcement illustrate a greater percentage of adolescent females acquire nicotine IVSA than males at the dose of 0.005 mg/kg (Lynch, 2009). However, similar rates for acquisition were observed at doses of 0.005 and 0.010 mg/kg, suggesting methodological and dose-dependent effects interact with sex to impact reinforcement during adolescence. In separate studies, it appears that males are more susceptible than female adolescents', as males initially exhibited higher nicotine self-administration and decreased as they aged; however, high nicotine self-administration persists in females as they reach adult ages (Levin et al., 2007, 2003). Nevertheless, these studies were done in separate years and not collectively, thus, direct sex

comparisons cannot be made. Our studies assessed male and female adolescents at a dose of 0.015 mg/kg/infusion collectively and observed no sex differences for nicotine IVSA. The sex dependent effects on reinforcement are likely mediated through a dose and/or paradigm difference.

Whereas our results suggest that male and female Sprague Dawley adolescent rats have equivalent nicotine self-administration and reward response at the 0.015 mg/kg/injection dose, more research is needed to understand how nicotine dose and sex contributes to nicotine-modulated behaviors using differing paradigms, ages, strains, and genotypes.

Extinction

Our current results illustrate that male and female Sprague Dawley rats have equivalent extinction learning, with a significant reduction of responding across sex at nicotine-paired lever pressing across time. While we observed no sex differences between adolescent males and females for the number of days required to meet extinction criteria or responding on the last day of extinction, prior research has shown that older adolescent and young adult (PN 70-85) females exhibit greater resistance to extinction when compared to males (Feltenstein et al., 2012; Tan et al., 2021). Whether these effects are related to age or methodological differences used need to be further evaluated. No other studies that we are aware of have tested sex-dependent differences during extinction learning after nicotine self-administration. Future studies are needed to determine at what age sex-dependent differences appear for extinction behavior and the mechanisms involved.

Reinstatement

Our studies illustrate that male and female adolescent rats exhibit significant cue-, nicotine-, and combined nicotine and cue-induced reinstatement and these findings are independent of sex. This finding in adolescents is consistent with those of with Feltenstein et al. (*2012*) who found no

sex differences in adult rats when reinstatement was initiated with nicotine-paired cue, nicotine alone, and pairing nicotine and cue. Similar to our studies, combination of nicotine and cue substantially enhanced nicotine-seeking behavior in adult males and females (Feltenstein et al., 2012). Very few studies have evaluated adolescent sex differences in cue-, drug, and combination of drug and cue. It has been shown that nicotine enhances the saliency of cue and other drugs of abuse in both adolescents and adults (Cardenas et al., 2021; Cardenas and Lotfipour, 2021; Mojica et al., 2014). Studies on the neuronal mechanisms underlying drug- and cue-induced reinstatement have implicated the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), dorsal striatum (DS), central nucleus of the amygdala (CeA), the bed of nucleus of the stria terminalis (BNST), the ventral pallidum (VP), and the ventral tegmental area (VTA). Glutamatergic mechanisms in the NAc have been implicated in the vulnerability to increase nicotine-seeking behavior in adult male rats as assessed by cue-induced reinstatement (Gipson et al., 2013). Inactivation of the mPFC attenuates drug- and cue-induced reinstatement for drug seeking, suggesting this region of the brain is critical for this behavior (Shaham et al., 2003). The VP has also been implicated in drug reinstatement providing evidence that this brain region can additionally regulate the neural circuits of drug seeking behavior (Farrell et al., 2019). Future studies aim to evaluate neuronal activation within these brain regions during drug seeking behavior in adolescent male and female rats to determine underlying mechanisms. Overall, our findings demonstrate the feasibility to evaluate reinstatement of nicotine and other drugs of abuse during the adolescent developmental period. The current study highlights the need for future research on potential sex- and genotype differences of nicotine-seeking behavior.

Limitations

Sexual maturation has been shown to occur sooner in adolescent females as compared to males in rodents by approximately 10 days (Sengupta, 2013; Spear, 2000; Yuan et al., 2015). Our current studies have not aged-matched based on sexual maturation, which could be a limitation of the work. Further, since studies have not extensively compared strain differences, this is still unknown, and a minor limitation in the work reviewed.

Conclusion

Taken together, our data demonstrate equivalent male and female behavior in the acquisition of natural rewards and nicotine seeking behavior using a reinstatement model in male and female adolescent rats. Our results suggest that adolescent males and females may be at equal risk for the addictive effects of nicotine exposure, assessed by acquisition and progressive schedules of self-administration, days to extinction, and reinstatement condition. How such factors change across age to induce the known sex-dependent effects appear during adulthood need to be studied further. Indeed, our laboratory has recently shown that genetic mechanisms in the human 3'-UTR of the α6 nAChR subunit can impact adolescent sub-chronic nicotine pretreatment effects on locomotor and anxiety related behaviors at higher doses than those used in our current studies (0.030 mg/kg/injection x 2 across four days) in a genotype- and sex-specific manner (Cardenas et al., 2022). Thus, a number of factors may be involved in whether sex-dependent effects are observed during adolescence. This study adds to the literature by indicating the importance of equivalent factors impacting nicotine seeking behavior in male and female adolescent rats. Future studies are needed to evaluate the biological mechanisms involved.

Chapter 3:

Sex- and Genotype-dependent Nicotine Plus Cue-primed Reinstatement is Enhanced in Adolescent Sprague Dawley Rats Containing the Human *CHRNA*6 3'-UTR Polymorphism (rs2304297)

Introduction

In recent years the prevalence of adolescent electronic nicotine use has dramatically increased (Miech et al., 2022; Wang et al., 2019). Adolescent electronic nicotine use remains a public health concern given that its use can progress into combustible cigarette smoking and conditioned the developing brain for addiction to other drugs of abuse (Creamer et al., 2021; Ren and Lotfipour, 2019; Soneji et al., 2017a; Yuan et al., 2015). Young people are highly sensitive to nicotine, exhibiting symptoms of dependence soon after smoking initiation and before the start of daily smoking (DiFranza et al., 2007). Further, electronic cigarette (e-cigarettes) cessation interest has increased among adolescents. In 2019 an estimated 57.5% middle and high school (about 3.5 million) students using tobacco/nicotine made an attempt to quit (Wang TW et al., 2019). The prevalence of unsuccessful quit attempts among middle and high school students who had either used e-cigarettes or cigarettes, was higher in 2020 than previous years (Medical Association, 2022).

Nicotine is the principal reinforcing constituent in tobacco products responsible for drug seeking and addiction. Nicotine directly activates neuronal nicotinic acetylcholine receptors (nAChR), which are ligand-gated ion channels consisting of five membrane-spanning subunits located on several neurotransmitter systems in the brain, most notably dopaminergic neurons (le Novère et al., 2002). In the mammalian brain the predominant and most prevalent nAChRs implicated in the addictive properties of nicotine are those containing α4 and β2 subunits

(Corrigal et al., 1991; Picciotto et al., 1998; Tapper et al., 2004). These subunits can independently partner with α6* nAChR subunits to makeup α6β2β3 (non-α4) and α6α4β2β3 nAChRs (Champtiaux et al., 2003; Changeux et al., 1998; Gotti et al., 2010; le Novère et al., 2002; Picciotto et al., 1998; Salminen et al., 2007). α6* nAChRs are largely localize in expression on the cell body or terminal regions of dopaminergic neurons and act as critical modulators of dopamine release in reward regions of the brain (Changeux et al., 1998; Gotti et al., 2007; le Novère et al., 2002; Salminen et al., 2007). α6* nAChRs reach maximum mRNA expression in the ventral tegmental area (VTA) and substantia nigra (SNg) during adolescence (Azam et al., 2007). α6 knockout (KO) mice do not self-administer nicotine even with an extensive range of doses, an effect which can be rescued using lentiviral re-expression of α6 nAChR subunits directly within the VTA (Pons et al., 2008). Further, administration of alpha(α)-conotoxin MII, an α6β2β3 antagonist, into the nucleus accumbens shell attenuate nicotine self-administration in rats (Brunzell et al., 2010). These studies suggest the α6* nAChRs in the VTA and/or its projections e.g., nucleus accumbens and prefrontal cortex, are necessary and sufficient to establish nicotine self-administration. Evidence also demonstrates that a selective α6 nAChRs antagonist, bPiDDB, is able to decrease nicotine-induced reinstatement in nicotine self-administering rats (Dwoskin et al., 2009). Drug paired cue are likely important factors in such behavioral effects, as intra-VTA infusion of an α6 nAChRs antagonist, α-conotoxin MII, blocks the rewarding effects of cue paired with a drug reinforcer (Brunzell, 2012; Löf et al., 2007). Such studies are important as cue and drug-induced reinstatement is a preclinical model of drug-seeking behavior (See reviews (Chiamulera, 2005; Epstein et al., 2006; Shaham et al., 2003)) and highlight the importance of α6* nAChRs critical involvement.

Clinically, a large body of literature highlights that a single nucleotide polymorphism (SNP) in the α6 nAChR subunit (encoded by the *CHRNA*6 gene) is associated with nicotine/tobacco use and related problems (Cannon et al., 2014; Ehringer et al., 2010; Fletcher, 2012; Nicole R. Hoft et al., 2009; N. R. Hoft et al., 2009; Lee et al., 2012; Lotfipour et al., 2010; Pedneault et al., 2014; Pugach et al., 2017; Rigbi et al., 2008; Thorgeirsson et al., 2010; Zeiger et al., 2008). The human *CHRNA*6 SNP is located in the 3'-untranslated region (UTR), a genomic region known to regulate mRNA stability, localization, and translation (Mayr, 2019; Mayya and Duchaine, 2019). The α6 subunit nAChR subunit SNP has been associated with increased cigarette smoking and drug experimentation during adolescence with $\alpha6^{GG}$- more impacted than $\alpha6^{CC}$-allele carriers (Lotfipour et al., 2010). Additionally, a clinical study revealed a sex-treatment interaction for rs230497, with a two-fold greater abstinence in the bupropion arm in males versus females by end of treatment, although not surpassing Bonferroni corrections (Lee et al., 2012). Sex heterogeneity has been observed as a critical factor to be considered with α6 nAChRs, the *CHRNA*6 gene and the *CHRNA*6 3′-UTR SNP in nicotine addiction and other neurological diseases (Bureau et al., 2019; Lee et al., 2012; Moen et al., 2021; Moen and Lee, 2021; Wieskopf et al., 2015). The *CHRNA*6 3′-UTR SNP has been associated with nicotine dependence in males (Wen et al., 2017). Further, other *CHRNA*6 SNPs in general show greater nicotine dependence associations in males than females (Bureau et al., 2019). As such, clinical findings provide evidence for the *CHRNA*6 3'-UTR SNP contributing to the susceptibility of tobacco/nicotine dependence with the strong likelihood of sex-dependent effects. Our prior pre-clinical data support this hypothesis (Cardenas et al., 2022).

Mechanisms underlying how the *CHRNA*6 3'-UTR mediates adolescent substance use are not known. To investigate the human *CHRNA*6 3'-UTR SNP *in vivo*, our lab generated a novel,

humanized rodent line. Our lab replaced the entire *CHRNA*6 3'-UTR of the rat line with the human *CHRNA*6 3'-UTR, generating a translational model of α6 nAChRs (Cardenas et al., 2022). Our laboratories recent results using this novel rat line confirm *in vivo* functionality of the *CHRNA*6 3'-UTR SNP, with genotype- and sex-dependent effects observed on adolescent nicotine-induced behaviors (Cardenas et al., 2022). The aim of our current study is to assess sex- and genotype-dependent effects on the influence of nicotine self-administration, progressive ratio, extinction and reinstatement of nicotine-seeking behavior in adolescent males and females containing the humanized *CHRNA*6 3'-UTR SNP.

Methods

Generation of Human *CHRNA*6 3'-UTR SNP Rodents

Human *CHRNA*6 3'-UTR SNP knock-in rats were designed and created with CRISPR/Cas9 gene editing techniques by Cyagen Biosciences as described in Cardenas et al. 2022 (Cardenas et al., 2022). Briefly, donor vectors and sgRNA were designed to target the 3'-UTR of the rat *CHRNA*6 gene (GenBank accession number: NM_057184.1; Ensembl: ENSRNOG00000012283). Donor vectors contained the human *CHRNA*6 3'-UTR (559 nucleotides) with either the minor SNP rs2304297 allele, C, or major SNP rs2304297 allele, G, at nucleotide position 123 and replaced the rat 3'-UTR (95 nucleotides).

Animals

Male and female wild type (WT) Sprague-Dawley rats were purchased from Charles river and bred in house with human *CHRNA*6 3'-UTR^{C123G} SNP rats. Upon weaning at postnatal day (PN) 21, animals were transferred out of the mother's cage and separated by sex (Figure 3.1). All animals were handled for 3-days prior to experimentation and group-housed throughout the experiment. All rats were maintained on a 12-hr light/dark cycle (lights on at 07:00 am). All

experimental procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Animals were handled for 2 min daily before testing began. No more than one animal per litter per experimental group was used to avoid potential confounds. Rats were minimally food-restricted beginning two days prior to operant conditioning to increase the motivation to learn the self-administration paradigm similar to our prior study (Carreño and Lotfipour, 2022). Grouped-housed (2 per cage) adolescent rats were fed 15-25 g of food to maintain normal growth during self-administration testing (Mojica et al., 2014). Animal weights based on postnatal days are presented in Figure 3.2, with postnatal day across condition highlighted in Figure 3.3. Attrition rates for animals in our current study are shown in Table 3-1. Similar to our previous wild type Sprague Dawley nicotine reinstatement studies in adolescent rats (Carreño and Lotfipour, 2022) we did not administer vaginal swabs in the present study to assess estrous cycle (Donny et al., 2000; Feltenstein et al., 2012; Lynch, 2009). The age range of adolescence was defined based on prior studies of Spear (Spear, 2000).

Table 3-1 Attrition Rates for *CHRNA*6 3'-UTR SNP rats in this study.

Condition	$a6^{CC}$ F	$a6^{GG}$ F	$a6^{CC}$ M	$a6^{GG}$ M
Food Reinforcement			1	2
Surgery Complication	5	4	2	4
NICIVSA	10	8	14	9
Reinstatement Outliers	2	1	2	3
Reinstatement Testing (Final N)	10	14	11	8

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers. The required number of responses at the reinforced (Reinf)

lever turned on a cue light over the lever, turned off the house light, and activated an externally mounted syringe pump that infused drug. During the infusion (5.6 s yielding 100 µl of solution) and timeout period (20 s) the cue light remained illuminated, and the house light remained off. After the timeout period, the house light turned on and signaled the availability of a reinforcer. Responses on the non-reinforced (NonReinf) lever were recorded but had no consequences. Procedures modeled previous work (Cross et al., 2020; Gellner et al., 2016).

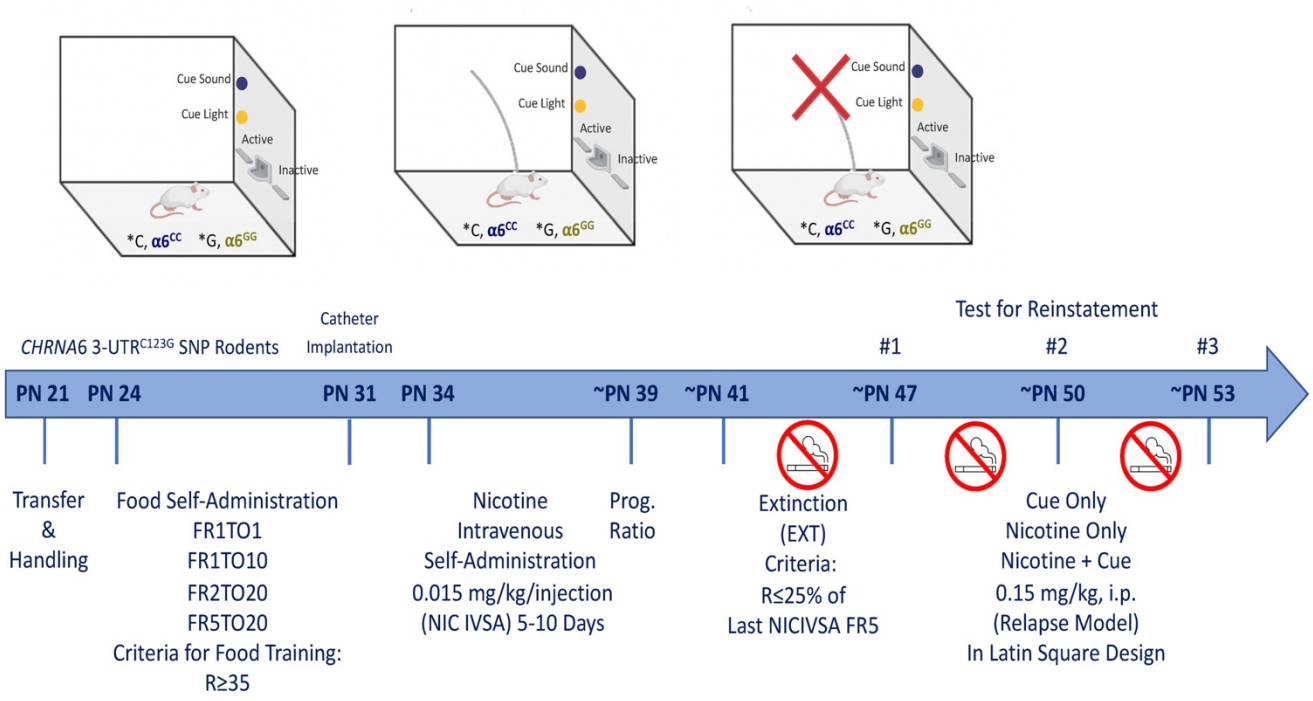

Figure 3.1 Experimental paradigm. Male and female *CHRNA6* 3'-UTR knock-in underwent food self-administration, catheter implantation, nicotine self-administration, progressive ratio, extinction, and cue-, nicotine-, and nicotine + cue reinstatement.

Food Self-Administration

To facilitate learning, male, and female adolescents (PN 24) were trained twice per day in a 30 min session to lever press for food pellets (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) in a lever pressing operant testing chambers (Med Associates, St. Albans, VT) based on previous studies (Costello et al., 2014; Cross et al., 2020) (Figure 3.1). One wall of the chamber contained two levers, a cue light over each, and a house light. The right lever was assigned as the active (Reinf) lever, each response at which was rewarded with delivery of food and presentation of cue light and tone. The left lever was inactive (NonReinf) had no consequences but was recorded as a measure of nonspecific activity. The animals started at an FR1TO1 (fixed-ratio 1, 1s timeout) schedule of reinforcement, followed by FR1TO10, FR2TO20 and finally FR5TO20, progressing upon earning 35 reinforcers.

Surgery

Following successful acquisition of food training, rats were anesthetized with Equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al., 2005; Cardenas et al., 2021; Cardenas and Lotfipour, 2021) (Figure 3.1). After surgery, rats were given the analgesic carprofen (5mg.kg, subcutaneous). During the 2-3-day recovery period, catheters were flushed daily with heparinized saline solution (1 ml of 1000 units/ml heparin into 30 ml of bacteriostatic saline) to maintain patency. Catheter patency was tested for rapid (5-10 s) anesthesia by infusing propofol (5 mg/kg, i.v.) before and after the completion of self-administration experiments. Only animals showing rapid anesthesia were included in analyses.

Nicotine intravenous self-administration and extinction

Animals (PN 34) intravenous self-administered (IVSA) nicotine (0.015 mg/kg/infusion) at an FR5 schedule for 1-h daily session for a minimum of 5 days, or until they reached stable

responding (Reinforced responses (Reinf) within 20% of the mean over the last 3 days; R ≥ 2 x NonReinforced responses; R ≥ 5) (Carreño and Lotfipour, 2022) (Figure 3.1). A compound stimulus, light and tone, we paired upon delivery of nicotine infusion. A dose of 0.015 mg/kg/infusion was chosen based on previous adult and adolescent studies (Cross et al., 2020; Gellner et al., 2016). Baseline responding was defined as the average reinforced responses over the last three days of self-administration. Rats were then allowed to respond to at the dose of 0.015 mg/kg/infusion on Progressive Ratio (PR) schedule (~PN 39). The PR schedule of reinforcement is a measure of motivation to obtain the drug (Richardson and Roberts, 1996). sequence was determined using the exponential formula (5 exp (0.2 x infusion number)-5) such that the required responses per infusion were as followed: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492 (Richardson and Roberts, 1996). PR conditions were the same for FR sessions, with the exception that the sessions were 4-h duration. Breakpoint was achieved when >20 min of inactivity on the active lever elapsed. After reaching stable responding and two-day of PR schedule extinction-reinstatement testing began.

During extinction (~PN 41), animals were placed in the same operant testing chambers the animals were not connected to the infusion tubing, the house light remained on and responses on the levers were counted but had no consequences as such no cue or reward were delivered. Extinction sessions were 1-h per day for a minimum of 5 days, or until responding was reduced to 25% of baseline (Carreño and Lotfipour, 2022).

Cue and nicotine-induced reinstatement

After meeting extinction criteria, reinstatement testing began (~PN 47) (Carreño and Lotfipour, 2022). Nicotine-seeking was reinstated using three reinstatement conditions given in a within-subjects counterbalanced design: Cue, nicotine-primed alone, and nicotine-primed paired

with cue. Presentation of cue consisted of cue light illumination and sound in the testing chamber. Nicotine-prime injections contained 0.15 mg/kg nicotine and was administered intraperitoneally immediately before the reinstatement test. The nicotine-prime dose was chosen based on previous work (Cross et al., 2020; Shaham et al., 2003). Between reinstatement tests, animals were returned to extinction condition for a minimum of two days, or until extinction criteria were met. Reinstatement was defined as a significant increase in responding from the last day of extinction.

Data analysis

Data were analyzed using JMP (SAS Institute) software (Carreño and Lotfipour, 2022). Food acquisition was analyzed by a compound 4-way multivariate ANOVA for lever presses (Reinf and NonReinf) x sex (male and female) x Genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x FR schedule (FR1TO1, FR1TO10, FR2TO20, and FR5TO20) with repeated measures on lever presses and FR schedule, with Bonferroni corrected t-test post hoc comparisons. Nicotine self-administration data were analyzed by a compound 4-way multivariate ANOVA Reinf/Nonreinf responses x day x sex (male and female) genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x day (day 3-5) with repeated measures on Reinf/Nonreinf responses and day. Reinstatement data were analyzed as normalized reinforced responding. Mean responses for reinstatement condition were analyzed by a 3-way multivariate ANOVA for sex x genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x reinstatement condition (cue only, nicotine only, and nicotine plus cue), with repeated measure on reinstatement condition. Significant main effects were further analyzed with 1-way ANOVAs and Bonferroni-corrected paired or unpaired t-tests, as appropriate. Food reinstatement data were analyzed as normalized reinforced responding. Mean responses for reinstatement condition were analyzed by a 3-way multivariate ANOVA for Sex x Genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x Reinstatement Condition (cue only, food only, and food plus cue), with a repeated

measure on reinstatement condition. Significant main effects were further analyzed with 1-way ANOVAs and Bonferroni-corrected paired or unpaired t-tests, as appropriate.

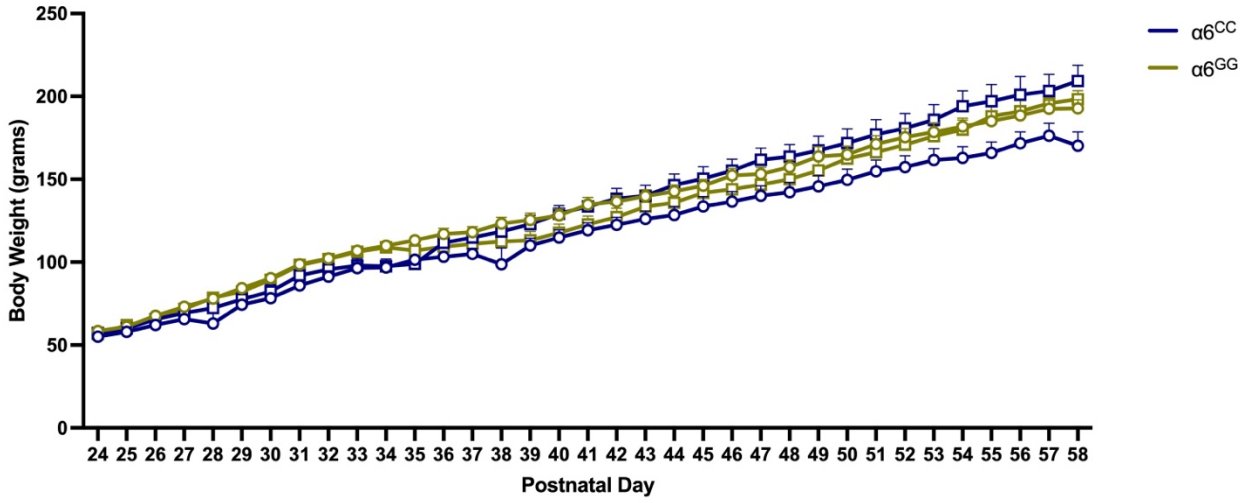

Figure 3.2 Body weight (g) across postnatal days. Male and female $\alpha6^{GG}$ and $\alpha6^{CC}$. Circles represent females and squares represent males.

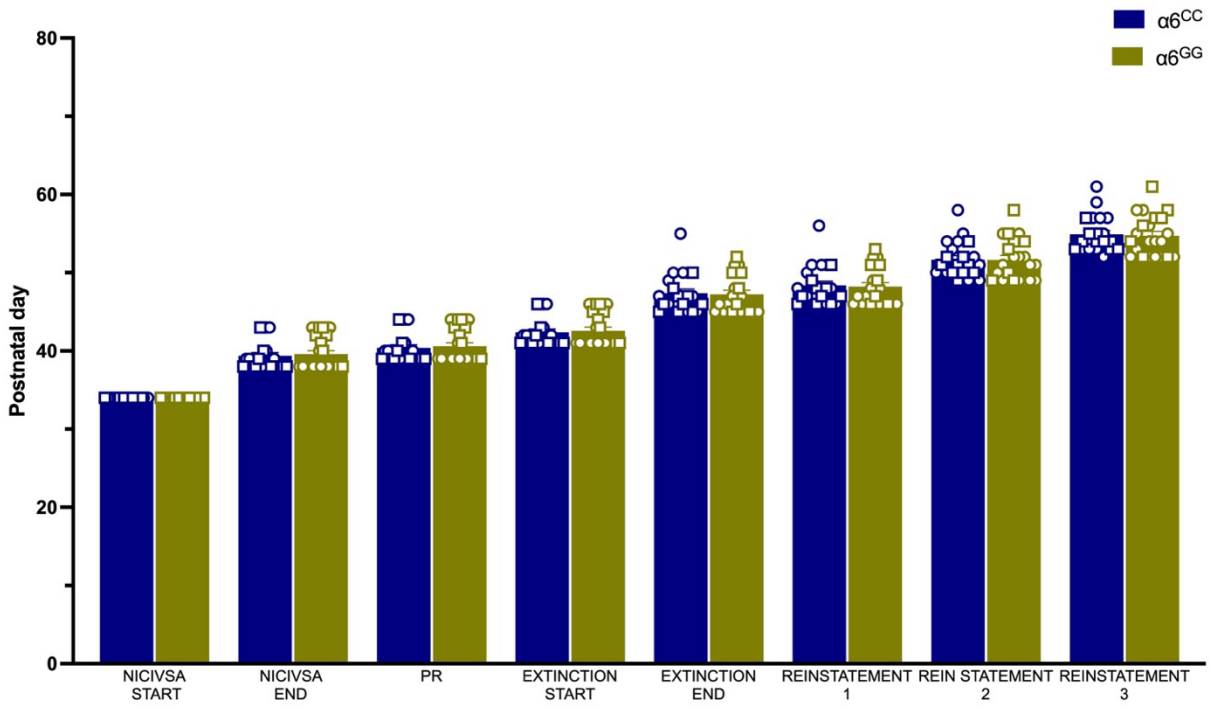

Figure 3.3 Postnatal day across nicotine self-administration, extinction, and reinstatement. No sex differences for the age of initiation of behavior for nicotine self-administration, progressive ratio, extinction, and reinstatement. Mean ± SEM postnatal days across study conditions for male and female $\alpha6^{GG}$ and $\alpha6^{CC}$. Circles represent females and squares represents males

Results

The *CHRNA6* 3'-UTR SNP Knock-in does impact food self-administration in male and female rats.

To facilitate acquisition of lever press adolescent male and female $\alpha6^{GG}$ and $\alpha6^{CC}$ we evaluated food responses at Fixed Ratio (FR)1 timeout (TO)1 and then escalated to higher schedules of reinforcement at FR1TO10, FR2TO20, and FR5TO20. A 4-way ANOVA revealed a significance for Reinf/NonReinf lever presses [$F(1,39)=1169.4560$, $p<0.0001$] and FR schedule [$F(3,117)=295.4014$, $p<0.0001$]. A significant interaction was observed between Reinf/NonReinf lever presses x FR schedule [$F(1,117)=302.9465$, $p<0.0001$]. Data are collapsed by sex since there was no sex or genotype interaction, but graphs (Figure 3.4a and b) are shown separately by sex

and genotype for clarity. Male and female adolescent $\alpha6^{GG}$ and $\alpha6^{CC}$ equally learn to press a lever for a natural reward.

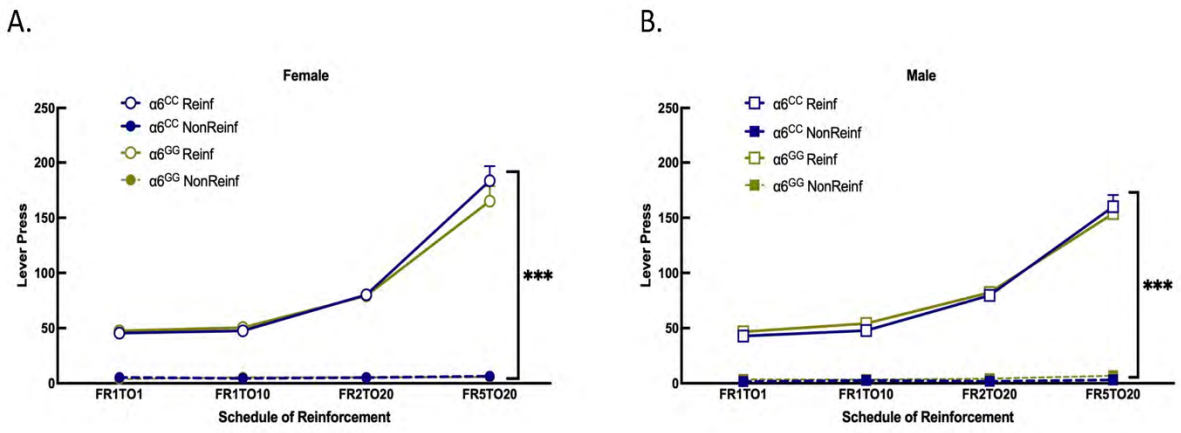

Figure 3.4 The human *CHRNA6* 3'-UTR knock-in does not impact food self-administration. Female (**A**) and male (**B**), $\alpha6^{CC}$ and $\alpha6^{GG}$, mean daily 30 min responses ± SEM for food self-administration at Fixed Ratio (FR)1 Time out (TO)1, FR2TO10, FR2TO20, FR5TO20 schedules of reinforcement. *** p <0.0001 Reinforced (Rein) vs. Non-Reinforced (Nonreinf) responses. N = 6-14/group. Circles represent females and squares represents males.

No sex or genotype differences for intravenous self-administration, progressive ratio, and extinction in male and female adolescent *CHRNA6* 3'-UTR SNP Knock-in rats.

During nicotine intravenous self-administration there was a significant effect of Reinf/Nonreinf responses for days 1-5 [(F1,39)=183.4430, p=0.0001)], Day [F(4,36)=11.7757, p=0.0001], interaction between Reinf/Nonreinf responses and Day [F(4,36)=19.2790, p=0.0001] (Figure 3.5a and b). No sex or genotype effects were observed for total nicotine intake (Figure 3.3c) or breakpoint values (Figure 3.5d). Following stable nicotine self-administration, nicotine-seeking behavior was extinguished by removal of nicotine and associated cue. All animals significantly reduced their responding on the reinforced lever beginning on Day 1 and continued throughout extinction (Figure 3.6a and b). No sex differences were observed for days to meet

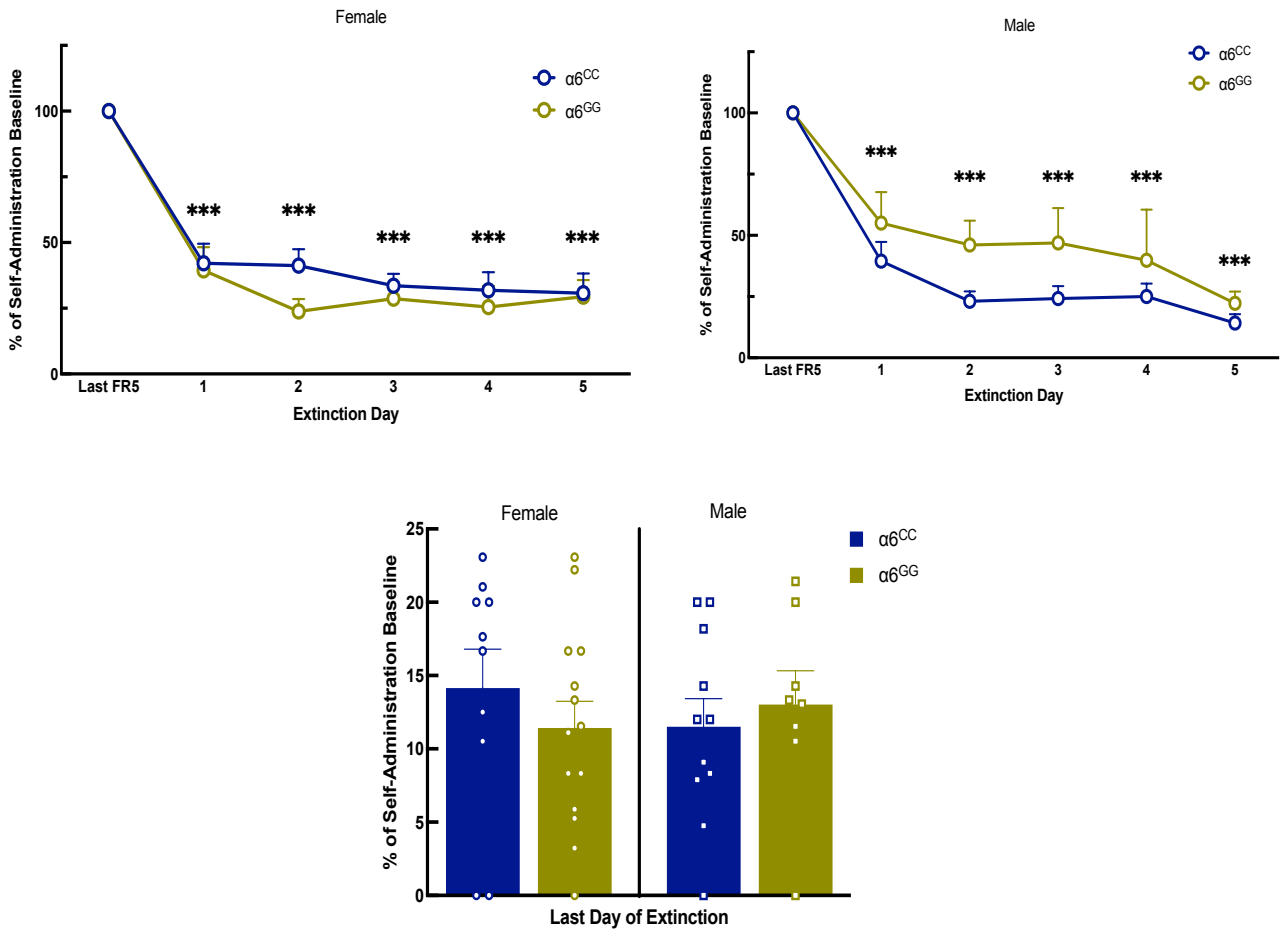

Figure 3.5 The human *CHRNA6* 3'-UTR SNP does not influence extinction. After completion of nicotine self-administration, female (A) and male (B), a6^{CC} and a6^{GG}, were allowed to respond on the reinforced and non-reinforced lever without schedule consequence (e.g., infusion of nicotine, cue light and tone). Data are presented as a mean±SEM percent of the last day of nicotine self-administration responding. No genotype or sex differences were observed for days to meet extinction criteria (C). ***p < 0.001 vs. Last FR5. N = 8–14/group. Circles represent females and squares represent males.

extinction criteria as shown in Figure 3.6c, males and females showing equivalent 25% or on less last day of extinction responding with an average of 6 days.

Sex- and genotype-dependent effects on reinstatement of nicotine in the *CHRNA6* 3'-UTR SNP adolescent rats.

Following extinction, animals were triggered to reinstate to nicotine-seeking behavior with cue-, nicotine- and combination of nicotine plus cue (Figure 3.7a and b). For reinstatement overall ANOVA reveal a Sex x Genotype interaction [$F(1,38)=8.0052$, $p=0.0074$], data was separated by sex and genotype. Main effects of reinstatement stimuli were found [$F(3,36)=26.0462$, $p<0.0001$]

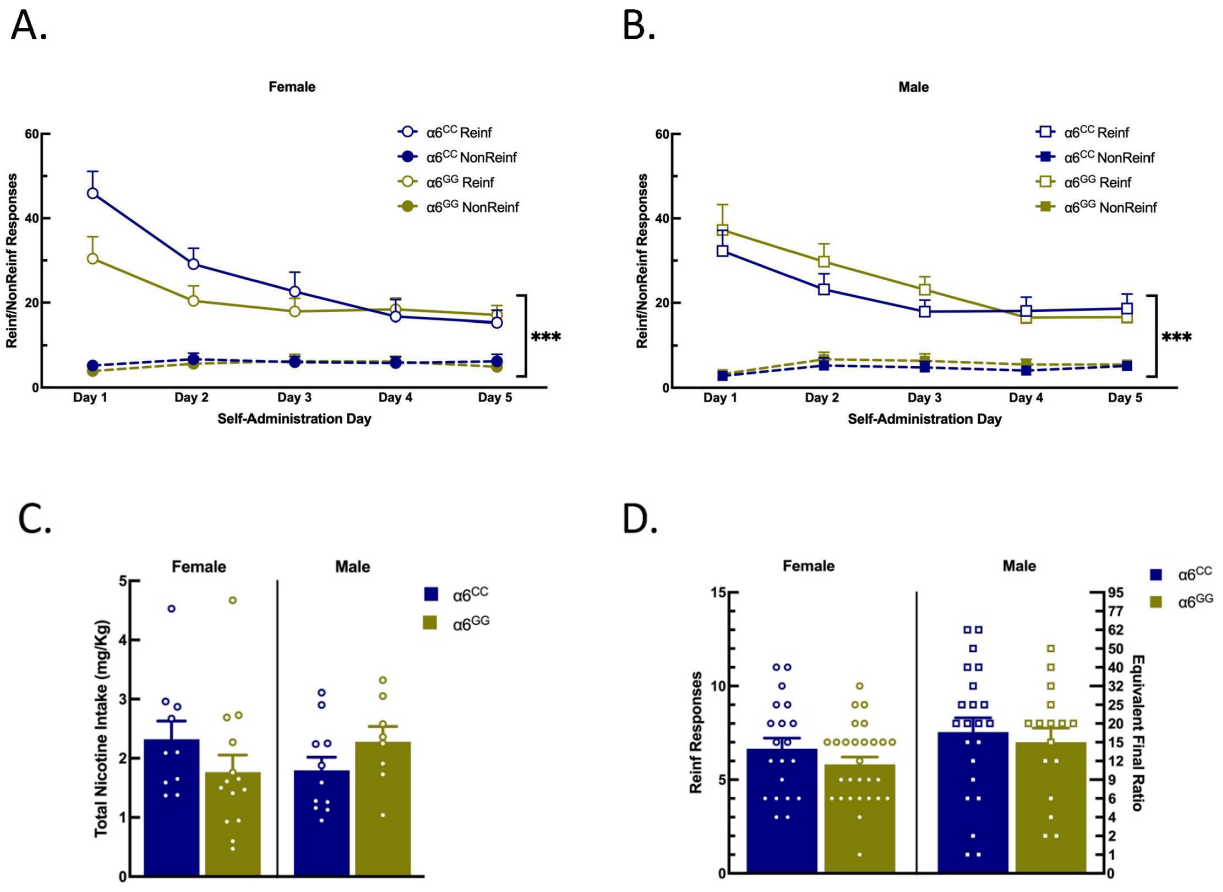

Figure 3.6 The human *CHRNA6* 3'-UTR knock-in does not impact nicotine self-administration, progressive ratio, and nicotine intake. Female (**A**) and male (**B**), $\alpha6^{CC}$ and $\alpha6^{GG}$, mean daily 1-h responses ± SEM for nicotine self-administration at FR5TO20 schedule of reinforcement. No genotype of sex differences was observed for nicotine intake (**C**) or breakpoint values (**D**).*** $p < 0.001$. N = 6-14/group. Circles represent females and squares represents males.

and a trend for Reinstatement Stimuli x Sex x Genotype [(F(3,36)=2.6865, p=0.061]. Further one-way ANOVA for nicotine + cue reveal [F(1,39)=10.0598, p=0.0030] and a one-tail t-test reveal male $\alpha6^{GG}$ show enhanced nicotine + cue primed reinstatement as compared to $\alpha6^{CC}$ (p=0.01) (Figure 3.7b). In females $\alpha6^{CC}$ rats show a trend for enhanced nicotine + cue primed reinstatement as compared with $\alpha6^{GG}$ rats (p=0.08) (Figure 3.7a).

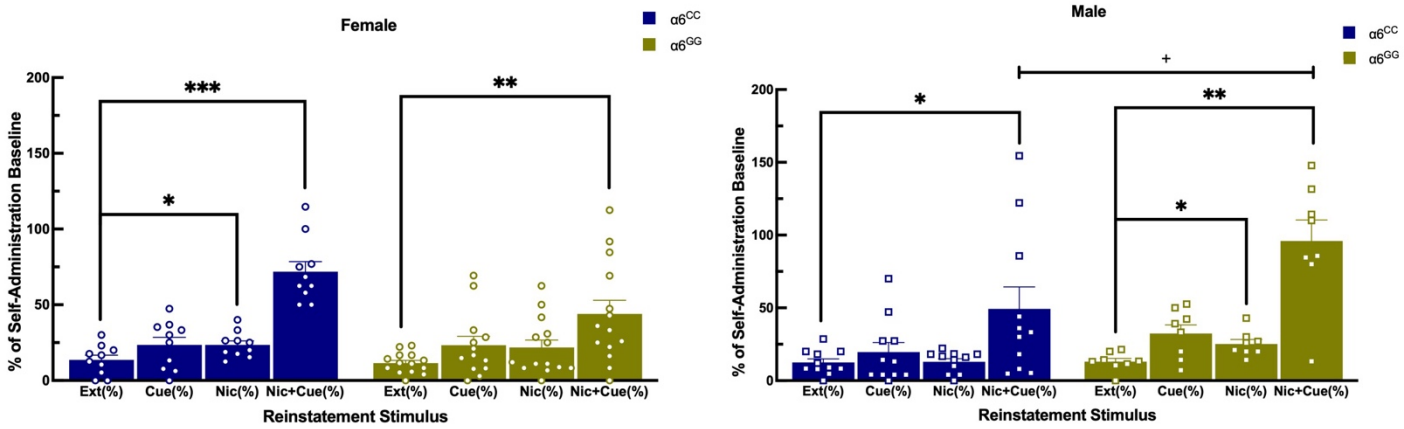

Figure 3.7 Sex and genotype dependent effects of reinstatement of nicotine in humanized *CHRNA6* 3'-UTR SNP rats. a6 3'-UTR SNP genotype- and sex-dependently influences nicotine + cue primed reinstatement, with males more impacted than females based on genotype (A,B).*** p < 0.001, **p < 0.01, *p < 0.05 vs. Extinction; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. Nicotine + Cue; ++p = 0.01 Male $a6^{GG}$ compared with $a6^{CC}$. N = 8–14/group. Circles represent females and squares represent males.

Discussion

We have previously demonstrated equivalent adolescent WT Sprague Dawley male and female behavior in the acquisition of a natural and drug reward and nicotine seeking behavior using a reinstatement model (Carreño and Lotfipour, 2022). In the current study, we investigate the role of humanized *CHRNA6* 3'-UTR SNP knock-in in male and female adolescent rats. Our results show no sex or genotype effects for food self-administration, nicotine self-administration, progressive ratio, and extinction. We observe a sex and genotype bidirectional effect during

reinstatement testing specifically, $\alpha6^{GG}$ males exhibit enhanced nicotine + cue reinstatement when compared to $\alpha6^{CC}$ males and a trending for $\alpha6^{CC}$ when compared to $\alpha6^{GG}$ females. Our study is the first to use an *in vivo* translational model of nicotine behavioral phenotype during adolescence. The current study uses an age range for male and female rats between PN 28-42 as the typical period of adolescence. Ages in the "gray zone", i.e., earlier than PN 28 and later PN 42 have been considered as part of adolescence up to around PN 60 (Spear, 2000). Thus, our studies were done during adolescence, a gradual transition period of soft events with sexual maturity as part of a developmental milestone (Spear, 2000).

The *CHRNA*6 3'-UTR SNP Knock-in does not influence a natural reward

The *CHRNA*6 3'-UTR SNP knock-in does not influence the ability for male and female, $\alpha6^{GG}$ and $\alpha6^{CC}$, adolescents to acquire and maintain food self-administration. Our results are in accord with our recent manuscript using a separate cohort characterizing the *CHRNA*6 3'-UTR SNP knock-in rats (Cardenas et al., 2022) and in adolescent wild type Sprague Dawley rats (Carreño and Lotfipour, 2022). These studies show that age matched male and female wild type and *CHRNA*6 3'-UTR SNP knock-in rats do not exhibit sex effects. Comparing food reinforcement between *CHRNA*6 3'-UTR SNP and wild type rats show similar behavior at different schedules of reinforcement, with the exception of slightly higher FR5TO20 for the *CHRNA*6 3'-UTR SNP rats (Carreño and Lotfipour, 2022). Assessing palatable food conditioned place preference (CPP) in α6 KO and WT C57BL/6J(B6) mice, exhibited similar place preference scores for the context associated with palatable food suggesting the α6 inactivation does not result in the incentive value of a natural reward (Sanjakdar et al., 2015). Intra-VTA perfusion of α-Cntx MII did not yield an effect on food self-administration in adult male rats on a fixed-ratio schedule of reinforcement (Gotti et al., 2010). Additionally, administration of a range of doses for r-bPiDI (2.47-74 μmol/kg;

s.c.), a selective small molecule antagonist of α6β2* nAChR, a tertiary amino analog, 1,10-bis(3-methyl-5-6,-dihydropyridin-1(2H)decane (r-bPiDI), derived from N,N-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI), did not alter the number of food pellets earned in male rats (Beckmann et al., 2015). Future studies should examine the role of a6* nAChR subunit in food-seeking behavior given the mechanism for food self-administration may differ from food-induced reinstatement.

The *CHRNA*6 3'-UTR SNP Knock-in does not influence nicotine intravenous self-administration, progressive ratio, and extinction.

In our current studies, the *CHRNA*6 3'-UTR SNP knock-in does not influence nicotine self-administration in adolescent (PN 34) males and females $\alpha6^{GG}$ and $\alpha6^{CC}$ at a dose of 0.015 mg/kg/injection on a fixed ratio and progressive ratio schedule of reinforcement. Our results are consistent with adolescent male and female Sprague Dawley WT rats (PN 34), which exhibit similar nicotine self-administration in an equivalent paradigm and show lack of sex effect (Carreño and Lotfipour, 2022). Comparing *CHRNA*6 3-UTR SNP to age matched wild type rats show higher nicotine self-administration and nicotine intake (Carreño and Lotfipour, 2022). This is likely influenced by the higher responses at FR5TO20 for food reinforcement prior to nicotine self-administration, as progressive ratio values were similar between *CHRNA*6 3′-UTR SNP and age matched wild type rats (Carreño and Lotfipour, 2022). Decreased nicotine self-administration in both, fixed ratio and progressive ratio have been observed with microinjections into either the nucleus accumbens shell or the ventral tegmental area with an α6β2* nAChR antagonist in rats (Brunzell et al., 2010; Gotti et al., 2010). Intra-VTA α-Cntx MII pretreatment significantly reduced responding for nicotine self-administration at a dose of 0.03 mg/kg/infusion in adult male WT Sprague Dawley rats suggesting the role of α6* nAChR in nicotine-reinforcing properties (Gotti

et al., 2010). Further, in adult rats, nicotine self-administration was decreased with a selective antagonist of α6β2* nAChR, r-bPiDI, at a dose of 0.03 mg/kg/infusion (Beckmann et al., 2015). Additionally, bPiDI prevented the acquisition of nicotine self-administration in WT and α4-S248F mice, mutant α4* nAChR that are insensitive to blockade by mecamylamine, a non-selective nicotinic receptor antagonist (Madsen et al., 2014). These studies suggest the involvement in α6* nAChR in nicotine reinforcement, particularly at higher nicotine doses that were not assessed in our current studies. Future studies are needed to examine dose-, sex-, or genotype-dependent effects, along with α6* nAChR blockade to suppress nicotine self-administration in the adolescent humanized *CHRNA*6 3'-UTR SNP rats. Whereas our results suggest that adolescent male and female $\alpha6^{GG}$ and $\alpha6^{CC}$ have equivalent nicotine self-administration and reward response at the 0.015 mg/kg/injection, a dose response is warranted. It is possible that nicotine pharmacodynamics and pharmacokinetic properties may be dose-dependently shifted in male and female humanized *CHRNA*6 3'-UTR SNP rats, which could be assessed in future studies.

Our results illustrate that male and female adolescent containing the *CHRNA*6 3'-UTR SNP exhibit equivalent extinction learning. These effects are similar to age matched wild type rats (Carreño and Lotfipour, 2022). Both male and females, $\alpha6^{GG}$ and $\alpha6^{CC}$, exhibited extinguished lever-pressing behavior beginning at day 1 and decreasing over time. Self-administration, extinction/reinstatement paradigm in adolescent males (PN 45) and young adult (PN 58) provides an understanding of the molecular mechanisms of extinction learning revealing key structures involved including the mPFC, OFC, Nucleus accumbens (NAc, core and shell) and amygdala (Funk et al., 2016). Further research is needed to understand how dose-dependent nicotine levels alter learning and memory processing during extinction in the humanized male and female *CHRNA*6 3'-UTR SNP rats.

Sex- and genotype dependent effects with a greater impact in $\alpha6^{GG}$ males versus $\alpha6^{CC}$ males.

For nicotine-induced reinstatement, male $\alpha6^{GG}$ and female $\alpha6^{CC}$ rats illustrated enhanced nicotine combined with cue responding with males being more impacted. Cue only failed to reinstate responding in all groups. In age matched wild type rats, cue-induced reinstatement was observed in both males and females (Carreño and Lotfipour, 2022). Male $\alpha6^{GG}$ and female $\alpha6^{CC}$ rats show that a non-contingent administration of nicotine during extinction of nicotine self-administration reinstates responding. These results are in accord with our recent studies using adolescent male and female wild type Sprague Dawley rats (Carreño and Lotfipour, 2022). There is mounting evidence for significant strain-dependent differences for nicotine self-administration (Leyrer-Jackson et al., 2021; Matta et al., 2007). Whereas we observe no sex or genotype differences for natural-, drug-reward and extinction between wild type Sprague Dawley and the *CHRNA*6 3'-UTR SNP rats with a Sprague Dawley genetic background, our reinstatement results suggest otherwise. The strain difference between the WT Sprague Dawley and the *CHRNA*6 3'-UTR SNP is the replacement of the rat *CHRNA*6 3'-UTR with the human *CHRNA*6 3'-UTR. Polymorphisms in 3'-UTR are known to be associated with neurological disorders and behaviors via involvement of micro(mi)RNAs and RNA binding protein (RBP) by influencing mRNA translation (Bae and Miura, 2020; Egervari et al., 2016). The human *CHRNA*6 3'-UTR may bind to miRNA present in humans, but not rodents. The technology to understand the mechanism of the *CHRNA*6 3'-UTR across species in mediating nicotine-induced behavioral sensitization is limited (Mayr, 2017).

Assessing α6* nAChR mRNA and protein expression could shed light into lack of sex or genotype behavioral differences. Sub-chronically treated humanized *CHRNA*6 3'-UTR SNP rats, following a 4-day nicotine pretreatment paradigm, found no sex-, genotype- or nicotine-dependent

alterations in α6 nAChR mRNA expression; however, behavior test showed $\alpha6^{CC}$ females and $\alpha6^{GG}$ males exhibited nicotine induced locomotor and anxiolytic behavior compared to their saline-treated counterparts (Cardenas et al., 2022). Further, in adult male and female mice with fluorescently tagged α4 and α6 nAChRs showed no sex differences for CPP scores at 0.5 mg/kg nicotine s.c. however, there was a significant correlation between upregulation of α4α6, but not α6 (non-α4) nAChRs and reward related behavior in males versus females (Akers et al., 2020). Future studies are essential in assessing neuronal activation, α6* nAChR mRNA and protein expression following nicotine + cue reinstatement in adolescent *CHRNA*6 3'-UTR SNP male and female rats.

Conclusion

Taken together, our data suggest no genotype and/or sex effects were observed for natural food reinforcement, five-day total drug intake for nicotine self-administration, progressive ratio schedules of reinforcement, extinction behavior, or other parameters of reinstatement (i.e., cue-primed). Our results suggest that adolescent male $\alpha6^{GG}$ (and potentially female $\alpha6^{CC}$) rats may be at risk for addictive effects as assessed by reinstatement paradigm. Future studies will need to evaluate the role of nicotine dose, age, and sex-dependent effects during adulthood. Further assessments of the neurobiological mechanisms involved are also warranted in order to identify what drives the nicotine-induced effects in our *CHRNA*6 3'-UTR SNP line in a sex- and genotype dependent manner. Such work may assist in translational studies in humans for improved prevention and intervention strategies to curb nicotine-seeking behavior.

Chapter 4:

Limbic Brain Tissue Neurotransmitter Levels Sex- and Genotype Dependently Predict Nicotine Seeking Behavior in *CHRNA*6 3'-UTR Single Nucleotide Polymorphism Rats

Introduction

The etiology of nicotine addiction is multifactorial including the dysfunction of mesostriatal and mesolimbic circuits and their regulation of dopamine (DA). The mesocorticolimbic DA circuits undergo substantial development during the adolescent period (Dwyer et al., 2019; Dwyer, J.B., McQuown, Leslie, 2009; Howe et al., 2021; Spear, 2000). Moreover, genetic factors can significantly alter smoking initiation and persistence, including genes associated with nicotine metabolic capacity, modulation of nicotinic acetylcholine receptors, and dopaminergic activity (Mutschler et al., 2021). Nicotine exposure during adolescence can perturb the normal development and neuronal nAChRs, essentially altering the function and pharmacology of receptor subunits and changing the reward-related neurotransmitter content.

Genetic association studies have indicated a single nucleotide polymorphism (SNP) in the 3'-UTR of the alpha(α)6 nAChR subunit gene (*CHRNA*6) to be associated with increased cigarette smoking and drug experimentation during adolescence (N. R. Hoft et al., 2009; Lotfipour et al., 2010; Pugach et al., 2017; Arielle S. Selya et al., 2018; Zeiger et al., 2008). Our lab has recapitulated the *CHRNA*6 3'-UTR SNP into a rat line generating, generating $\alpha6^{GG}$ and $\alpha6^{CC}$ allele carriers (Cardenas et al., 2022). Our published results from our genetic knock-in of the human *CHRNA*6 3'-UTR SNP shows that while it does not impact baseline behaviors, sex- and genotype-dependent effects in sub-chronic, but not acute nicotine-induced behaviors are observed (Cardenas et al., 2022). Further, assessing the *CHRNA*6 3'-UTR SNP knock-in rats in a reinstatement model of nicotine-seeking behavior found sex- and genotype-dependent effects on the reinstatement of

nicotine plus associated cue with a greater impact in $\alpha6^{GG}$ males when compared to $\alpha6^{CC}$ males and a trend in the opposite direction in the females (Carreño and Lotfipour, 2023). Mechanisms mediating nicotine-induced behavioral responses in our *CHRNA*6 3'-UTR SNP during adolescents are unknown.

In this chapter, we assessed neurotransmitters in brain regions known to regulate drug reward including the prefrontal cortex (PFC), dorsal caudate putamen (dCPu), nucleus accumbens (NAc), basal lateral amygdala (BLA), interpeduncular nucleus (IPN), and ventral tegmental area (VTA). Neurotransmitters quantified are norepinephrine (NE), epinephrine (Epi), dopamine (DA), serotonin (5-HT), and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in naïve and nicotine-seeking adolescent male and female rats containing the *CHRNA*6 3'-UTR SNP knock-in. We hypothesize that nicotine will alter neurotransmitters, particularly DA and DA turnover levels, in limbic brain regions mediating nicotine seeking behavior (e.g., PFC, NAc shell) (Epstein et al., 2006; Gasbarri et al., 2017; Mcfarland and Kalivas, 2001; Shaham et al., 2003; Venniro et al., 2016) in a sex- and genotype-dependent manner with a greater impact in male $\alpha6^{GG}$ rats.

Methods

Animals

Male and female wild type (WT) Sprague-Dawley rats were purchased from Charles river and bred in house with human *CHRNA*6 3'-UTR^{C123G} SNP rats as described in Chapter 2 and 3.

Tissue Catecholamine Levels

Brains tissue from naïve animals, aged PN 32, were immediately removed, rapidly frozen in -20°C 2-methylbutane and stored at -80°C until use. Brain tissue was sectioned at 300µm on a cryostat set to -12°C (Leica, Deer Park, IL, USA) (Dwyer et al., 2019). Brain sections taken

contained the mPFC, CPu, NAc, BLA, VTA, IPN, LC, which were identified with a rat brain atlas (Paxinos and Watson, 1989). These brain regions of the corticostriatal-limbic system were assessed due to their involvement in reward and motivated behaviors (Capriles et al., 2003; Epstein et al., 2006; Gasbarri et al., 2017; Mcfarland and Kalivas, 2001; Shaham et al., 2003; Tian et al., 2022; Venniro et al., 2016). Brain sections were briefly frozen on dry ice before tissue samples were dissected bilaterally with a 1 mm diameter tissue punch (Integra, Mansfield, MA, USA). Tissue were expelled into 300 ml of ice-cold 0.1 M perchloric acid and homogenized. Samples were centrifuged at 10 000g for10 min, and the resulting pellets resuspended in 100 ml of 0.1 M NaOH overnight before measuring the protein content using a BCA protein assay kit (Pierce, Rockford,IL, USA). The supernatants were used for the measurement of NE, Epi, DA, 5-HT and metabolites DOPAC, HVA and 5-HIAA using high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD). Remaining tissue sections were examined anatomically after tissue puncture to verify correct localization of tissue samples.

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers as described in Chapter 2 and 3.

Food Self-Administration

Male and female adolescents (PN 24) were trained lever pressing operant testing chambers (Med Associates, St. Albans, VT) based on previous studies (Costello et al., 2014; Cross et al., 2020) and as described in Chapter 2 and 3.

Surgery

Following successful acquisition of food training, rats were anesthetized with Equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al.,

2005; Cardenas et al., 2021; Cardenas and Lotfipour, 2021) as described in Chapter 2 and 3. Only animals showing rapid anesthesia were included in analyses.

Nicotine intravenous self-administration and extinction

Animals (PN 34) intravenously self-administered (IVSA) and continued through progressive ratio and extinction as described in Chapter 2 ad 3.

Cue and nicotine-induced reinstatement

After meeting extinction criteria, reinstatement testing began (~PN 47) (Carreño and Lotfipour, 2022) as described in Chapter 2 and 3. In brief, nicotine-seeking was reinstated using nicotine-primed paired with cue. Presentation of cue consisted of cue light illumination and sound in the testing chamber. Nicotine-prime injections contained 0.15 mg/kg nicotine and were administered intraperitoneally immediately before the reinstatement test. Upon completion of the reinstatement test animals were quickly decapitated. Brain tissue was immediately removed, rapidly frozen in -20°C 2-methylbutane and stored at -80°C until use.

Statistical Analysis

Data were analyzed using JMP (SAS Institute) software (Carreño and Lotfipour, 2022). All data are expressed as mean ± SEM. Each neurotransmitter, metabolite, and metabolite ratio was analyzed separately in each brain region. Tissue level neurotransmitter was analyzed by a three-way ANOVA for neurotransmitter concentration x sex x genotype with repeated measures by neurotransmitter concentration. Significant main effects were analyzed with 1-way ANOVAs and Bonferroni-corrected paired or unpaired t-tests, as appropriate. Food acquisition was analyzed by a compound 4-way multivariate ANOVA for lever presses (Reinf and NonReinf) x sex (male and female) x Genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x FR schedule (FR1TO1, FR1TO10, FR2TO20, and FR5TO20) with repeated measures on lever presses and FR schedule, with Bonferroni corrected

t-test post hoc comparisons. Nicotine self-administration data were analyzed by a compound 4-way multivariate ANOVA Reinf/Nonreinf responses x day x sex (male and female) genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x day (day 3-5) with repeated measures on Reinf/Nonreinf responses and day. Reinstatement data were analyzed as normalized reinforced responding. Mean responses for reinstatement condition were analyzed by a 3-way multivariate ANOVA for sex x genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x reinstatement condition (cue only, nicotine only, and nicotine plus cue), with repeated measure on reinstatement condition. Significant main effects were further analyzed with 1-way ANOVAs and Bonferroni-corrected paired or unpaired t-tests, as appropriate. Food reinstatement data were analyzed as normalized reinforced responding. Mean responses for reinstatement condition were analyzed by ANOVA for sex x genotype ($\alpha6^{GG}$ and $\alpha6^{CC}$) x reinstatement condition (cue only, food only, and food plus cue). Significant main effects were further analyzed with 1-way ANOVAs and Bonferroni-corrected paired or unpaired t-tests, as appropriate.

Pearson's correlation coefficient were assessed to compare the neurotransmitter content or turnover ratio vs nicotine + cue seeking behavior response, reporting the RSquare and p-values with a false discovery rate (FDR) of $p<0.1$.

Results

Enhanced tissue DA and other neurotransmitter levels in $\alpha6^{GG}$ as compared with $\alpha6^{CC}$ male naïve adolescent brains with opposite effects in $\alpha6^{CC}$ females in distinct limbic brain regions during adolescence in *CHRNA6* 3'-UTR SNP rats.

Adolescent (PN 32) male and female, $\alpha6^{GG}$ and $\alpha6^{CC}$, tissue level neurotransmitter (NE, EPI, DA, 5-HT and their metabolites (DOPAC, HVA, 5-HIAA) were assessed in key regions of the reward circuitry including the mPFC, CPu, NAc core and shell, BLA, VTA, IPN, and LC (Figure 4.1a-p). We observed differences specific to each region analyzed. Overall male $\alpha6^{GG}$

exhibited greater DA content in the PFC, CPu, and NAc core when compared to males $\alpha6^{CC}$ rats. These effects were not observed in females.

A three-way ANOVA revealed a significance for neurotransmitters for all regions [F(55, 825)=57.0743, p=0.0001]. Subsequently, brains regions were analyzed separately. In the PFC, a main effect for Sex [F(1,30)=5.0924, 0.0315], and Neurotransmitter Levels [F(6,25)=12.0905, p=0.0001], were observed, but not interaction with Sex or Genotype. *Post hoc* analysis revealed NE ($p<0.01$), DA ($p<0.05$), and DOPAC ($p<0.05$) neurotransmitter tissue levels differences in adolescent $\alpha6^{GG}$ males when compared to $\alpha6^{CC}$ males. Neurotransmitter differences were not observed in adolescent female $\alpha6^{CC}$ or $\alpha6^{GG}$ rats in the PFC (Figure 4.1 a-b).

For the CPu, at baseline, we found significant differences in neurotransmitter levels [F(6,27)=53.668, p=0.0001] and Sex x Genotype interaction [F(1,35)=7.2884, p=0.0106]. *Post hoc* analysis revealed greater DA in $\alpha6^{GG}$ males than $\alpha6^{GG}$ males ($p<0.05$) (Figure 4.1 c-d). Within the NAc core, a three-way ANOVA revealed a main effect for neurotransmitter level [F(6,27)=38.7504, p=0.0001] and an interaction for Sex x Genotype [F(1,35)=3.9955, p=0.0534] for DA. Post hoc analysis revealed greater DA in $\alpha6^{GG}$ males than $\alpha6^{GG}$ males ($p<0.05$) (Figure 4.1 e-f). In the NAc shell, we found significant effect for neurotransmitter level [F(6,27)=24.1707, p=0.0001] . We evaluated neurotransmitters separately, revealing a trend for Sex x Genotype effect [F(1,33)=3.8648, p=0.058] for HVA. *Post hoc* analysis revealed greater DA in $\alpha6^{GG}$ males when compared to $\alpha6^{GG}$ males ($p<0.05$) (Figure 4.1 g-h). There were no genotype differences in females.

Basolateral amygdala, a critical region for reward behaviors via projections to the nucleus accumbens, showed differences in neurotransmitter levels [F(6,25)=20.4597, p=0.0001], and although no genotype or sex differences were observed, data were separated for clarity purposes (Figure 4.1 i-j). For the VTA and IPN, significant differences in neurotransmitter content were

found [F(6,25)=24.3230, p=0.0001] and [F(6,27)=26.9032, p=0.0001], respectively. No sex or genotype differences were observed in *post hoc* analysis (Figure 4.1 k-l and m-n). In the LC, differences in neurotransmitters were found [F(6,26)=36.1523, p=0.0001]. As we evaluated neurotransmitter separately, NE and DOPAC tissue levels revealed a Sex x Genotype interaction effect [F(1,35)=8.8545, p=0.0053] and [F(1,33)=4.4798, p=0.0419, respectively. Females $\alpha6^{CC}$ exhibited greater NE and DOPAC when compared to $\alpha6^{GG}$ females. Males $\alpha6^{GG}$ exhibited greater NE than $\alpha6^{CC}$ males (Figure 4.1 o-p).

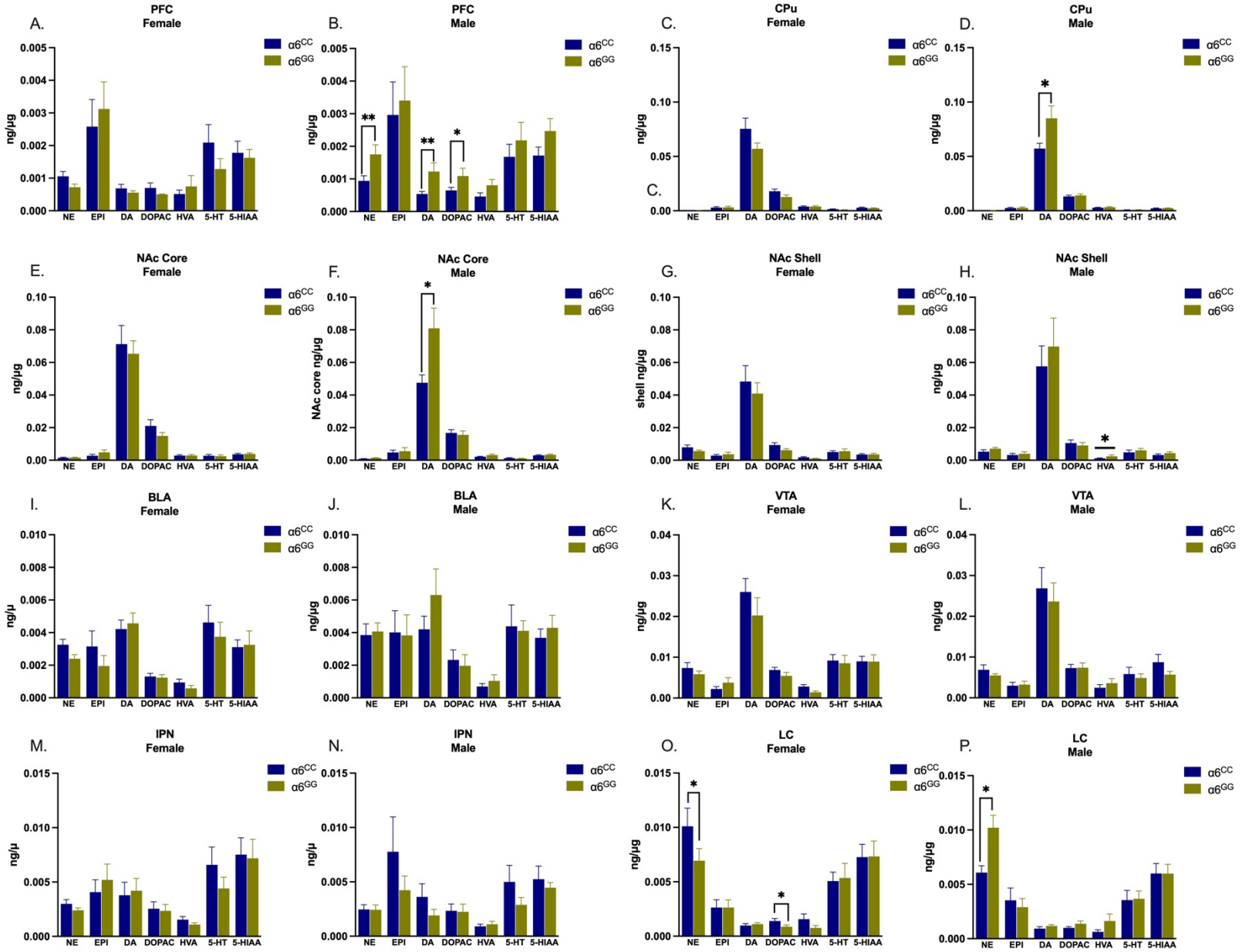

Figure 4.1 Neurotransmitter profile in naïve male and female, α6^{GG} and α6^{CC} adolescent (PN 32) rats. (A-B)Prefrontal cortex (PFC), (C-D) caudate putamen (CPu), (E-H) nucleus accumbens (NAc) core and shell, (I-J) basolateral amygdala (BLA), (K-L) ventral tegmental Area (VTA), (M-N) Interpeduncular nucleus (IPN), (O-P) Locus Coeruleus (LC). *p< 0.05; **p<0.01. All data are presented as mean ±SEM. N = 8-10/group

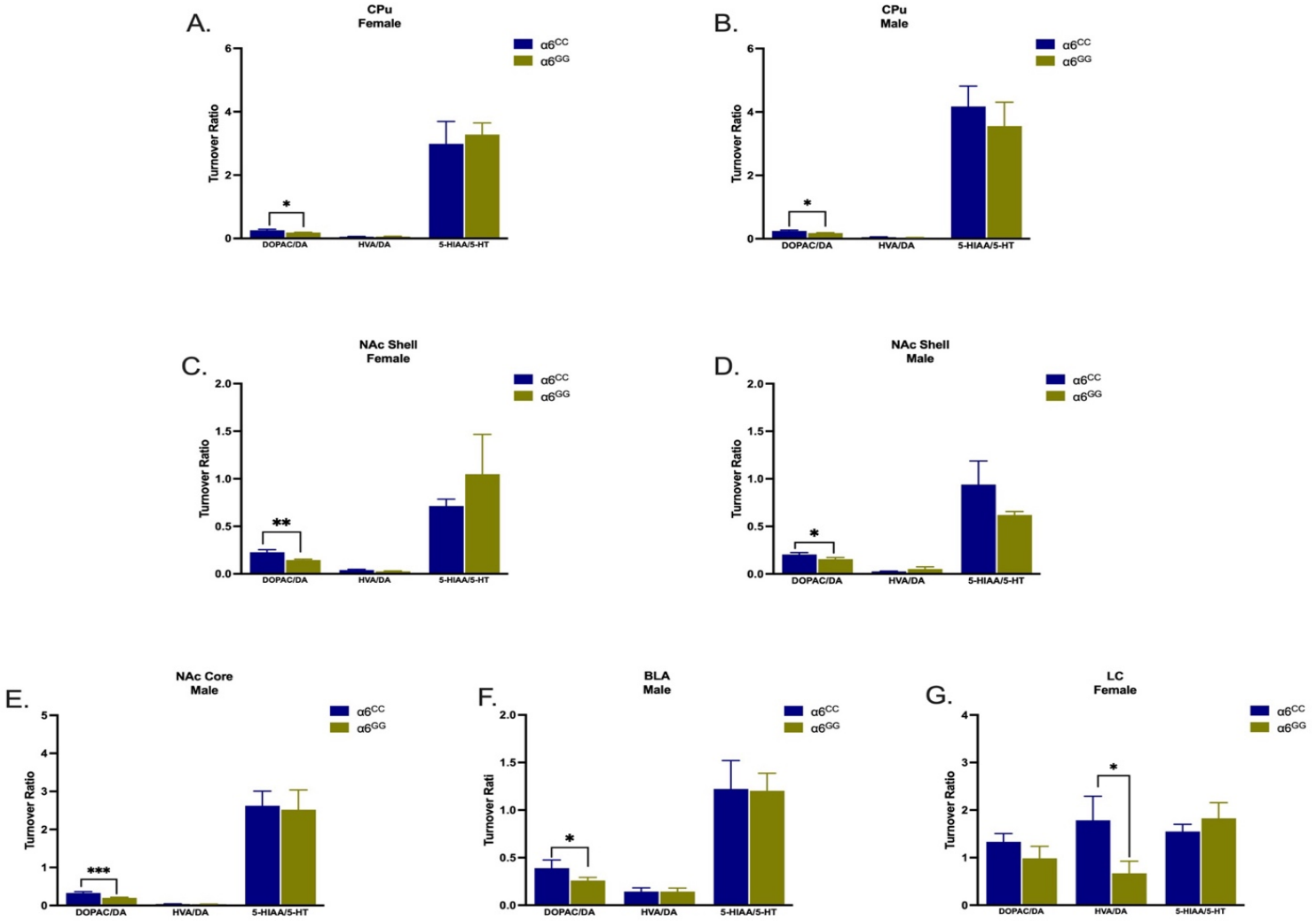

Figure 4.2 Catecholamine turnover in naïve male and female $\alpha6^{GG}$ and $\alpha6^{CC}$ adolescent (PN 32) rats. 3,4-dihrydoxyphenylacetic acid/dopamine (DOPAC/DA) observed in males and females $\alpha6^{CC}$ when compared to males and females $\alpha6^{GG}$ ($p<0.05$) in Caudate putamen (CPu) (A-B), Nucleus Accumbens (NAc) Shell (C-D). In the NAc Core male $\alpha6^{CC}$ exhibit significant turnover ratio when compared to $\alpha6^{GG}$ ($p<0.001$) (E). In the BLA, $\alpha6^{CC}$ females showed significant greater turnover ratio for DOPAC/DA when compared to $\alpha6^{GG}$ females (F). Homovanillic acid (HVA)/Dopamine (DA) was greater in females $\alpha6^{CC}$ than $\alpha6^{GG}$ females ($p<0.05$) (G). All data are presented as mean ± SEM. N = 8-10/group

Baseline turnover

Genotype [F(1,35)=8.7014, p=0.0056] differences were observed in the CPu, with *post hoc* analysis revealing greater DOPAC/DA turnover in both male and female $\alpha6^{CC}$ rats when compared to males and female $\alpha6^{GG}$ rats (Figure 4.2a-b). The nucleus accumbens core and shell showed similar results, with a Genotype main effect [F(1,35)=3.34, p=0.002 and F(1,35)=10.6089, p=0.0025, respectively], with *post hoc* analyses revealing male and female $\alpha6^{CC}$ rats showing greater DOPAC/DA turnover when compared to $\alpha6^{GG}$ male and female rats in the NAc shell (Figure 4.2c-d) and only in male $\alpha6^{CC}$ significantly greater than $\alpha6^{GG}$ male in the NAc core ($p<0.01$) (Figure 4.2e) . Male $\alpha6^{CC}$ DOPAC/DA turnover was greater in the BLA when compared to male $\alpha6^{GG}$ ($p<0.05$) (Figure 4.2f) . HVA/DA turnover was enhanced in the LC for female $\alpha6^{CC}$ as compared with $\alpha6^{GG}$ rats ($p<0.05$) (Figure 4.2g). The results illustrate that baseline neurotransmitter rates are mainly genotype dependent, with DOPAC/DA levels enhanced in $\alpha6^{CC}$ as compared with $\alpha6^{GG}$ rats in the CPu and NAc. However, some unique genotype and sex-dependent effects are also observed in the NAc core, BLA and LC.

Sex- and genotype-dependent effects on tissue neurotransmitter levels are diminished in animals tested in nicotine plus cue induced reinstatement, with exception of a few brain regions and neurotransmitters.

We have replicated behavioral results from our recent publication in which we report sex and genotype directional effects if nicotine-seeking behavior (Carreño and Lotfipour, 2023). In our published results (Chapter 3), we show male $\alpha6^{GG}$ exhibit enhanced nicotine + cue seeking behavior when compared to male $\alpha6^{CC}$ ($p<0.05$). In our current study, nicotine plus cue, main effects for reinstatement stimuli F(1,30)=21.4724, p=0.0001 were observed. A one-way ANOVA revealed Sex x Genotype [F(1,14)=10.1195, p=0.0067] interaction, Sex and genotype-dependent

effects are impacted in males $\alpha6^{GG}$ when compared to males $\alpha6^{CC}$ ($p<0.01$) for nicotine + cue reinstatement. In our current studies, similar to our published results (Carreño and Lotfipour, 2023) we observed no sex or genotype effects for food acquisition, nicotine self-administration, breakpoint for progressive ratio or extinction (Figure 4.3A-F).

A subset of male and female $\alpha6^{CC}$ and $\alpha6^{GG}$ adolescents from the reinstatement behavioral testing were analyzed for neurochemical profile in regions of the reward pathway similar to baseline studies. In the PFC main effect for neurotransmitter level was found [$F(6,14)=13.8552$, $p=0.0001$]. A one-way ANOVA revealed a trend effect for Sex x Genotype [$F(1,19)=3.3984$, $p=0.0809$] for 5-HT and sex x genotype interaction for 5-HIAA, $F(1,19)=4.0426$, $p=0.0588$. $\alpha6^{GG}$ females exhibited greater 5-HT and 5-HIAA ($p<0.05$) when compared to $\alpha6^{GG}$ (Figure 4.4 a-b). Although sex and genotype differences were not found for all neurotransmitters, we separated the data based on *a priori* hypothesis. In the CPu, an effect for neurotransmitter levels [$F(6,84)=3.6737$, $p=0.0001$] and interaction for Neurotransmitter Levels x Sex [$F(6,84)=3.6737$, $p=0.0027$] were found. *Post hoc* analysis revealed no sex or genotype differences across neurotransmitters in the CPu (Figure 4.4 c-d). In the NAc core, differences in neurotransmitter level [$F(1,102)=88.6527$, $p=0.0001$] was observed. A one-way ANOVA revealed a Sex x Genotype interaction for 5-HIAA [$F(1,20)=88.6527$, $p=0.001$] and a trend for HVA [$F(1,20)=3.9127$, $p=0.0619$]. $\alpha6^{CC}$ females exhibit greater HVA and 5-HIAA when compared to $\alpha6^{GG}$ females ($p<0.05$) (Figure 4.4 e-f). Within the NAc shell, a main effect of neurotransmitter level was observed [$F(1,102)=34.4029$, $p=0.0001$]. A one-way ANOVA revealed a sex effect for HVA [$F(1,24)=6.8524$, $p=0.0151$] and a trend for Sex x Genotype [$F(1,24)=3.8501$, $p=0.0614$]. *Post hoc* analysis revealed $\alpha6^{CC}$ females to have enhanced HVA levels when compared to females $\alpha6^{GG}$ ($p<0.05$). Within the LC, neurotransmitter level differences were observed [$F(6,7)=1.9390$,

p=0.0009] and Neurotransmitter Level x Sex [(F6,7)=4.1882, p=0.0413]. *Post hoc* analysis did not reveal sex or genotype differences. Overall, our studies illustrate that many of the sex x genotype dependent effects observed for baseline neurotransmitter levels are blunted in animals tested for reinstatement. Further, metabolic turnover observed for baseline are eliminated in animals tested for reinstatement (data not shown). DA content in naïve and reinstatement male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$, in regions of the reward pathway are shown in Table 4-1.

Exploring the relationship between neurotransmitter content and nicotine plus cue behavioral response.

Positive correlations for turnover ratio and behavioral response were observed in $\alpha6^{GG}$ males for HVA/DA in the mPFC, NAc shell, BLA, where greater turnover ratio equates to greater reinstatement response. Summary of these results can be found in Table 4.2 and Figure 4.5.

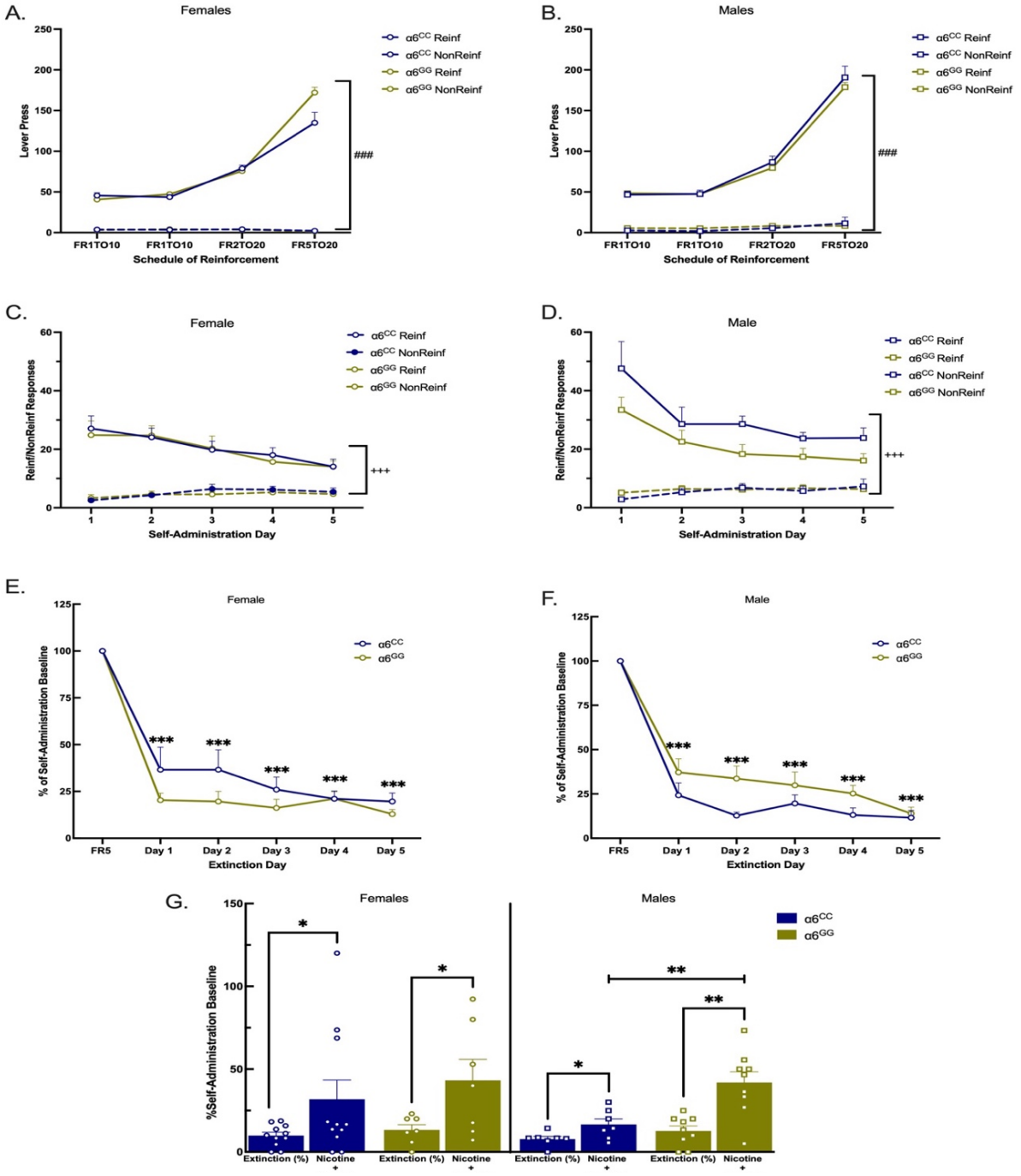

Figure 4.3 Sex and genotype dependent effects of humanized CHRNA6 3'-UTR SNP rats. Female (A) and male (B), $\alpha6^{CC}$ and $\alpha6^{GG}$ mean daily 30 min responses ± SEM for food self-administration at Fixed Ratio (FR)1 Time out (TO), FR1TO10, FR2TO20, FR5TO20 schedules of reinforcement. ***p<0.0001 Reinf vs NonReinf Reinf responses. Female (C) and male (D), $\alpha6^{CC}$ and $\alpha6^{GG}$ mean 1-hr daily responses ± SEM for nicotine self-administration at FR5TO20 schedule of reinforcement. ***p<0.0001 Reinf vs NonReinf. Female (E) and male (F), $\alpha6^{CC}$ and $\alpha6^{GG}$ extinction 1-hr daily responses ± SEM percent of the last day of nicotine self-administration responding. G. Sex and genotype effects of reinstatement of nicotine-seeking in humanized *CHRNA6* 3'-UTR SNP with $\alpha6^{GG}$ more impacted when compared to $\alpha6^{CC}$ males. *p<0.05 vs extinction, **p<0.01 vs extinction; ++ p<0.01 $\alpha6^{GG}$ vs $\alpha6^{CC}$. N=7-11/group

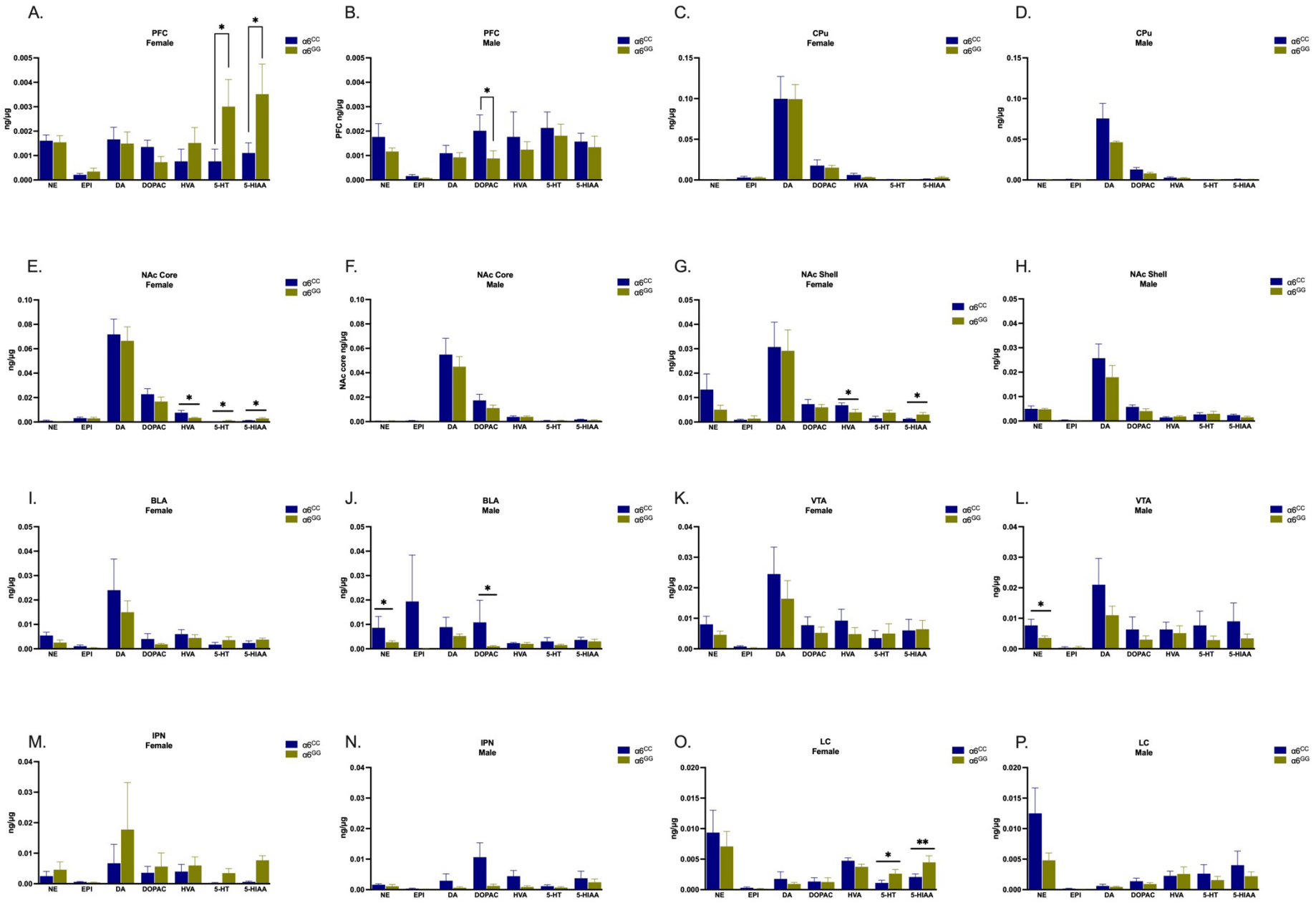

Figure 4.4 Neurotransmitter profile in a subset of animals after nicotine plus cue testing. *p<0.05, **p<0.01. Prefrontal cortex (PFC); caudate putamen (CPu), nucleus accumbens (NAc) core and shell; basolateral amygdala (BLA); Ventral Tegmental Area (VTA); Interpeduncular nucleus (IPN); Locus Coeruleus (LC). *p<0.05, **p<0.01. All data are presented as mean ± SEM. N = 5-9/group.

Table 4-1 Dopamine content (ng/μg) in male and female, $\alpha6^{CC}$ and $\alpha6^{CC}$ in regions of the reward pathway.

	Female		Male	
	$\alpha6^{CC}$	$\alpha6^{GG}$	$\alpha6^{CC}$	$\alpha6^{GG}$
PFC, Naïve	0.000655 ± 0.000176	0.000478 ± 0.002	0.000548 ± 0.000176	0.00126 ± 0.000176**
PFC, Nicotine + Cue	0.00151 ± 0.00431	0.00140 ± 0.00431	0.000978 ± 0.00431	0.00686 ± 0.00352**
CPu, Naïve	0.0767 ± 0.00913	0.057 ± 0.00913	0.0568 ± 0.00913	0.0878 ± 0.00913
CPu, Nicotine + Cue	0.0987 ± 0.0213	0.126 ± 0.0213	0.0725 ± 0.0213	0.0612 ± 0.0174
NAc Core, Naïve	0.0629 ± 0.0093	0.0653 ± 0.0088	0.0475 ± 0.0093	0.074 ± 0.0098*
NAc Core, Nicotine + Cue	0.0705 ± 0.0109	0.0639 ± 0.0109	0.0506 ± 0.0109	0.0501 ± 0.00803
NAc Shell, Naïve	0.0483 ± 0.012	0.0421 ± 0.013	0.0533 ± 0.0127	0.0653 ± 0.0135
NAc Shell , Nicotine + Cue	0.0376 ± 0.0106	0.0283 ± 0.0106	0.03 ± 0.0106	0.0262 ± 0.00822
BLA, Naïve	0.00422 ± 0.001	0.00489 ± 0.001	0.0042 ± 0.001	0.0063 ± 0.001
BLA, Nicotine + Cue	0.0318 ± 0.00654	0.0157 ± 0.00654	0.00853 ± 0.00654	0.00693 ± 0.00506
VTA, Naïve	0.0234 ± 0.004	0.016 ± 0.004	0.0236 ± 0.004	0.0212 ± 0.004
VTA, Nicotine + Cue	0.0324 ± 0.00533	0.0187 ± 0.00527	0.0171 ± 0.00645	0.0113 ± 0.00456
IPN, Naïve	0.0036 ± 0.0011	0.00417 ± 0.001	0.0036 ± 0.011	0.00019 ± 0.001**
IPN, Nicotine + Cue	0.00507 ± 0.00785	0.0128 ± 0.0064	0.0029 ± 0.00906	0.0011 ± 0.007
LC, Naïve	0.0009 ± 0.00019	0.0011 ± 0.0002	0.000685 ± 0.0002	0.0001 ± 0.0002
LC, Nicotine + Cue	0.00176 ± 0.0006	0.00093 ± 0.00554	0.000613 ± 0.00064	0.000444 ± 0.00039

Compiled naïve and nicotine + cue reinstatement baseline concentration ± SEM for adolescent male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$. **p<0.01 naïve $\alpha6^{GG}$ vs $\alpha6^{CC}$; *p<0.05 naïve $\alpha6^{GG}$ vs $\alpha6^{CC}$

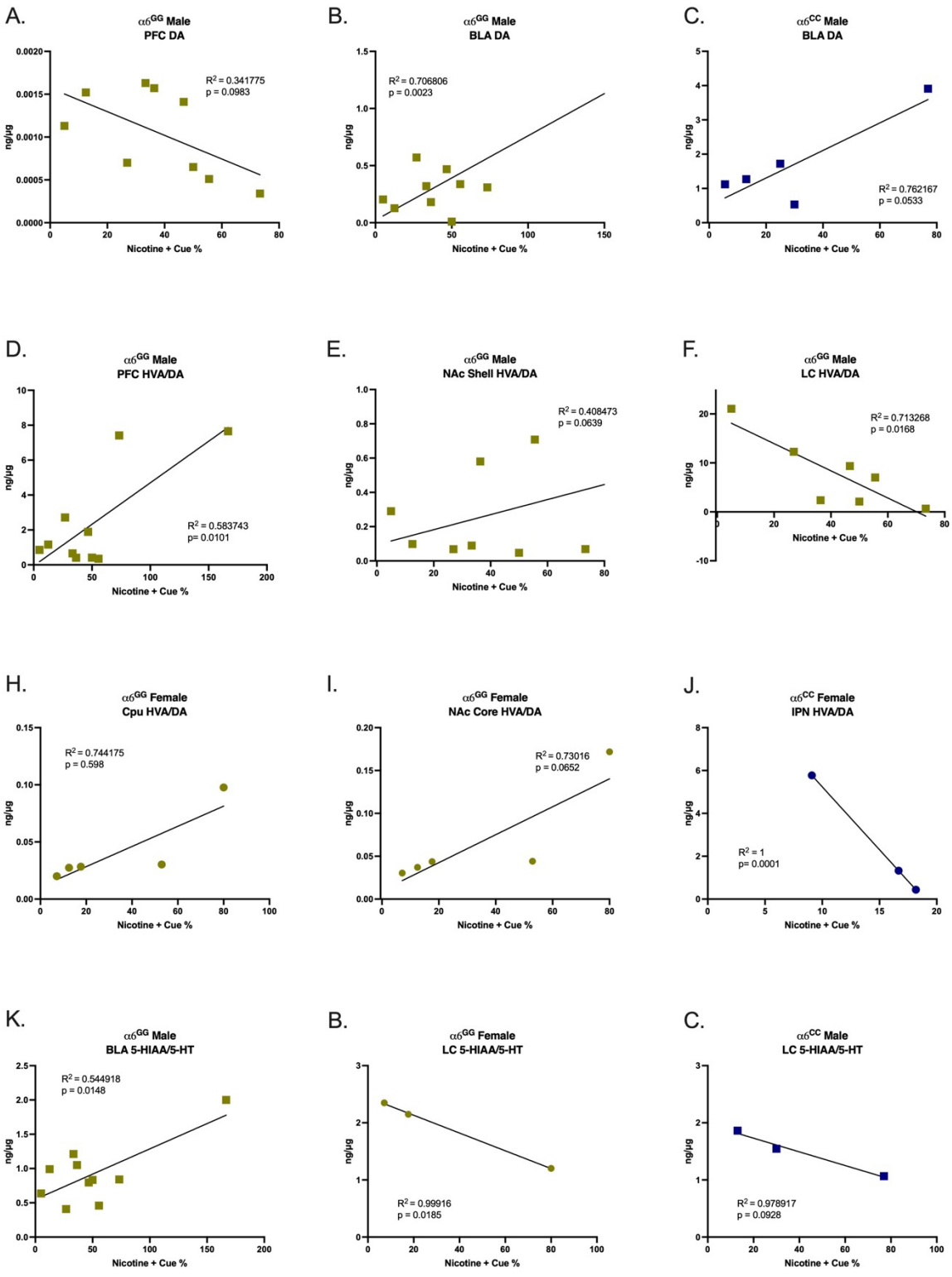

Figure 4.5 Correlation data between neurotransmitter concentration or turnover ratio and behavior, nicotine + cue reinstatement responding. Prefrontal cortex (PFC); caudate putamen (CPu), nucleus accumbens (NAc) core and shell; basolateral amygdala (BLA); Ventral Tegmental Area (VTA); Interpeduncular nucleus (IPN); Locus Coeruleus (LC). All data are presented as mean ± SEM. N = 5-7/group.

Table 4-2 Neurotransmitter correlation and nicotine + cue-induced reinstatement behavior in rats containing the human *CHRNA*6 3'-UTR SNP

Genotype	Sex	Brain Region	NT	R^2	P value	Directionality
$\alpha6^{GG}$	Male	PFC	Dopamine	0.341775	0.0983	Negative
$\alpha6^{GG}$	Male	BLA	Dopamine	0.706806	0.0023	Positive
$\alpha6^{CC}$	Male	BLA	Dopamine	0.762167	0.0533	Positive
$\alpha6^{GG}$	Male	PFC	HVA/DA	0.583743	0.0101	Positive
$\alpha6^{GG}$	Male	NAc Shell	HVA/DA	0.406473	0.0639	Positive
$\alpha6^{GG}$	Male	LC	HVA/DA	0.713268	0.0168	Negative
$\alpha6^{GG}$	Male	BLA	5-HIAA/5-HT	0.544918	0.0148	Positive
$\alpha6^{CC}$	Male	LC	5-HIAA/5-HT	0.978917	0.0928	Negative
$\alpha6^{GG}$	Female	CPu	HVA/DA	0.744175	0.0598	Positive
$\alpha6^{GG}$	Female	NAc Core	HVA/DA	0.73016	0.0652	Positive
$\alpha6^{GG}$	Female	LC	5-HIAA/5-HT	0.99918	0.0185	Negative
$\alpha6^{CC}$	Female	IPN	HVA/DA	1	0.0001	Negative

Discussion

The present studies illustrate baseline neurotransmitter levels are genotype and sex-dependent in several brain regions in adolescent naïve *CHRNA*6 3'-UTR SNP rats. In particular DA is greater in $\alpha6^{GG}$ males in the PFC, Cpu, and NAc core as compared to $\alpha6^{CC}$ males. NE is greater in the LC in $\alpha6^{CC}$ females and $\alpha6^{GG}$ males as compared to their counterparts. Genotype and sex dependent effects are blunted during reinstatement. However, when assessing correlations between reinstatement and tissue level neurotransmitters, I observe that extracellular DA turnover (HVA/DA) in the NAc shell and PFC increases with nicotine seeking behavior in $\alpha6^{GG}$ males exclusively. These effects are opposite in the LC. The results provide a mechanism for how dopamine turnover tissue levels could mediate nicotine seeking behavior in our *CHRNA*6 3'-UTR SNP rats.

Prior results have illustrated that the PFC and NAc shell are critical for reinstatement behavior (Lubbers et al., 2014; McFarland and Kalivas, 2001; Shaham et al., 2003). In particular, DA levels in the PL region of the PFC and the shell, but not core have been shown to trigger reinstatement (Bossert et al., 2012, 2007, 2006). Our findings are the first to illustrate that HVA/DA turnover tissue levels in the PFC and the NAc shell can predict reinstatement behavior via a potential α6 nicotinic receptor subunit in *CHRNA*6 3'-UTR SNP rats. Whether these effects are indeed driven by the α6 subunit needs to be further evaluated. One surprising result was the opposite correlations observed for the LC, where a negative association was observed with HVA/DA turnover and reinstatement behavior. The findings suggest the LC could mediate reinstatement behavior and would require additional studies to test this hypothesis.

We currently do not know why our naïve adolescent animals exhibit genotype and sex dependent effects in baseline tissue neurotransmitter levels that get blunted during nicotine-

seeking behavior. Indeed, we found heightened DA levels in male $\alpha6^{GG}$ as compared with $\alpha6^{CC}$ male rats in several limbic brain regions. In addition, $\alpha6^{GG}$ males and $\alpha6^{CC}$ females exhibit greater NE in the LC. The LC is a small nucleus located in the brainstem that provides noradrenergic neurotransmitter system in the brain (Poe et al., 2020). NE has been implicated in drug-seeking behavior. In addition to cues previously associated with drug, noncontingent administration of the drug and stress can reinstate drug-seeking behavior. Activation of kappa-opioid receptors (KORs) in monoamine circuitry results in stress-induced reinstatement of drug-seeking in conditioned place preference (CPP) and self-administration models. Administration of NE alone has been shown to reinstate cocaine seeking in adult male Long-Evans rats (Brown et al., 2009). In mice, norbinaltorphimine (NorBNI), a selective KOR antagonist, pretreatment significantly attenuated stress-induced reinstatement of nicotine-CPP but had no effect on nicotine prime reinstatement (Jackson et al., 2013). KOR-induced reinstatement of cocaine CPP with U50,488, a KOR agonist, was significantly attenuated by injections of NorBNI directly in the LC in mice (Al-Hasani et al., 2013). Injections of NorBNI in the LC did not alter conditioning as the control and NorBNI groups formed place preference to cocaine with similar degree (Al-Hasani et al., 2013). Furthermore, it has been shown that stress can release dynorphin, neuropeptide, which activate KORs with the dopaminergic nuclei to produce drug-seeking and negative affect-like behavior (Bruchas et al., 2010; Graziane et al., 2013; Tejeda et al., 2013; Van't Veer et al., 2013). In addition, KOR agonist can inhibit DA release and alter behavioral effects within NAc and VTA (Ebner et al., 2010; Graziane et al., 2013; Shippenberg et al., 2007). These studies highlight KOR function in dopaminergic circuits which may play a role in reinstatement and KOR-mediated behaviors. Future studies are needed to evaluate the role of KORs in the *CHRNA6* 3'-UTR SNP rats.

In the PFC, differences in NE, DA, and DOPAC, were observed in which male $\alpha6^{GG}$ neurotransmitter content was greater when compared male $\alpha6^{CC}$. Such differences were not observed in females. What these other effects mean in association with behavior is not known, as there were no direct correlations with behavior. Prior studies have illustrated that maternal nicotine can alter DA content and metabolism in the adolescent prefrontal cortex (Dwyer et al., 2019). Differences of the prelimbic and the infralimbic have been posited to orchestrate differing roles in conditioning responding (Riaz et al., 2019.). The canonical view postulates the PL is required for goal-directed behavior ("go") whereas the IL regulates behavioral inhibition ("no go") (Capriles et al., 2003; Mcfarland and Kalivas, 2001; Moorman and Aston-Jones, 2015; Peters et al., 2009). Further, PL and IL regulate goal-directed responding and habitual responding, respectively (Shipman et al., 2018). Conflicting evidence suggest the PL may serve an inhibitory role for reward and drug-seeking behavior. Age-dependent structural alterations of pyramidal neurons from the PL were observed after nicotine self-administration in which a subpopulation of PL pyramidal cells in adolescents (PN 29) were altered as compared to adults (PN 80) suggesting that cortical function may be differentially affected (Bergstrom et al., 2008). Future studies should assess neurochemical changes in the IL before and after nicotine administration in adolescent male and female rodents containing the *CHRNA*6 3'-UTR SNP.

This study investigated how neurotransmitter levels affect nicotine + cue reinstatement behavior in the *CHRNA*6 3'-UTR SNP rats. Taken together, results indicate that baseline neurotransmitter levels are dependent on genotype and sex, with DA and NE levels being greater in certain brain regions of $\alpha6^{GG}$ males and $\alpha6^{CC}$ females. During reinstatement, genotype and sex-dependent effects become blunted, but extracellular DA turnover in the PFC and NAc shell increases with nicotine seeking behavior in $\alpha6^{GG}$ males. The LC may mediate reinstatement

behavior, and KORs in dopaminergic circuits may play a role in reinstatement. Given that α6 mRNA expression peaks during adolescence (Azam et al., 2007) and nicotine dose-dependently affect sex-specific behaviors (Lenoir et al., 2015) evaluation of adults containing the *CHRNA*6 3'-UTR SNP are warranted. Future studies are needed to evaluate α6 mRNA expression in the *CHRNA*6 3'-UTR SNP rats after nicotine + cue reinstatement. Adolescent nicotine sub-chronic exposure does not interact with sex or genotype to influence α6 mRNA expression in the VTA, SNg, and IPN in the *CHRNA*6 3'-UTR SNP rats (Cardenas et al., 2022). In addition, *cfos*, an immediate early gene, will need to be evaluated in nicotine + cue reinstatement in the *CHRNA*6 3'-UTR SNP rats to determine if altered *cfos* expression in limbic brain regions during reinstatement are altered.

Chapter 5:

Sex and Genotype Dependent Drug-Induced DA Release in Adolescent *CHRNA*6 3'-UTR SNP Rats

Introduction

E-cigarettes are the most prevalent tobacco product used by adolescents. From 2017 – 2019 e-cigarette use among middle and high school students increased from 9 - 16.5 percentage points, which accounts for the largest increases ever recorded for any substance among adolescents (Johnston et al., 2022). There is mounting evidence suggesting e-cigarette use predicts future cigarette experimentation (Miech et al., 2017; Soneji et al., 2017b). In addition, substantial epidemiological, clinical and preclinical studies have associated enhanced use of drugs of abuse following nicotine exposure during adolescence (Ren and Lotfipour, 2019). During adolescence, the brain undergoes critical maturational process that have important implications for behavior (Spear, 2000; Yuan et al., 2015). Changes in the levels of neurotransmitters dopamine (DA) and serotonin (5-HT) in the limbic system make adolescents more susceptible to rewards and stress. Considerable research has implicated the DA system in the ventral tegmental area (VTA) as a direct mediator of nicotine reward signal (Corrigall et al., 1994, 1992; Picciotto and Corrigall, 2002). It is paramount to understand mechanisms underlying nicotine effects and to identify possible therapeutic targets.

Nicotine primary action is to bind, activate and desensitize nicotinic acetylcholine receptors (nAChRs) in the brain and in the periphery (Stolerman and Jarvis, 1995). In the central nervous system, nAChRs are composed of homomeric α7 and heteromeric α (α2-α10) and β (β2-β4) subunits which are regionally and temporally expressed (Champtiaux et al., 2002; Hendrickson et al., 2013; Lykhmus et al., 2017; Zoli et al., 2015). The α6 nAChR subunit reaches peak mRNA

expression in dopaminergic cell bodies in the VTA and substantia nigra (SNg) during adolescence (Azam, L, Chen, Y, and Leslie, 2007). In addition, the α6 nAChR subunit is localized in the mesolimbic and nigrostriatal DA pathway which may indicate a role of α6*-containing nAChRs in nicotine-induced behaviors and DA release (* denotes other subunits) (Azam, Chen, and Leslie, 2007; Jackson et al., 2009; le Novère et al., 2002; Pons et al., 2008; Zoli et al., 2015). Neurobiological actions of nicotine are similar to other psychomotor stimulants including cocaine and methamphetamine (Laviolette and van der Kooy, 2004). It has been hypothesized that nicotine addiction is mediated by DA release in the mesolimbic system via local action at somatodendritic sites in the VTA (Di Chiara and Imperato, 1988; Nisell et al., 1994). Indeed, injections of 6-hydroxydopamine into the nucleus accumbens (NAc) blocked nicotine self-administration in adult rats (Corrigall et al., 1994). In addition, the functional role of α6* nAChRs *in vivo* have shown their involvement in nicotine-induced locomotion (Cardenas et al., 2022; Drenan et al., 2008) and nicotine self-administration (Brunzell et al., 2010; Carreño and Lotfipour, 2023; Pons et al., 2008).

Nicotine and methamphetamine (Meth) are commonly used together (Goldsamt et al., 2005). Meth is a potent analog of the stimulant, amphetamine. *In vitro* studies have shown that Meth evokes greater efflux of DA via dopamine transporter (DAT) than its counterpart, amphetamine (Goodwin et al., 2009). *In vivo* studies have shown that nicotine pretreatment enhances the acquisition of Meth self-administration and increases its intake in adolescent males, but not female rats (Cardenas and Lotfipour, 2021; Dao et al., 2011). In addition, repeated administration of methamphetamine and nicotine in mice produced locomotor sensitization effects and a symmetrical cross-sensitization (Kuriban, 1999). How nicotine and methamphetamine modulate DA release in the NAc in our novel *CHRNA*6 3'-UTR SNP adolescent is unknown.

In this chapter, we assessed the humanized *CHRNA*6 3'-UTR SNP knock-in rats in intravenous administration of nicotine and Meth on DA release. Meth is used as an alternative tool to assess the impacts of DA transmission in our rat lines. Given prior results that adolescent nicotine exposure can sex and age-dependently enhance meth self-administration (Cardenas and Lotfipour, 2021), our current studies evaluated the combined interactions in our *CHRNA*6 3'-UTR SNP rat lines. Given challenges to measure DA release in male and female adolescents during drug-seeking behavior, we modeled nicotine initiation, using a low-dose nicotine pretreatment paradigm (Cardenas et al., 2021; Cardenas and Lotfipour, 2021; Dao et al., 2011; Linker et al., 2020; McQuown et al., 2007). Subsequently, male and female adolescent rats (PN 31) underwent in vivo analytical quantification of neurotransmitters collected from the interstitial fluid from the NAc shell. DA, and its metabolites, 3,4-Dihydroxyphenulacetic acid (DOPAC), and homovanillic acid (HVA) from the extracellular space were measured by high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) methods. Our studies test the hypothesis that sex- and genotype-dependent effects will be observed for drug-induced DA release in adolescent *CHRNA*6 3'-UTR SNP rats.

Methods

Animals

Male and female wild type (WT) Sprague–Dawley rats were purchased from Charles River and bred in house with human *CHRNA*6 3′-UTR SNP rodents. As described in Chapter 2 and 3.

Drugs

Nicotine tartrate (Glentham Life Sciences, Corsham, Wiltshire, UK) was calculated as a base, dissolved in saline, with a final pH of 7.2–7.4. Methamphetamine (National Institute of Drug Abuse) was dissolved in saline and filtered via 0.22 μm sterile filters (VWR). Pentobarbital (Sigma

Aldrich, St. Louis, MO, USA) was dissolved in saline, propylene glycol, and ethanol to make Nembutal. Nembutal was further diluted to make Equithesin. Carprofen (Zoetis, Parsippany, NJ, USA) was diluted in saline. Nicotine, Equithesin, and carprofen were filtered via 0.22 μm sterile filters (VWR, Radnor, PA, USA). Propofol (Medvet, Mettawa, IL, USA) in a 5 mg/kg, intravenous (i.v.) was administered to test for catheter patency.

Surgical Implantation of Intravenous Catheter

Catheter construction and implantation was as described previously (Belluzzi et al., 2005). Animals were anesthetized with Equithesin (0.0035 ml/g body weight), and a chronic catheter was surgically implanted into the right external jugular vein. The catheter was passed subcutaneously from the animal's back to the jugular vein where the tubing was inserted. The cannula assembly was mounted on the animal's back and was sealed to prevent clogging and to keep a closed system. The wounds were closed with wound clips, antiseptic ointment was applied to the wounds, and carprofen (5 mg/kg, subcutaneous) was injected to prevent infection. The animals were kept in a warm cage for postsurgical observation until they emerged from anesthesia. Catheter patency was tested for rapid (5-10 s) anesthesia by infusion propofol (5 mg/kg, i.v.) before and after completion of in vivo microdialysis experiments (Figure 5.1).

Nicotine Pretreatment

Starting on PN 28 rats were administered nicotine (2 x 0.03 mg/kg/0.1 mL, I.V.) or saline injections spaced one minute apart daily for 3 days (Figure 5.1).

***In Vivo* Microdialysis**

Stereotaxic surgery

Immediately after implantation of the intravenous catheter, animals for microdialysis were im-planted with a cranial guide cannula. Animals were placed in a stereotaxic frame (Stoelting

Co., Chicago, Il, USA), their skulls revealed and drilled to expose the dura. A chronic guide cannula (Bioanalytical Systems, Inc., West Lafayette, IN) was stereotaxically implanted 2.0 mm above the target area, fixed to the skull with acrylic dental cement, and sealed with a dummy cannula. Anatomical coordinates for adolescent animals PN 28-29 were estimated from the adult atlas (Paxinos and Watson, 1989), then empirically determined in a preliminary experiment with histological confirmation. The following guide cannula coordinates were measured from the dura; nucleus accumbens shell AP, + 2.1 mm; ML, ±0.6 mm; DV,-6 mm.

Microdialysis

Animals were given 3 days to recover with daily handling after surgeries. Catheters were flushed daily. Intravenous catheter patency was tested by propofol 1 day before the experiment. On the experimental day, the dummy cannula was replaced with a 2 mm microdialysis probe with a 30 kDa cut-off membranes (MD-2200; Bioanalytical Systems, Inc., West Lafayette, IN). The probe membrane extended 2 mm beyond the tip of the guide canula. The quality of probes was tested in vitro before the experiment with an average recovery of 10 % (data not shown). Microdialysis was carried out under a free-moving condition in Culex NxT with Raturn – Multi Animal (Bioanalytical Systems, Inc., West Lafayette, IN), with the probe continuously perfused with artificial cerebrospinal fluid (Ringers Solution 147 mM NaCl, 2.2 mM $CaCl_2$, 4 mM KCl) at a constant flow rate of 1.1 ml/min delivered by a Empis infusion pump (CX-300; Bioanalytical Systems, Inc., West Lafayette, IN). Dialysate was collected into a Honeycomb refrigerated fraction collector (MD-1201; Bioanalytical Systems, Inc., West Lafayette, IN) that maintained the samples at 4°C. After a 4 h of perfusion to establish an equilibration between the probes' internal and external environment, microdialysis samples were collected every 20 min. When DA levels reached a stable baseline, animals were given two 0.100 ml injections (i.v.) of saline, 1 min apart.

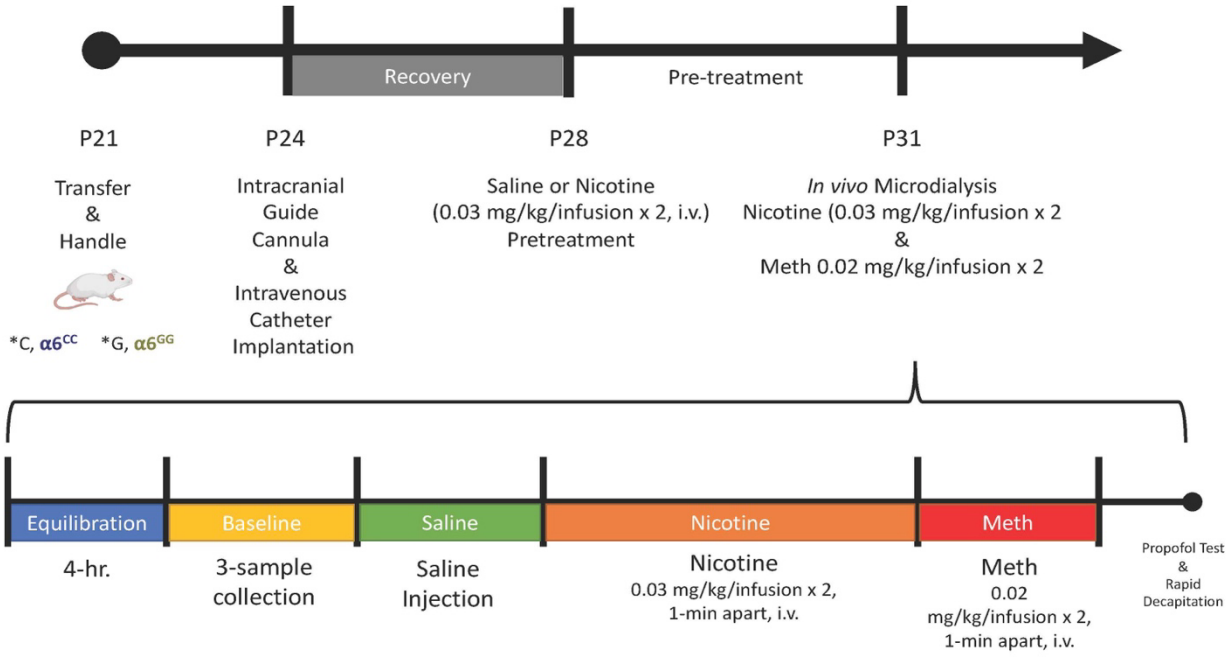

Figure 5.1 Experimental timeline.

After 100 min, nicotine (0.3 mg/kg/.100 ml injection, i.v.) was injected twice at a 1 min interval and samples were collected for another 200 min. After 220 min, methamphetamine (0.2 mg/kg/0.100 mL injection, i.v.) was injected twice at 1 min interval and samples were collected for another 100 min (Figure 1). DA and its metabolite levels were quantified by HPLC-ED. The position of microdialysis probes was verified histologically and mapped onto relevant atlas sections (Paxinos and Watson, 1986).

HPLC-ECD detection

Microdialysate samples (20ml) were automatically injected by an ESA 542 refrigerated auto-sampler onto a 1503.2 mm ODS C18column (ESA Inc.,Chelmsford MA) connected to an ESA 580 HPLC pump. The column was kept at 351C and perfused by MD-TM mobile phase (ESA, Chelmsford, MA) at a rate of 0.6 ml/min. DA and metabolite levels were determined by an electrochemical ESA 5600 detector with an ESA 5020 guard cell with the dominant potential of 300 mV. The sensitivity of the detector is 500 fg. Measurements were analyzed using

CoulArray for Windows Software 2.0 (ESA Inc., Chelmsford, MA, USA). Standard curves were generated with catecholamine (ThermoScientific, Wlatham, MA), DOPAC, and HVA (Sigma-Aldrich, St Louis, MO) standards, and levels in experimental samples were determined from the curve and expressed as ng per 20 ml, unadjusted for recovery, as there were no significant differences in probe recovery. Basal levels of DA and its metabolites were determined by averaging the samples before nicotine injection. Nicotine- and methamphetamine-induced changes in DA and its metabolite levels were expressed as area under the curve (AUC)

Behavioral Activity

The Culex NxT with Raturn – Multi Animal (Bioanalytical Systems, Inc., West Lafayette, IN) is equipped with a sensor assembly consisting of a left (clockwise activity) and right (counterclockwise activity) sensor that monitors the animal's movement. The movement is recorded by the Culex NxT with Raturn – Multi Animal software. The clockwise and counterclockwise and up activity was combined to form "cumulative time spent".

Statistics

Microdialysate samples, were analyzed by a four-way ANOVA for Sex x Genotype x Neurotransmitter x Time. Significant main effects or interactions were further tested by t-test with the Bonferroni adjustment for multiple comparisons. Pearson's correlation coefficients were assessed to compare the neurotransmitter content or turnover ratio vs cumulative time spent moving (sum of clockwise, counterclockwise and up, in seconds), reporting the RSquare and p-values, with a false discovery rate (FDR) of $p < 0.1$.

Results

An overall interaction for DA release was found for Time x Genotype x Sex x Pretreatment [$F(13,559)=2.68$, $p=0.0012$]. Thus, we evaluated these parameters separately (Figure 2). No sex or genotype differences were observed for DA baseline in acute and sub-chronic condition (Table 5.1; Figure 2a and b). Nicotine and meth induced DA release is enhanced in $\alpha6^{CC}$ females, primarily in acute condition as compared to $\alpha6^{CC}$ females. Nicotine and Meth induced DA release is enhanced in $\alpha6^{GG}$ males independent of nicotine pretreatment, but no genotype differences were observed.

For DOPAC, a DA metabolite, showed Time x Genotype x Sex x Pretreatment interactions [$F(13,520)= 1.8302$, $p=0.0036$], thus evaluating all groups separately. Baseline differences were observed for sub-chronic $\alpha6^{CC}$ males as compared to $\alpha6^{GG}$ males with greater DOPAC baseline for $\alpha6^{CC}$ males ($p<0.05$) (Table 5.2; Figure 3a and b). No significant DOPAC/DA and HVA/DA differences were observed (Table 5.2). Nicotine altered DOPAC in acute males and females $\alpha6^{CC}$ and female $\alpha6^{GG}$, but not acute $\alpha6^{GG}$ males (Figure 3c-f). On the contrary, sub-chronic $\alpha6^{GG}$ exhibited greater nicotine-induced DOPAC changes ($p<0.05$), and no differences in female $\alpha6^{GG}$ and male and female $\alpha6^{CC}$. Meth injection negatively altered DOPAC extracellular levels in all groups with significant changes from saline injections in saline pre-treated $\alpha6^{GG}$ males and sub-chronic $\alpha6^{CC}$ males.

HVA, a secondary DA metabolite showed a Time x Genotype x Sex x Pretreatment interactions $F(13,520)= 2.1027$, $p=0.0128$, therefore, we evaluated all group separately. No baseline differences were observed for HVA (Table 5.3; Figure 4 a and b). Nicotine altered HVA levels in acute pretreated $\alpha6^{CC}$ females, but not in acute pre-treated $\alpha6^{CC}$ males and $\alpha6^{GG}$ males

and females (Figure 4c-j). Meth altered HVA levels with the most impact in acute pretreated $\alpha6^{GG}$ females and sub-chronic pretreated $\alpha6^{CC}$ and $\alpha6^{GG}$ males (Figure 4c-j). Overall, the findings suggest important sex, genotype, and nicotine pretreatment effects impact drug-induced DA release and its metabolites within adolescent *CHRNA*6 3'-UTR SNP rats.

When testing locomotion, a Time x Genotype x Sex x Pretreatment effect was again observed $F(13,585)=1.8206$, $p=0.00369$. As such, we evaluated activity for these various parameters separately. *Post hoc* analysis showed similar to locomotor activity is enhanced in $\alpha6^{GG}$ males independent of nicotine pretreatment. In contrast, nicotine induced locomotor activity is enhanced in $\alpha6^{CC}$ females primarily in acute pretreated groups, while these are independent of genotype in the sub-chronic nicotine treated female rats. We also observe some unique associations when assessing DA levels and locomotion associations, which are summarized in Figure 5.5 e-g. The effects are dependent on drug, pretreatment and sex, with only three groups showing significant relationships.

Table 5-1 Adolescent male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$, Dopamine, DOPAC, and HVA baseline concentration (ng/ml).

		Female		Male	
		$\alpha6^{CC}$	$\alpha6^{GG}$	$\alpha6^{CC}$	$\alpha6^{GG}$
DA	Acute	0.1034 ± 0.042	0.1252 ± 0.039	0.0970 ± 0.036	0.1717 ± 0.034
	Sub-chronic	0.1367 ± 0.039	0.0737 ± 0.039	0.1353 ± 0.036	0.1248 ± 0.034
DOPAC	Acute	60.15 ± 15.43	41.05 ± 14.08	30.21 ± 13.04	51.13 ± 12.20
	Sub-chronic	46.05 ± 14.08	43.23 ± 14.08	59.77 ± 13.04	24.69 ± 12.20*
HVA	Acute	34.20 ± 7.22	27.51 ± 6.59	22.32 ± 6.11	13.90 ± 5.72
	Sub-chronic	21.01 ± 6.59	30.03 ± 6.59	17.46 ± 6.11	15.39 ± 5.72

Compiled acute and sub-chronic baseline concentration ± SEM for adolescent male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$. *$p<0.05$ $\alpha6^{GG}$ vs $\alpha6^{CC}$ sub-chronic.

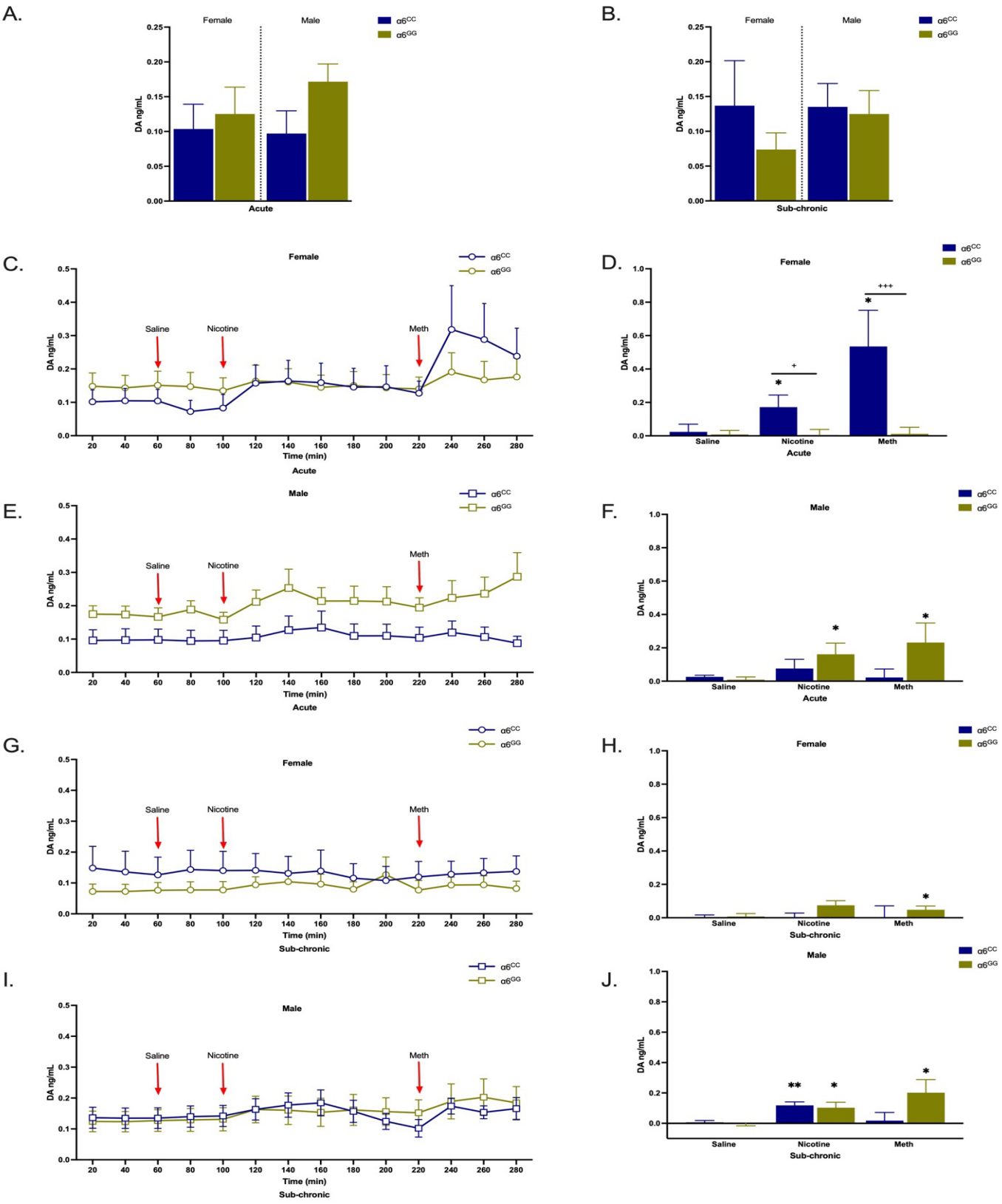

Figure 5.2 Extracellular DA levels in male and female $\alpha6^{GG}$ and $\alpha6^{CC}$. Acute (a, c and e) and sub-chronic (b, g, and i) pre-treatment. Samples were collected every 20 min. Saline, nicotine (0.03 mg/kg x 2, i.v.) and methamphetamine (0.02 mg/kg x 2, i.v.) were injected at 60, 100, and 220 mins, respectively. Area under the curve (AUC) revealed differences in a sex and genotype dependent manner. *p< 0.05 vs saline, **p<0.01 vs saline. N = 5-8/group.

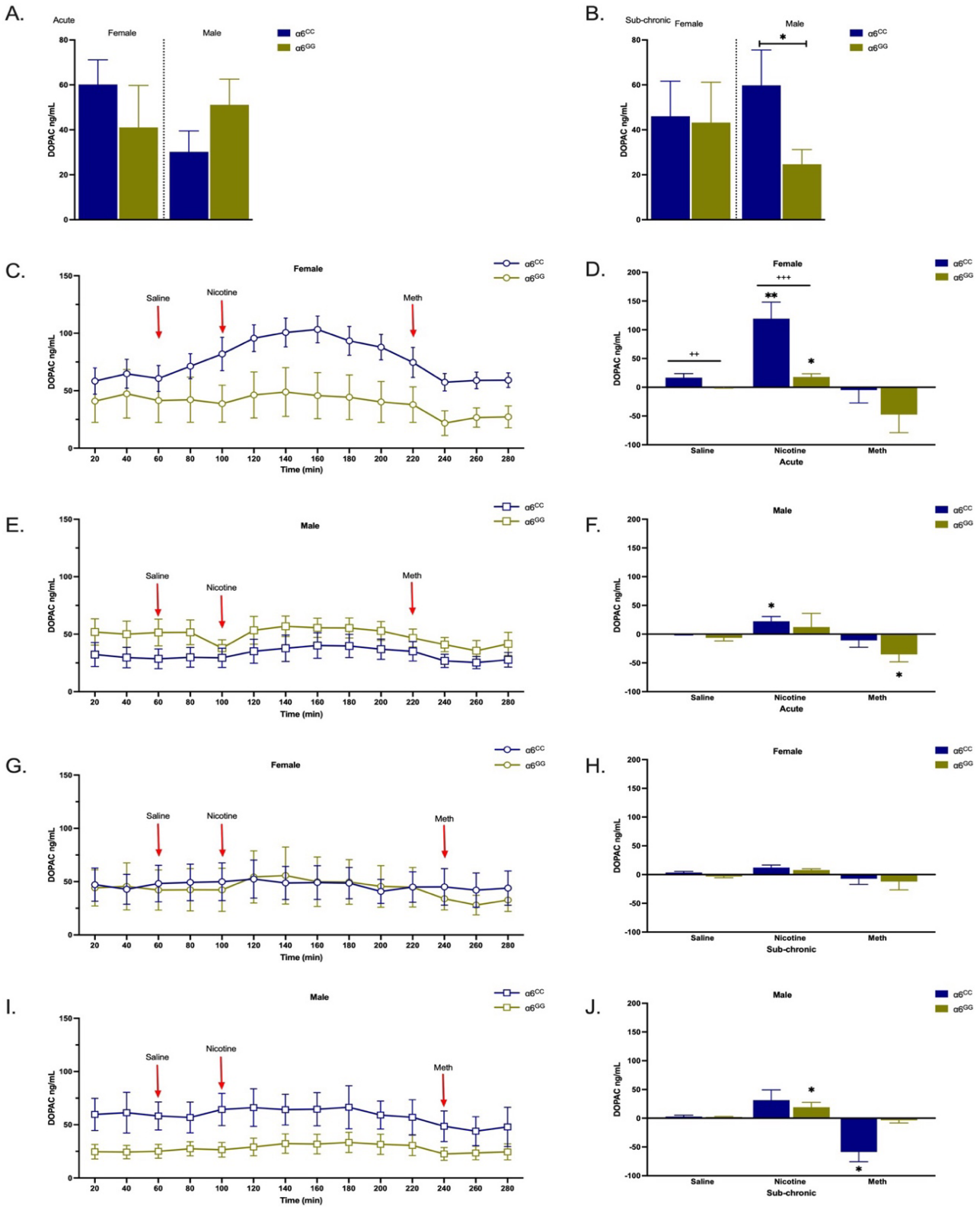

Figure 5.3 Extracellular 3,4,-Dihydrophenyalacetic acid (DOPAC) in in male and female $\alpha6^{GG}$ and $\alpha6^{CC}$. Acute (a, c and e) and sub-chronic (b, g, and i) pre-treatment. Samples were collected every 20 min. Saline, nicotine (0.03 mg/kg x 2, i.v.) and methamphetamine (0.02 mg/kg x 2, i.v.) were injected at 60, 100, and 220 mins, respectively. Area under the curve (AUC) revealed differences in a sex and genotype dependent manner. *p< 0.05 vs saline, **p<0.01 vs saline. N = 5-8/group.

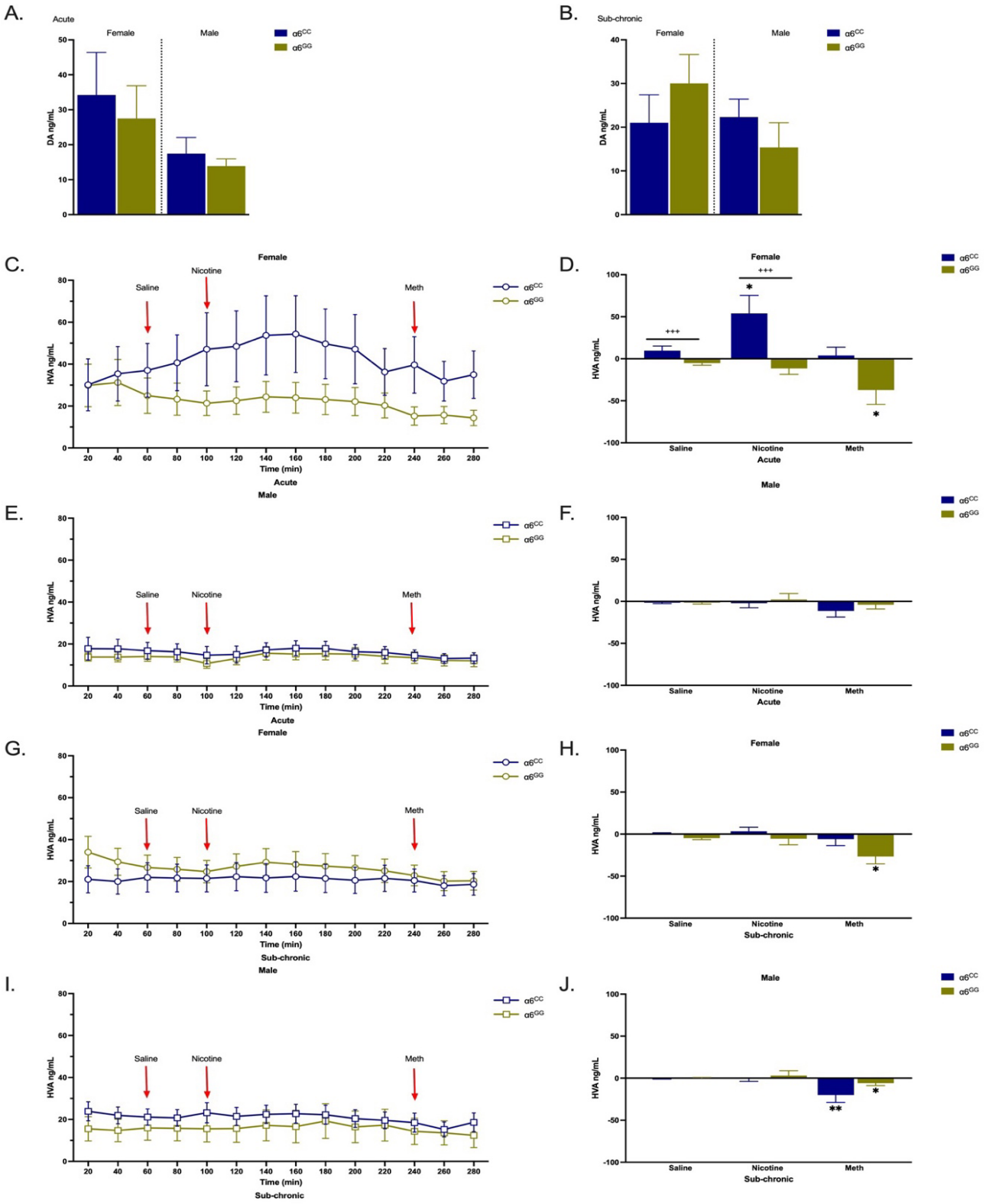

Figure 5.4 in Homovanillic acid (HVA) in male and female $\alpha6^{GG}$ and $\alpha6^{CC}$. Acute (a, c and e) and sub-chronic (b, g, and i) pre-treatment. Samples were collected every 20 min. Saline, nicotine (0.03 mg/kg x 2, i.v.) and methamphetamine (0.02 mg/kg x 2, i.v.) were injected at 60, 100, and 220 mins, respectively. Area under the curve (AUC) revealed differences in a sex and genotype dependent manner. *p< 0.05 vs saline, **p<0.01 vs saline. +++p< 0.001 $\alpha6^{CC}$ vs $\alpha6^{GG}$**.** N = 5-8/group.

Table 5-2 Adolescent male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$, turnover DOPAC/DA and HVA/DA baseline concentration (ng/mL).

		Female		Male	
		$\alpha6^{CC}$	$\alpha6^{GG}$	$\alpha6^{CC}$	$\alpha6^{GG}$
DOPAC/DA	Acute	894.60 ± 420.08	1132.97 ± 383.48	533.20 ± 355.0342	339.43 ± 332.1041
	Sub-chronic	695.46 ± 383.481	743.76 ± 383.48	903.25 ± 355.0342	323.44 ± 332.1041
HVA/DA	Acute	396.91 ± 195.16	669.59 ± 178.16	302.17 ± 164.94	85.48 ± 154.28
	Sub-chronic	241.08 ± 178.16	743.76 ± 383.48	253.33 ± 164.94	164.04 ± 154.28

Compiled acute and sub-chronic DOPAC/DA and HVA/DA turnover baseline concentration ± SEM for adolescent male and female, $\alpha6^{CC}$ and $\alpha6^{GG}$.

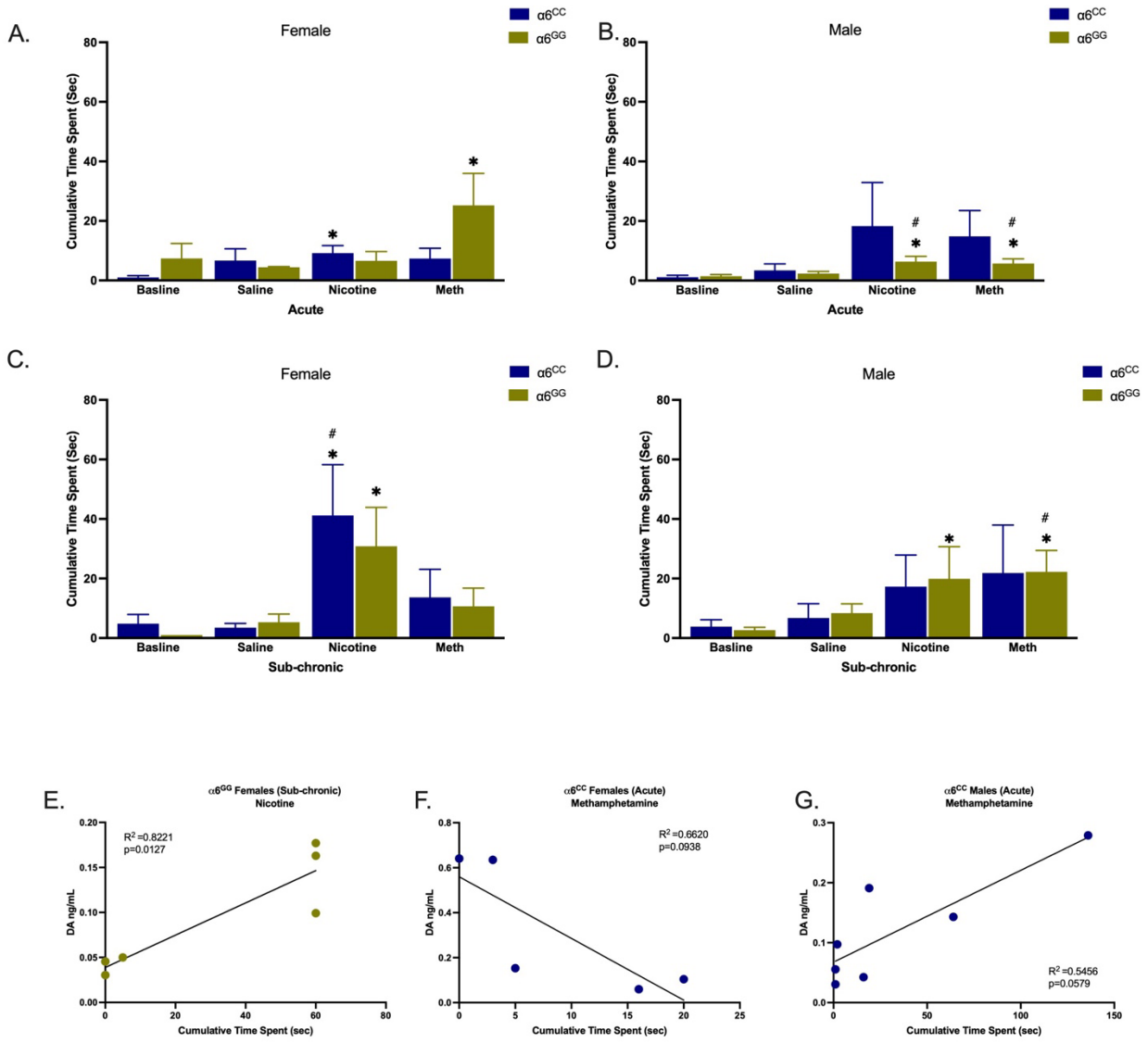

Figure 5.5 Locomotion activity and correlation between DA release and cumulative time spent. A-B. Acute nicotine and methamphetamine cumulative total time spent in seconds in in male and female, $\alpha6^{GG}$ and $\alpha6^{CC}$. C-D Sub-chronic nicotine and methamphetamine cumulative total time spent in seconds in male and female, $\alpha6^{GG}$ and $\alpha6^{CC}$. E-G. Correlation between nicotine DA release and cumulative time spent (seconds). *p<0.05 vs baseline; #p<0.01 vs saline. N = 5-8/group

Discussion

Overall, my results illustrate that drug induced DA release is enhanced in $\alpha6^{CC}$ females, as compared to $\alpha6^{GG}$ females, after acute but not sub-chronic pretreatment. No genotype differences were observed for adolescents $\alpha6^{GG}$ and $\alpha6^{CC}$ males with or without nicotine pretreatment. Additional studies are needed to evaluate genotype differences between male $\alpha6^{GG}$ and $\alpha6^{CC}$ rats. The findings provide additional evidence to suggest that *CHRNA*6 3'-UTR SNP is functional in dopaminergic neurons and regulates the release of DA levels in males and females differently. The sex and genotype effects were also observed for locomotor behavior, which resemble prior published work using a similar paradigm (Cardenas et al., 2022). While only minimal correlations were observed with NAc shell DA levels and locomotor activity, this makes sense as the NAc core is thought to better mediate activity (Boye et al., 2001; Kelsey and Willmore, 2006; Sellings and Clarke, 2003). Future studies could assess these relationships when evaluating reinstatement behavior to determine whether *in vivo* release of DA can predict reinstatement.

Behavioral studies in rodents with the *CHRNA*6 3'-UTR SNP knock-in have showed that $\alpha6^{CC}$ females and $\alpha6^{GG}$ males displayed nicotine-induced enhanced locomotor and anxiolytic behavior when compared to their saline-treated counterparts (Cardenas et al., 2022). Further, unpublished studies from the lab show that adolescent sub-chronic nicotine exposure affects Meth acquisition and maintenance in a sex- and genotype-dependent manner e.g., nicotine-treated $\alpha6^{CC}$ and saline-treated $\alpha6^{GG}$ female displayed preference for reinforced versus nonreinforced responding for Meth self-administrations for days 1-5. In contrast, nicotine pretreatment enhanced Meth self-administration in both adolescent male $\alpha6^{CC}$ and $\alpha6^{GG}$ rats, independent of genotype (unpublished Cardenas, 2022). These results suggests that the α6 nAChR subunit is important for adolescent nicotine-induced Meth self-administration. My microdialysis data support these results,

as genotype effects are only observed in females after acute nicotine pretreatment. While previous studies have evaluated α6 nAChR subunit expression in the *CHRNA*6 3'-UTR SNP rats via *in situ* hybridization after sub-chronic nicotine exposure the results yield no sex or genotype effects (Cardenas et al., 2022). Such studies are needed to understand if α6 nAChR subunit expression are needed to evaluate sex and genotype differences.

Females are more sensitive than males to acute and prolonged effects of psychoactive drugs including nicotine (Becker and Hu, 2008; Flores et al., 2017). In females, this is regulated by ovarian hormones, which can enhance dopamine release in the striatum (Cummings et al., 2014; Shams et al., 2016). Adolescence is a developmental period that extends beyond puberty with brain maturation occurring independent of pubertal influences (Cross et al., 2017; Spear, 2000; Yuan et al., 2015). DA neurons in the VTA are more extensive in females than in males and this effect is eliminated via gonadectomy of males (Johnson et al., 2010). In addition, striatal DA receptors exhibit overproduction followed by pruning in adolescent males (Andersen, 2002; Azam et al., 2007). A minor increase in striatal DA receptors has been observed for females. While we observe sex differences in females $\alpha6^{CC}$ as compared to $\alpha6^{GG}$, primarily in the acute condition, how ovarian hormones modulate DA release in the *CHRNA*6 3'-UTR knock-in is unknown. Future studies should evaluate sex differences in the *CHRNA*6 3'-UTR rat line in adolescents and adults male and females.

Our findings suggest that hyperdopaminergic mechanisms may mediate locomotor activity. In our exploratory work I was able to quantify movements (up, clockwise, counterclockwise) in the microdialysis chamber during administration of nicotine and meth. A positive correlation between DA release and behavior measurement was observed for sub-chronic $\alpha6^{GG}$ females with an $R^2 = 0.8221$, and p-value of 0.0127. Projection of the α6 subunit in the NAc shell suggests

possible involvement in reward whereas their occurrence is the striatum could imply a role in locomotor activity (Le Novère et al., 1999; Quik and McIntosh, 2006). The differential effects of DA release and locomotor activity in the present study are not necessarily correlated. Several studies have provided an explanation for these differential results by elaborating the neuroanatomical network of the midbrain dopamine system in connection with sub-circuits responsible for drug-induced reward and locomotion (Figure 5.6) (Gotti et al., 2010; Janhunen and Ahtee, 2007).

The mesolimbic and mesostriatal pathways are two major circuitries in the midbrain dopamine system. The mesolimbic pathway that originates from the VTA and terminates in the NAc shell is highly implicated in reward processing. The mesostriatal DA pathway which originates from the SNg and projects into the dorsal striatum (caudate putamen) involved in locomotor activity. Differences in expression patterns and pharmacological properties of nAChRs subtype differ between the mesolimbic and mesostriatal dopamine pathways as illustrated in Figure 5.6 (Janhunen and Ahtee, 2007). Literature suggests the α6β2 is prevalent in the ventral striatum. The α6β2 may explain differences in either systemic and intravenous nicotine administration of the mesostriatal and mesolimbic pathways. Nicotinic receptors containing either α6 (non-α4) and α4α6β2 may have different pharmacological and physiological properties. For example, the α4α6β2 receptor has higher affinity for nicotinic agonist, αCtx MII, than their counterpart α6β2 (Salminen et al., 2007). Additionally, α4α6β2β3, make up 50-60% of the α6* nAChR subtype expressed in dopaminergic nerve terminals in mouse striatum, which is more sensitive to activation by nicotine than other nAChRs subtypes (Salminen et al., 2007). Further, Intra-αCtx MII and αCtx-PIA, α6 nAChR subunit antagonist, perfused in the VTA inhibited nicotine DA release in the NAc and habituated locomotion (Gotti et al., 2010). Mice expressing a

gain of function, $\alpha6^{L9S}$, mutation exhibit spontaneous locomotion that is exaggerated by low dose of systemic nicotine (Drenan et al., 2008). Thus, intravenous nicotine administration in our model can potentially explain the lack of locomotor effect and DA release in the nucleus accumbens. How such mechanisms may mediate reinstatement behavior need to be assessed in future studies. Further, to identify the role of α6 nAChRs mediating these mechanisms, future studies could assess intra-NAc shell or -VTA nAChR antagonist such as α-Ctx MII to test the neuroanatomical locus as contributing to elevated nicotine reward seeking behavior in the *CHRNA*6 3'-UTR SNP knock-in rats. Such studies could provide additional insights as to how the *CHRNA*6 3'-UTR SNP modifies dopaminergic circuits, which may assist in improving prevention and intervention strategies in the future.

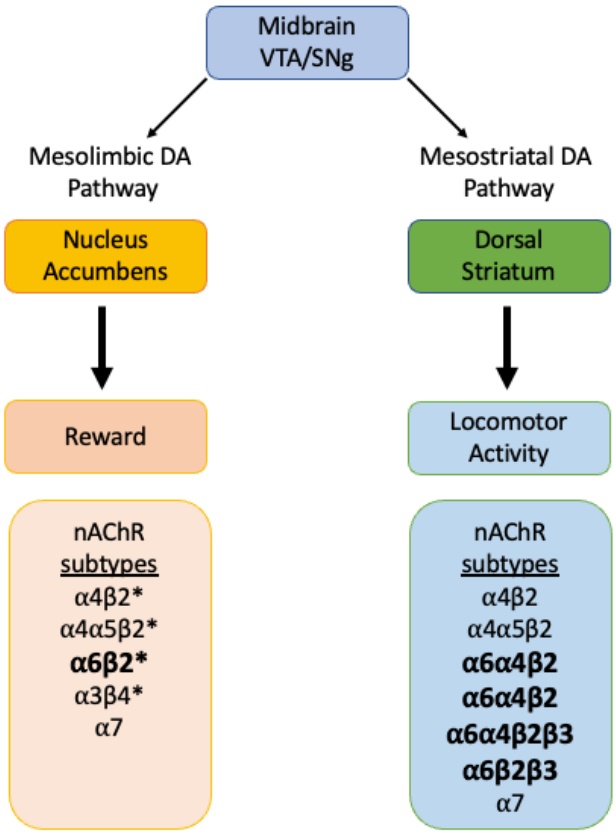

Figure 5.6 Nicotinic acetylcholine receptors in the mesolimbic and mesostriatal pathways. The mesolimbic pathway that originates from the VTA and terminates in the nucleus accumbens is highly implicated in reward processing. The mesostriatal dopamine pathway that originates from the SNg and projects to the dorsal striatum is involved in the control of locomotor activity. Expression patterns and pharmacological properties of nAChR subtypes differ between mesolimbic and mesostriatal dopamine pathways, suggesting differential contributions of nAChR to the functions of the two pathways The nAChR subtypes expressed in the two dopamine pathways include but not limited to the one shown in the table. * denotes other subunits. Modified from Calabresi and Di Fillippo, Neuron, 2008

Chapter 6:

General Discussion: Implications and Future Directions

Summary

This dissertation evaluated sex and genetic effects to understand the role of *CHRNA*6 3'-UTR SNP in nicotine-self-administration, reward, extinction, and reinstatement behavior. Research herein demonstrated no genotype or sex effects impacting natural rewards, nicotine self-administration, extinction, and nicotine-seeking in male and female wild type Sprague Dawley rats. Subsequently, evaluating nicotine-seeking behavior in our novel *CHRNA*6 3'-UTR SNP knock-in rat line revealed sex and genotype dependent effects. Male $\alpha6^{GG}$ rats exhibited potentiated nicotine + cue-induced reinstatement when compared to males $\alpha6^{CC}$. To understand the mechanisms involved, neurochemical profiles in naïve and nicotine-seeking male and female adolescents containing the *CHRNA*6 3'-UTR SNP were evaluated in brain reward circuitry. Our results illustrate neurotransmitter levels are genotype and sex-dependent in several brains regions in adolescent naïve *CHRNA*6 3'-UTR SNP rats, whereas these effects are blunted during reinstatement. Correlations between behavior and tissue level neurotransmitters, showed that extracellular DA turnover in the NAc shell and PFC increases with nicotine seeking behavior in $\alpha6^{GG}$ males exclusively. These correlation effects are opposite in the LC. DA may be a key player in tissue levels mediating nicotine seeking behavior in our *CHRNA*6 3'-UTR SNP rats. NE is greater in the LC in $\alpha6^{GG}$ males and $\alpha6^{CC}$ females suggesting the LC may mediate reinstatement behavior. Finally, I assessed the functional impact of the *CHRNA*6 3'-UTR SNP via drug-induced DA release in our humanized *CHRNA*6 3'-UTR SNP male and female adolescents rats, within the primary drug reward region of the brain, i.e., NAc shell. Drug-induced DA release is enhanced in acute and sub-chronic treated male $\alpha6^{GG}$ as measured from the change of baseline however, no

genotype differences were observed for males. Conversely, in females, acute, but not sub-chronic enhanced drug induced DA release in $\alpha6^{CC}$ but not $\alpha6^{GG}$ rats. Taken together, these findings provide evidence to suggest the *CHRNA*6 3'-UTR SNP is functional in dopaminergic neurons and regulates the release of DA levels uniquely in males and females.

α6-Containing Nicotinic Receptors and Nicotine-Induced Behavior

Based on our data, it is expected that antagonizing α6β2* nAChRs in the VTA with local infusions of α-Ctx-MII or α-Ctx-PIA would block the effect of nicotine self-administration and DA release. α-Ctx-MII, is a putative selective antagonist of α6β2 and α3β2 nAChRs (Azam and McIntosh, 2005; Cartier et al., 1996; Salminen et al., 2007), whereas α-Ctx-PIA, an alpha-conotoxin derivative which is more selective for α6β2 (Dowell et al., 2003). This will further support the role of α6* nAChRs in mediating drug reward phenotypes.

Several stressors e.g., intermittent foot shock, acute food deprivation, and yohimbine, an alpha-2 adrenergic receptor antagonist, have been shown to induce drug-seeking behavior in male adult and adolescents rats (Bertholomey et al., 2016; Buczkowski et al., 2014; Chen et al., 2015; Gellner et al., 2023; Lê et al., 2005; Shaham et al., 2003; Yuan et al., 2017). How stress, either physical or through pharmacological agents, will reinstate drug-seeking behavior in male and female adolescents containing the *CHRNA*6 3'-UTR should further be explore. Naïve adolescent female $\alpha6^{CC}$ and male $\alpha6^{GG}$ exhibit greater NE in the locus coeruleus when compared to female $\alpha6^{GG}$ and male $\alpha6^{CC}$, respectively. The locus coeruleus, a noradrenergic system, is the main source of noradrenaline in the central nervous system (Gina R. Poe et al., 2020). Locus coeruleus neurons innervate anatomically and functionally distinct regions including the prefrontal cortex and thalamus and midbrain. In addition, DA has been identified as a co-transmitter in LC noradrenaline cells (Devoto et al., 2005, 2001). DA-NE corelease in reward circuitry regions especially in the

PFC, will further need to evaluate and may provide a mechanism for stress and reward action of psychostimulants.

The α6β2* nAChRs have been associated with nicotine use and dependence in human. Candidate gene studies demonstrate gene cluster encoding α6 and β3 subunits are associated with nicotine dependence such as cigarettes per day, positive subjective response to nicotine, and smoking initiation (Culverhouse et al., 2014; Nicole R. Hoft et al., 2009; Saccone et al., 2010, 2009; Thorgeirsson et al., 2008; Wang et al., 2014; Zeiger et al., 2008). Future studies examining withdrawal in *CHRNA*6 3'-UTR SNP rats would complement this existing literature to evaluate how the genetic variant affects behaviors associated with dependence. Following 7-14 days of continuous nicotine exposure, withdrawal can be evaluated following the removal of nicotine or administration of a nicotinic receptor antagonist, i.e., mecamylamine (Kenny and Markou, 2001; Malin, 2001). Negative affective states produced by withdrawal such as physical signs and anxiety-like behavior can be assessed in our *CHRNA*6 3'-UTR SNP rodents. α6 nAChRs are known to impact affective (emotional), but not somatic (physical) withdrawal symptoms (Jackson et al., 2009). These findings will further imply that targeting α6* nAChRs may prove successful for treatment of tobacco dependence.

The interaction between prenatal exposure to maternal cigarette smoking and adolescence lifetime smoking has also been observed with greater impact on the GG-allele carriers (Lotfipour et al., 2010). Further, preclinical models of gestational nicotine exposure have shown to alter the adolescent dopaminergic system in corticostriatal circuits (Dwyer et al., 2019). Impact of gestational nicotine exposure in the adolescent *CHRNA*6 3'-UTR SNP rats will assist in understanding the effects on early developmental nicotine exposure as well as the genetic predisposition in substance use, via an altered DA system.

Mechanisms mediating α6-containing Nicotinic Receptors

Mechanisms mediating nicotine + cue seeking behavior suggest DA alterations to be a key component. Naïve adolescent animals exhibit genotype and sex dependent effects at baseline, suggesting a hyper-excitability of α6* nAChRs in response to endogenous Acetylcholine (ACh) (Cohen et al., 2012). How such factors can contribute to behavior requires further assessment. Future studies should test the role of ACh, the endogenous agonist of nicotinic receptors and assessing its impact on locomotion, DA release and DA neuron firing patterns.

Future studies should quantify neurotransmitter release via *in vivo* microdialysis, DA sensors, and neuronal activity during nicotine + cue reinstatement. Discerning how α6β2* nAChRs in the mesolimbic pathway may regulate nicotine reward should be explored. Potentially nicotine activates α6β2* nAChRs on DA terminals in the nucleus accumbens to promote nicotine reward, which modulated by α6β2* nAChRs-mediated DA release. Equally nicotine activates α6β2* nAChRs in the VTA neuron soma to drive DA release, promoting nicotine reward. Future studies should investigate intra-NAc shell α-Ctx effect on DA release and nicotine reward-seeking behavior in *CHRNA*6 3'-UTR SNP rats. Administration of an α6 antagonists directly into the VTA should attenuate nicotine reinforcement (Corrigall et al., 1994; Nisell et al., 1994).

Activation of KOR and NE receptors systems in dopaminergic circuits results in stress-induced reinstatement of drug-seeking. It is paramount to assess stress-induced reinstatement in adolescent *CHRNA*6 3'-UTR SNP rats. It is unknown how stress and LC NE cell firing and dynorphin/KOR signaling occurs in the CHRNA6 3'-UTR SNP line.

Previously it was demonstrated that nicotine pretreatment, genotype, and sex do not alter the α6 subunit nAChR mRNA expression in our novel *CHRNA*6 3'-UTR SNP rats (Cardenas et al., 2022). Nicotine is known to cause upregulation in chronic administration. Nicotine-induced

upregulation has been observed for α4β2* and α7 nAChRs in the midbrain, hippocampus, and cortex persisting into adulthood (*signifies other nAChR subunits) (Doura et al., 2008). In wild type adolescents (PN 42) chronic nicotine treatment down-regulated α6 expression (Doura et al., 2008). It is not known if α6 nAChR mRNA expression is altered during the reinstatement of nicotine + cue or other behavioral parameters. Future studies will need to evaluate α6 nAChR mRNA and protein receptor density.

General Conclusion

Overall, the studies in this dissertation provide evidence the *CHRNA*6 3'-UTR SNP is functional *in vivo*, promotes neurochemical differences and associated behaviors that are related to nicotine addiction in adolescents. Given the increasing use of e-cigarettes among adolescents, a vulnerable population in developing nicotine and subsequent drug dependence, it is critical to identify genetic predisposition, but also novel therapeutic targets. Preclinical studies presented herein advances our understanding of the mechanisms underlying nicotine addiction phenotypes based on the *CHRNA*6 3'-UTR SNP, which may assist in improved therapeutic interventions in the future.

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