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Sex Differences In The Mechanisms That Modulate The Neurochemical Effects Of Nicotine

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SEX DIFFERENCES IN THE MECHANISMS THAT MODULATE THE NEUROCHEMICAL EFFECTS OF NICOTINE

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Dedication

Dedicated to my family: Jeffrey, Maria Nelly, and Luis Enrique Uribe,

and my girlfriend, Paige D. Williams.

Thank you for your never-ending love and support.

SEX DIFFERENCES IN THE MECHANISMS THAT MODULATE THE NEUROCHEMICAL EFFECTS OF NICOTINE

by

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DISSERTATION

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Abstract

Prior behavioral studies in our laboratory revealed that overexpression of the stress peptide, corticotrophin-releasing factor (CRF), in the mesocorticolimbic reward pathway enhanced the reinforcing effects of nicotine. The latter effect was greater in female versus male rats, suggesting that females are uniquely susceptible to the effects of stress on nicotine reinforcement. The goal of this dissertation was to examine the neurochemical mechanisms by which overexpression of CRF in the nucleus accumbens (NAc), a terminal region of the mesocorticolimbic pathway, modulates the behavioral effects of nicotine. Specifically, we examined whether nicotine-induced dopamine levels are altered by CRF overexpression via excitatory (glutamate) and/or inhibitory (γ-aminobutyric acid; GABA) amino acid control of dopamine release in the NAc of female versus male rats. The animals first received intra-NAc infusion of a viral vector (AAV2/5-CRF) to overexpress CRF mRNA in one hemisphere. In the contralateral NAc, a control viral vector (AAV2/5-GFP) was infused. Three weeks later, the rats were implanted with dialysis probes in the NAc of each hemisphere. The following day, dialysate samples were collected during baseline and then following administration of two doses of nicotine (0.3 and 0.6 mg/kg/expressed as base). Dopamine, GABA, and glutamate levels were assessed using liquid chromatography-mass spectrometry procedures. The results revealed that during baseline, there were no sex differences in any of our neurochemical measures following intra-NAc infusion of the control virus. Our statistical analysis revealed that only glutamate levels were significantly altered by CRF overexpression in the NAc. Specifically, in males, overexpression of CRF produced an *increase* in NAc glutamate levels during baseline and following nicotine administration. However, in females, overexpression of CRF produced a *decrease* in NAc glutamate levels during baseline and following nicotine administration. These data suggest that stress systems promote excitatory glutamatergic regulation of the NAc in a sex-

dependent manner. Future studies are needed to examine the role of different glutamate receptors in the NAc in modulating the behavioral effects of nicotine in female versus male rats.

Keywords: tobacco use, sex differences, microdialysis, dopamine, amino acids, CRF

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Chapter 1: Introduction

1.1 Tobacco use is a public health burden

High rates of tobacco use are a major economic and health concern. According to the Center for Disease Control (CDC) and the World Health Organization (WHO), tobacco use is the leading cause of preventable death and disease in the United States. With approximately 1.1 billion smokers worldwide, the WHO has predicted that by 2030, tobacco use will cause an estimated 9 million deaths per year (WHO, 2011). Approximately 16 million people in the United States live with a disease that is caused by smoking, such as chronic obstructive pulmonary disease, emphysema, bronchitis, and various types of cancer (CDC, 2016). Xu et al (2015) found the annual healthcare costs associated with tobacco smoking are more than $170 billion dollars, with public healthcare such as Medicare and Medicaid accounting for more than 60% of those expenditures.

Women appear to be more susceptible to use tobacco products despite the known health consequences. Although smoking rates are similar in men and women, females are less likely to stop smoking and are more susceptible to the negative health consequences associated with long-term smoking as compared to men (Lombardi et al., 2011; Surgeon General, 2014). Women report more often than men that they smoke in response to negative affective states (Perkins et al., 1999, 2012, and 2013; Stanton, 1995). Women also display stronger anxiety, depression, and intense craving during smoking abstinence as compared to men (Leventhal et al., 2007; Panagiotakopoulos & Neigh, 2014; Pang et al., 2018; Schnoll et al., 2007). Women with a prior history of an anxiety disorder are also more likely to smoke and show higher relapse rates when compared to men (Brook et al., 2012). Comparatively, there is a stronger co-morbid association between stress disorders and smoking in women relative to men (Mykletun et al., 2008). Women with post-traumatic stress disorder report higher ratings of negative affect during abstinence (Dedert et al., 2012) and display greater expectancies that smoking will relieve the negative affective states

(Langdon and Leventhal, 2014). In general, women exhibit lower smoking cessation rates because of a more severe withdrawal syndrome and, in part, the lack of efficacy and benefit of nicotine replacement therapy as compared to men (Cepeda-Benito et al., 2004; Perkins 2001; Perkins and Scott, 2008; Piper et al., 2010).

Although there are over 4,800 chemicals in tobacco products, nicotine is the major habit-forming compound in tobacco products (Balfour, 2004). Nicotine is a mild stimulant and when consumed, induces a feeling of euphoria, alertness, relaxation, and motor activation (Heishman et al., 2010; Hukkanen et al., 2005; Le Foll and Goldberg, 2009). Indeed, nicotine is readily self-administered by humans, dogs, rodents, and primates (Caille, 2012; Corrigall, 1999; Harvey et al., 2004; Katner et al., 2004; Risner et al., 1983). The recent rise in electronic nicotine delivery systems (i.e., ENDS) speaks to the strong motivational effects of nicotine (Drope et al., 2017; Yoong et al., 2019). Following cessation from chronic nicotine use, a withdrawal syndrome emerges that involves physical, affective, and cognitive aversive symptomatology. Importantly, it is the avoidance of these negative symptoms that promotes the continued use of nicotine products (O'Dell et al., 2014). Thus, the motivational effects of nicotine are modulated by its acute pleasurable effects and avoiding the negative aversive states that emerge during withdrawal from chronic nicotine use (Balfour, 2004; Mansvelder et al., 2002; Sohn et al., 2003).

1.2 Mesocorticolimbic pathway

The mesocorticolimbic pathway originates in the ventral tegmental area (VTA) and terminates in several forebrain structures including the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Aston-Jones and Harris, 2004; Carelli and Wightman, 2004; Koob, 1996). This pathway has been has been studied extensively with regard to modulating the rewarding effects of abused drugs such as nicotine, ethanol, cocaine, and morphine (Corrigall, 1999; DiChiara et al., 1988; Schuette et al., 2013). Lesions of the dopaminergic fibers in the NAc or administration of dopamine

antagonists into the NAc abolish the psychostimulant and rewarding effects of drugs (Bari and Pierce 2005; Kelly and Iversen, 1976; Roberts and Koob 1982). In addition, several behavioral animal models of drug addiction such as sensitization, conditioned place preference, and intravenous self-administration (IVSA) are associated with increased dopaminergic activity of the NAc (Beckmann et al., 2011; Cadoni and Di Chiara, 2000; Torres et al., 2015). Together, these studies suggest that the NAc is a critical structure that modulates the addictive properties of drugs of abuse.

Much work has established that the behavioral effects of nicotine are due to activation of nicotinic acetylcholine receptors (nAChRs) in the brain, particularly in the mesocorticolimbic pathway. nAChRs consist of pentameric transmembrane proteins of homomeric or heteromeric α or β receptor subunit complexes. Nicotine binds to nAChR subunits, resulting in channel activation and/or desensitization. There are different nAChRs expressed on the γ-aminobutyric acid (GABA) and glutamate inputs that regulate dopamine release in the NAc (Johnson et al., 1992; Kalivas et al., 1989; Kalivas et al., 1993; Mansvelder et al., 2003; Mansvelder & McGehee, 2002; Xu et al., 2006). Dopamine cell bodies in the VTA express α2-10 and β2-4 nAChRs subunits that project to the NAc (Mansvelder et al., 2003). In addition, within the VTA, GABAergic interneurons express α4β2 nAChRs and PFC glutamate terminals express α7 nAChRs. Following administration of nicotine, α4β2 sites on intra-VTA GABAergic interneurons are activated but then quickly desensitize (Mansvelder et al., 2002). As a result, VTA dopamine neurons receive less GABAergic inhibition. This effect of nicotine produces a reduction in inhibition that results in enhanced activation of dopamine neurons. Nicotine also binds and sensitizes α7 nAChRs on PFC glutamate terminals, causing a larger release of glutamate and a shift towards enhancement of VTA dopaminergic neurons. (Wooltorton et al., 2003). As a result, nicotine produces a shift from tonic inhibition towards excitation of VTA dopamine neurons that results in enhanced dopamine

transmission in the NAc. Overall, desensitization of the α4β2 sites on VTA GABA neurons leads to disinhibition of dopamine neurons thereby facilitating a more sustained increase in the release of dopamine in the NAc following nicotine administration.

1.3 NAc cytoarchitecture

The NAc is a heterogeneous structure that integrates information from limbic and cortical structures such as the frontal cortex, basolateral amygdala, hippocampus, and bed nucleus of stria terminalis (Xu et al., 2020). The NAc is composed of two distinct subdivisions that differ in projection targets, neurochemical profile, and cytoarchitecture (Wright and Groenewegen 1996). The NAc core is an important in fine tuning and guiding motor behavior (Everitt et al., 2008), whereas the NAc shell is important in the processing of emotional information related to environmental stimuli and motivation (Kelley, 2004; Park et al., 2019). The shell of the NAc is composed of GABAergic medium spiny neurons (>90%) that project back to the VTA, PFC, and the caudate putamen (Meredith et al, 2008). It also has local populations of cholinergic and GABAergic interneurons (<10%) (Castro and Bruchas 2019; Meredith 1999). There has been evidence that these local population interneurons can be categorized into three populations; 1) parvalbumin-positive neurons, 2) somatostatin-positive neurons, and 3) acetylcholine-releasing neurons (Kawaguchi, 1993; Tepper et al., 2010). There is also evidence that suggests the somatostatin-positive neurons are expressed most densely in the NAc shell (Ribeiro et al., 2018). Interestingly, while there is a decrease in the number of parvalbumin-positive neurons, there is a concomitant increase of somatostatin-positive neurons and acetylcholine-releasing neurons in the NAc shell (Castro and Bruchas 2019). This suggests that GABAergic and cholinergic interneurons are in greater number and exert greater action in the NAc shell than in the NAc core.

1.4 Stress

The hypothalamic-pituitary-adrenal (HPA) axis modulates the stress response to maintain or return to a homeostatic equilibrium in the body. The stress response begins with the activation of the paraventricular nucleus of the anterior hypothalamus, where parvocellular cells synthesize and release the stress peptide, corticotrophin-releasing factor (CRF), into the median eminence. CRF then travels through the portal system and binds to CRF receptors located on the anterior pituitary gland, causing the release of adrenocorticotropin hormone (ACTH) into the hypophysial portal circulation. Finally, ACTH binds to glucocorticoids receptors on the fasciculate cells of the adrenal cortex, stimulating the release of the stress hormone, cortisol in humans or corticosterone in rodents, and initiating the stress response (Vale et al., 1982; Thiel et al., 1999).

CRF has been implicated as a neuromodulator in extrahypothalamic brain regions that govern limbic and cognitive functions (Smith et al., 2006; Swanson et al., 1983). CRF binds to and exerts its actions through two distinct G-protein coupled receptors, CRF1 and CRF2. One of the roles of the CRF1 receptor is activating the HPA axis, resulting in changes of behavioral, affective, and cognitive function. Genetic knockout of CRF1 receptors in rodents results in the attenuation of anxiety-like behaviors such as freezing, avoidance, and startle responses (Heinrichs et al., 1997; Hikichi et al., 2000; Schulz et al., 1996; Smith et al., 1998). The distribution of CRF1 receptors in the brain reflects its many roles as studies have found its expression in the cortices, hypothalamus, and throughout the mesolimbic pathway (Albrechet-Souza et al., 2015; Van Pett et al., 2000). Although there is conflicting literature with the specific role of CRF2 receptors in stress (Ho et al., 2001; Takahashi et al., 2001), it is generally thought that CRF2 receptors reduce anxiety-like behavior (Bale et al., 2004). In contrast to CRF1, CRF2 receptors expression is restricted to discrete brain regions including the periaqueductal gray and lateral septum (Hauger et al., 2006; Sanders et al., 2016). Although there is expression of CRF2 receptors in the NAc, their distribution is sparse in comparison to CRF1 receptors, suggesting the actions of CRF1 largely control

neurotransmission in the mesocorticolimbic pathway (Hauger et al., 2006; Lemos et al., 2012; 2019).

In addition to initiating the stress response, CRF plays a neuromodulatory role in the NAc. There is a population of CRF mRNA positive neurons in the NAc, suggesting that the possible presence of local stress systems that modulate dopamine release and dopamine-dependent behaviors (Chen et al, 2012; Lemos et al., 2019; Merchenthaler et al., 1984; Peng et al., 2017). Stress systems in the NAc shell have been implicated in mediating the transition of causal drug use into dependence via potentiation of the rewarding effects of drugs and the emergence of a negative affective and emotional withdrawal syndrome (Koob 2010; Koob et al., 1996; Roberto et al., 2017). Both acute and chronic administration of drugs of abuse activates the HPA axis (Goeders 1997; Piazza et al., 1993; Piazza et al., 1997). Pre-clinical research has shown chronic nicotine use recruits stress systems in extrahypothalamic areas that promote nicotine use in rodent models (Koob and Kreek, 2007; Semba et al., 2004). Brain slices from male mice have shown that CRF increases dopamine release in the NAc (Lemos et al., 2012) and administration of yohimbine, a pharmacological stressor that increases CRF, reinstates extinguished nicotine IVSA in both female and male rats (Feltenstein et al., 2012). Overall, these data show that CRF systems in the NAc potentiate the behavioral effects of nicotine, as described below.

1.5 Dissertation Studies

Prior to my Dissertation studies, I co-authored a meta-analysis showing that intact females display higher levels of nicotine IVSA than males (Flores et al., 2019). To better understand the mechanisms of sex differences in the behavioral effects of nicotine, my subsequent work compared nicotine self-administration following viral overexpression of CRF in the NAc shell. The results

revealed that CRF overexpression in the NAc shell increased the reinforcing effects of nicotine in a sex- and ovarian hormone dependent manner (Uribe et al., 2020). Specifically, overexpression of CRF in the NAc selectively increased the reinforcing effects of nicotine in intact female versus male rats. The latter effect was absent in ovariectomized (OVX) females, suggesting that the potentiating effects of CRF overexpression in the NAc are ovarian-hormone dependent. Real time quantitative polymerase chain reaction (rt-qPCR) methods also revealed that CRF overexpression enhanced mRNA expression of CRF1 and CRF2 receptors to a greater extent in intact female versus male rats. Overexpression of CRF also produced a larger increase in β-arrestin2 (Arrb2) in male versus intact and OVX female rats. This is important, as Arrb2 is a protein that internalizes CRF receptors. These data suggest that CRF systems in the NAc play a role in modulating sex differences in the behavioral effects of nicotine. To verify that the effects of the viral vector were localized in the NAc (and not due to the vector being retrograde transported to the VTA), we collected coronal slices of the NAc shell and VTA. Subsequent immunohistochemistry analysis revealed co-localization of viral replication of green fluorescence protein (GFP) and the neuronal markers, neuronal nuclei (NeuN) and 4′,6-diamidino-2-phenylindole (DAPI), in the shell of the NAc. Viral infusions produced a stable and localized overexpression of GFP in the NAc shell and analysis of AAV2/5-mediated transduction efficiency in neurons revealed that a majority of GFP-positive cells were DAPI- and NeuN-positive (90%), suggesting that the cells that were transduced by the virus were neurons. Importantly, we did not observe any fluorescence in the VTA, suggesting that overexpression of CRF was isolated to the NAc and not back transported to the cell body region of the VTA. This prior work was important for validating our viral overexpression procedures. However, there were still remaining questions regarding the manner in which stress systems modulate the neurochemical environment of the NAc, particularly following nicotine administration in female and male rats.

To address this question, my dissertation work assessed whether overexpression of CRF in the NAc alters inhibitory (GABA) and excitatory (glutamate) amino acid regulation of dopamine release in female and male rats during *baseline* (**Aim 1**) and following *nicotine* administration (**Aim 2**). We examined dopamine, glutamate, and GABA in the NAc of female and male rats that received intra-NAc infusions of the viral vector, AAV-CRF. In the contralateral hemisphere, rats received intra-NAc infusions of the control vector, AAV-GFP. Three weeks later, dialysate levels of dopamine, glutamate, and GABA were assessed in the NAc of both hemispheres during baseline and then following nicotine administration. It was ***hypothesized*** that CRF overexpression would enhance dopamine release in the NAc to a greater extent in females that experience stronger nicotine reward than males based on our prior work published in Uribe et al., (2020). The ***rationale*** for our hypotheses is based on previous work showing acute activation of CRF increases dopamine levels in the NAc (Chen et al., 2012) and intra-NAc infusions of CRF produce place preference in naïve rats (Lemos et al., 2012). Another report found overexpression of CRF in the NAc selectively enhanced the reinforcing effects of nicotine in female versus male rats (Uribe et al., 2020). We also anticipated that the increase in dopamine levels would be modulated via greater excitatory glutamate and reduced GABA inhibition in the NAc.

Chapter 2: Materials & Methods

2.1 Adeno-associated virus

Advancements in the fields of neuroscience and virology have resulted in the application of viral vectors that can serve as tools to alter the expression of biological material in neurons. Viral vectors incorporate into the nucleus of the cell to alter expression levels of the gene of interest, such as CRF. In this way, viral vectors allow researchers to evaluate the functional role of an array of different genes in discrete brain regions in specific neurons that are infected (Aschauer et al., 2013). Different serotypes of the AAV vector determine the rate and efficiency at which the AAV infects the cell. We chose the AAV2/5 hybrid serotype because it transduces neurons effectively (Wu et al., 2006) and reliably maintains gene expression for up to 8 weeks (Qi et al., 2014).

Two viral vectors were obtained from a commercial vendor (Vector BioLabs, Philadelphia, PA, USA) and were made to a specific titer concentration in a previously published paper (Qi et al., 2014). The first viral vector is the AAV2/5-CRF. The viral vector has the rat CRF cDNA (GenBank accession number NM_031019) packaged into it. The second viral vector is the AAV2/5-GFP and will be the control vector. The viral vector has the humanized green fluorescent protein (GFP) cDNA packaged into it. GFP is an inert protein that has no biological effect and exhibits green fluorescence when exposed to 488 nm to 510 nm spectrum range. An advantage of a commercial vendor is the viral vectors can be obtained in a timely manner, have been shown to have a high transduction efficiency in neurons (>90%), and maintains reliable expression of CRF and GFP for 3-8 weeks.

The AAV follows a preprogrammed infection cycle (Synder and Moullier, 2011; Wu et al., 2006). The AAV enters into the nucleus of the cell, whereby it goes into the leading strand of the DNA and replicates using the cellular polymerase to replicate (Goncalves 2005). Replication of the virus leads to increases of mRNA of the gene of interest, leading to translation and increases

of the protein (Georgiadis et al., 2016). An important caveat is that while the AAV increases mRNA expression, it does not always lead to increases of protein expression (Anderson et al.,, 1997; Washburn et al., 2003). This may be due to several reasons such as degradation or differences in translation per mRNA (Li et al., 2017) . However, a prior study from our laboratory has shown inta-NAc infusion of the AAV2/5-GFP does result in increases of protein expression of GFP as shown by an increase in florescence (Uribe et al., 2020).

2.2 Neurochemical methods

In vivo microdialysis procedures estimate changes in levels of neurotransmitters in the extracellular space of a discrete brain region. Dialysis studies provide valuable insights into how the neurochemical environment changes in response to drug administration (Chefer et al., 2009). In this procedure, a dialysis probe with a semipermeable membrane is inserted into the brain region of interest. The membrane excludes proteins and molecules above a molecular weight that is dependent on the pore size of the membrane, allowing for the collection of the neurotransmitters (Hamberger et al., 1983; Kendrick 1989). A buffer made of artificial cerebrospinal fluid (aCSF) is infused through the inlet of the probe at a constant rate via a syringe pump. The infusion of aCSF perfusate creates a concentration gradient across the microdialysis membrane allowing for neurotransmitters to diffuse into the membrane. The dialysate is then collected in a vial from the outlet of the probe and is stored until it is analyzed using a variety of analytical methods.

Different methods have been used to measure neurotransmitter content in dialysate samples, including high performance liquid chromatography (HPLC) electrochemical detection, HPLC-fluorescence detection, capillary electrophoresis-laser induced fluorescence, and immunoassays (Zapata et al., 2009). A limitation of these methods is that they only measure one neurotransmitter at a time, lack sensitivity for detecting small changes in multiple neurotransmitter levels, and require specific conditions to be run that may interfere with the detection of certain electroactive

compounds. This can be limiting when trying to study dynamic changes and relationships among different neurotransmitter systems in brain regions where the content of these analytes is low. Thus, the present work employed liquid chromatography-mass spectrometry (LC/MS) coupled analysis. The rationale for using LC/MS is its capability for measuring many compounds in a complex sample due to its high level of sensitivity and resolution. Although not utilized in our protocol, additional rationale for using LC/MS includes the additional separation step where salts are removed, reducing the complexity of the sample and allowing to optimize conditions for the desired neurotransmitters (Annesley 2003; Zheng et al., 2012). Different compounds will be retained and released in the column and LC/MS coupled analysis also gives us the capability to detect neurotransmitters at a greater sensitivity when compared to traditional methods (Uutela et al., 2009).

2.3 Subjects

Adult female and male Wistar rats were obtained from an out-bred stock of rats (Envigo Inc; RRID:RGD_13508588) and were maintained on a reverse 12-hr light/dark cycle (lights on at 6:00 PM and off at 6:00 AM) in a humidity- and temperature- controlled vivarium (22°C). The rats were pair-housed in standard ventilated hanging cages (41.5 cm long × 17 cm wide × 21 cm high). The rats had *ad libitum* access to food and water throughout the study. Following intracranial infusions, rats were moved to a separate humidity- and temperature- controlled room. They were singly-housed and maintained on a regular 12-hr light/dark cycle (lights on at 6:00 AM and off at 6:00 PM). All procedures were approved by The University of Texas at El Paso Institutional Animal Care and Use Committee, and the 2011 Guide for Care and Use of Laboratory Animals (Institute of Laboratory, Animals Resources on Life Sciences, the National Research Council, and the National Academy of Sciences; reference number: A-201307-01). Although our hypotheses were not pre-registered or our sample size predetermined by a priori power analysis, the

experimental procedures, sample size, and statistical approach used in this report were based on prior published works (Carcoba et al., 2018; O'Dell et al., 2013).

2.4 Procedural summary

The procedures are summarized in **Figure 1**. Rats were anesthetized with an isoflurane/oxygen vapor mixture (1-3%) and were placed into a stereotaxic apparatus. The head of the rat was sliced and the skin was held open with hemostats. Once the head was placed into a flat skull position, two holes were drilled using the following coordinates from bregma for the NAc (AP = +1.7, ML = ±1.4 mm) using the rat brain atlas of Paxinos and Watson rat atlas (2004). The coordinates from bregma to infuse the viral vectors were: AP = +1.7, ML = ±1.4, DV = -9.1 mm (from skull). All rats received an infusion of the viral vector, AAV2/5-CRF, in the NAc of one hemisphere. The injections were made using a syringe pump (Harvard 22, Harvard Apparatus, Holliston, MA, USA) using an injection volume of 0.5 µls over a 3-min period. The injectors were left in place an additional 5-min to allow proper diffusion from the injector tip. In the contralateral NAc, rats received an infusion of the control virus, AAV2/5-GFP, to overexpress GFP. The hemisphere with the GFP viral vector will be the "control" hemisphere. Rats remained undisturbed for three weeks after intracranial infusions to allow for viral incubation and replication. Previous research has shown that AAV replication of our virus reaches maximal overexpression 2 weeks later, and remains constant for 8 weeks in brain tissue (Qi et al., 2014). Simple randomization for viral infusion occurred prior to the start of the experiment via a number labeling system for each rat. The viral infusions were also counter-balanced across brain hemispheres. The experimenters were unaware to the subjects' viral condition during dialysis testing and the sample preparation for the analysis of neurotransmitter content.

Dual microdialysis probes with an active membrane length of 2 mm were aimed into both NAc (model CMA11, Holliston, MA) using the coordinates as referenced above. The probes were

perfused for at least 1 hr prior to probe placements at a rate of 1.0 μL/min with artificial cerebrospinal fluid (aCSF) composed of 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 5.4 mM d-glucose, and 0.25 mM ascorbic acid and adjusted to a pH of 7.2–7.4. This allowed us to examine changes in neurotransmitter release following overexpression of CRF in one hemisphere and overexpression of GFP in the contralateral "control" hemisphere.

2.5 Dialysis testing

Following surgery, the rats were transferred to a Plexiglas® cage (24 cm long × 24 cm wide × 31 cm high) with food and water available throughout dialysis testing. Rats were left overnight to recover and the probes were flushed with aCSF at a rate of 0.1 μl/min. The following day, one hr before collection, the flow rate was adjusted to 0.6 μL/min for both probes prior to dialysate collection.

Our dialysis regimen is shown in **Figure 2**. It consisted of 20-min sampling periods during a baseline period and systemic administration of two doses of nicotine (0.3 and 0.6 mg/kg/base, subcutaneous). Nicotine hydrogen ditartrate (NIDA, Research Triangle, Bethesda, MA, USA) was dissolved in 0.9% sterile saline and adjusted to a pH level of approximately 7.4. All nicotine solutions were prepared according to the rats' body weight on the day of dialysis testing. These nicotine doses were chosen based on our previous work sex differences in nicotine intake in this dose range (Uribe et al., 2020). Dialysate was collected for three 20-min sampling periods (60 mins) for a baseline reading. Baseline levels were analyzed to give a basal and control level of neurotransmitters before the treatment regimen. Following baseline, the first dose of nicotine was injected and dialysate was collected for five 20-min sampling periods (100 mins) prior to the next injection of nicotine. The dialysate samples were immediately frozen on dry ice and then stored at −80 °C until assayed. At the end of dialysis testing, the rats were sacrificed using CO_2 inhalation and then decapitated to ensure euthanasia. The probes were then extracted and the brains were

removed, frozen, and sliced in 20 μm coronal sections for verification of probe placement. Probe placement was verified using gross observation of the probe track within the NAc shell using the Paxinos and Watson rat atlas (2004).

2.6 Mass spectroscopy analysis

Following dialysate collection, quantification of neurotransmitters was performed using an approach involving LC-MS analysis. The samples were analyzed using mass spectrometry analysis of dopamine, glutamate, and GABA in each NAc. The neurochemical analyses were performed using a Thermo Scientific UltiMate™ 3000 Standard Quaternary System with a Waters BEH C18 column (1 mm × 100 mm, 1.7 μm, 130 Å pore size) for separation. The autosampler was coupled to a TSQ Endura triple quadrupole MS. Our mass spectrometry methods incorporated calibration curves using standards ranging from 5, 50, 100, 500 and 1000 nM for GABA and glutamate, and 0.5, 5, 10, 50 and 100 nM for dopamine. The internal standard and sample derivatization procedures followed the methods described in Buczynski et al (2016) and Song et al (2012). Briefly, the composition of mobile phase A was 10 mM ammonium formate (0.15% (v/v) formic acid in water). Mobile phase B was 100% acetonitrile. The mobile phase gradient for the neurotransmitters lasted 10 minutes long and was: initial, 0% B; 0.1 min, 15% B; 2 min, 20% B; 2.3 min, 25% B; 2.31 min, 50% B; 5.31 min, 50% B; 5.57 min, 65 % B; 6.57 min, 65%B; 6.58 min, 0% B; 8.0 min, 0% B. Two blanks were run in-between to wash out any residue from the previous sample to ensure there is no carryover or contamination. The peaks were visually inspected to detect artifacts. Peak areas were obtained and analyzed for each neurotransmitter with their respective internal standard. Standard curves were used to calculate the concentration of all relevant neurotransmitters in each sample.

2.7 Statistics

Our initial approach to the data analysis involved an overall ANCOVA analysis that included all of our neurotransmitter measures. Since significant overall effects were not observed in this larger analysis, we proceeded with traditional ANOVA approaches with separate analyses of each neurotransmitter. Thus, separate mixed ANOVA analyses were used to compare group differences in baseline and following nicotine administration changes across time. To determine if the presence of the control virus caused changes in the release of neurotransmitters in the NAc of female and male rats, baseline levels of dopamine, GABA, and glutamate in the control hemispheres were assessed. The analysis compared baseline dialysate levels using two-way mixed ANOVAs with sex (female or male) as a between subject factor and time (20-min sampling periods) as a within subject factor. In order to provide a comparison of sex differences that accounted for potential differences in baseline and following nicotine administration levels produced by infusion of the viral vector, the data were converted to percent change from respective control levels [(CRF dialysate value/average control value) × 100]. The first level of analysis compared baseline levels using three-way mixed ANOVAs with virus (CRF or GFP) and sex (female or male) as between subject factors and time (20-min sampling periods) as a within subject factor. The second level of analysis compared dialysate levels following nicotine administration using three-way mixed ANOVAs with virus (CRF or GFP) and sex (female or male) as between subject factors and time (20-min sampling periods) as a within subject factor. For the repeated measures analyses with more than three levels, a test for the assumption of normality was employed using the Mauchly-Sphericity test. In cases where violations of normality occurred, a Huynh–Feldt correction factor modified the degrees of freedom, which resulted in an adjusted *F*-ratio. Where main or interaction effects were observed, planned comparisons were conducted using the Bonferroni correction factor, because it would yield a more conservative *p*-value for

multiple comparisons ($p \leq 0.05$). All statistical analyses were performed on SPSS version 26, and all of the graphs were generated using GraphPad Prism version 8.

Chapter 3: Results

3.1 Analysis of sex differences in GFP control rats

In order to best illustrate sex differences, we present the data as % change from respective GFP controls during baseline (**Figure 3**) and following nicotine administration (**Figure 4**). We first needed to examine whether females and males begin at an equivalent starting point. **Table 1** depicts the baseline levels in the control NAc hemisphere in female and male rats. In the control hemisphere, the analysis of dopamine revealed that there was no interaction between time and sex [F (2, 20) = 0.70, p = 0.51]. Also, there was no main effect of sex [F (1,10) = 0.11, p = 0.74]. The analysis of GABA revealed that there was no interaction between time and sex [F (1.57, 15.69) = 0.02, p = 0.96]. Also, there was no main effect of sex [F (1, 10) = 0.25, p = 0.88]. The analysis of glutamate revealed that there was no interaction between sex and time [F (2, 20) = 1.69, p = 0.21]. Also, there was no main effect of sex [F (1, 10) = 0.68, p = 0.43].

Table 1: Baseline levels of dopamine, GABA, and glutamate (mean ± SEM) following overexpression of the control virus (GFP) in the NAc of female and male rats.

Group	Dopamine (nM)	GABA (nM)	Glutamate (µM)
Female	16.1 (±1.8)	65.3 (±9.8)	7624.3 (±1079.4)
Male	14.9 (±4.5)	69.7 (±3.7)	6360.9 (±1178.9)

3.2 Aim 1: Baseline sex differences

Figure 3 depicts percent (%) change baseline values in dopamine, glutamate, and GABA in CRF overexpressing female and male rats. The analysis of dopamine revealed that there was no interaction between time, sex, and virus [F (2, 44) = 0.78, p = 0.47]. Also, there was no main effect of sex [F (1, 22) = 0.03, p = 0.87] or virus [F (1, 22) = 3.87, p = 0.062]. The analysis of glutamate revealed that there was no interaction between time, sex, and virus [F (2, 44) = 1.27, p = 0.29]. However, there was a interaction between sex and virus [F (1, 22) = 7.01, p = 0.02], with females showing a decrease in glutamate levels at all sampling points and males showing greater glutamate

levels at the first sample point when compared to their respective controls (*p<0.05). With regard to sex differences, CRF overexpressing males displayed greater glutamate release than CRF overexpressing females at all sampling periods. The analysis of GABA revealed that there was no interaction between time, sex, and virus [F (2, 44) = 0.19, p = 0.82]. Also, there was no main effect of sex [F (1, 22) = 0.27, p = 0.61] or virus [F (1, 22) = 2.29, p = 0.15].

3.3 Aim 2: Sex differences following nicotine administration

Figure 4 depicts percent (%) change in dopamine, glutamate, and GABA levels following administration of two nicotine doses (0.3 and 0.6 mg/kg/base) in CRF overexpressing female and male rats. The analysis of dopamine revealed that there was no interaction between time, sex, and virus [F (12, 264) = 1.05, p = 0.41]. Also, there was no main effect of sex [F (1, 22) = 1.25, p = 0.28] or virus [F (1, 22) = 2.01, p = 0.17]. The analysis of glutamate revealed that there was no interaction between time, sex, and virus [F (12, 264) = 0.99, p = 0.46]. However, there was a two-way interaction between sex and virus [F (1, 22) = 0.4.01, p = 0.05], with females showing a decrease in glutamate release at the 4th and 6th sampling period and male showing greater glutamate release at the 9th sampling period when compared to their respective controls. With regards to sex differences, CRF overexpressing males show greater glutamate during the 9th, 10th, and 12th sampling period when compared to CRF overexpressing females. The analysis of GABA revealed that there was no interaction between time, sex, and virus [F (12, 264) = 0.91, p = 0.54]. Also, there was no main effect of sex [F (1, 22) = 0.102, p = 0.75] or virus [F (1, 22) = 0.10, p = 0.76].

3.4 Probe verification

Our estimates of the probe placements were superimposed on digital images derived from the Paxinos and Watson atlas (2004). **Figure 5** illustrates our probe placements in the NAc of CRF overexpressing (red probe) and GFP controls (green probe). Each line reflects our estimate of the placement of the membrane sampling portion of the probe collected during sectioning. The left

panel reflects placements in female rats and the right panel reflects placements in the male rats. The diameter of the probe membrane was 0.24 mm wide, such that the estimated drawings of the probe span a range of sections wider than what is noted on a single plate in the diagram. The schematic illustrates that our probe placements were contained in the NAc shell. Two animals (2 females) were omitted from the study because of improperly placed microdialysis probes missing the NAc shell. At the end, females (n = 8) and males (n = 4) were included in the final analyses. We recognize that the number of male subjects is low, and this might have contributed to a lack of statistical power and significant results in the analyses of the selected neurotransmitters.

Chapter 4: Discussion

4.1 Summary

The main finding of this dissertation is that CRF differentially modulates the neurochemical effects of nicotine in the NAc of female and male rats. Overexpression of GFP did not alter dopamine, GABA, or glutamate in female versus male rats. During baseline, CRF overexpression altered glutamate release in sex-dependent manner, with male rats displaying an increase in glutamate and female rats displaying a decrease in glutamate levels. Similarly, following nicotine administration, CRF overexpression increased glutamate levels in males and decreased glutamate levels in females.

4.2 Aim 1: Sex differences during baseline

We first focused on baseline differences to assess the effects of CRF overexpression on tonic release of key neurotransmitters in the NAc. Our major finding was that during baseline, over-expression of CRF in the NAc significantly increased glutamate release in male rats. We suggest that CRF overexpression created a shift in the NAc towards a greater excitatory neurochemical environment. Consistent with this, prior work in male mice revealed that optogenetic activation of glutamatergic inputs into the NAc produced conditioned place preference (Britt et al., 2012). This suggests that enhanced glutamatergic input to the NAc elicits rewarding effects in male rats. Interestingly, the present study revealed that CRF overexpression in the NAc decreased baseline glutamate release in females relative to their GFP control group. Although the effects were not significant, both CRF overexpressing female and male rats displayed a trend for an increase in dopamine release when compared to control rats. This pattern of results came as a surprise as previous studies have shown CRF micro-infusion into the NAc increases dopamine levels (Chen et al., 2012) and produces place preference in naïve rats (Lemos et al., 2012). Furthermore, following stress induction, dopamine levels in the NAc shell are elevated (Kalivas et al., 1995; Wu

et al., 1999). The lack of significance may be related to the low sample size in the study. Although not statistically significant, both CRF female and male rats displayed a trend for a decrease in GABA levels (**Figure 3C**). There is evidence that CRF+ neurons colocalize with the GABAergic marker, GAD 65 and GAD 67, suggesting that CRF modulates GABA release (Chen et al., 2004; Yan et al., 1998). *Together, these data suggest that CRF overexpression altered the tonic release of amino acids in the NAc. This effect was observed in a sex-dependent manner, with male rats displaying greater excitatory glutamate release and females displaying reduced excitatory input to the NAc. Thus, CRF overexpression in the NAc altered the neurochemical environment towards greater excitatory potential in males prior to nicotine administration, as described below.*

4.3 Aim 2: Sex differences following nicotine administration

Figure 6 below summarizes our results regarding the effects of CRF overexpression on key neurotransmitters in the NAc following phasic changes in synaptic release following nicotine administration. The results revealed that following nicotine administration, CRF overexpression increased glutamate in males, whereas glutamate levels were decreased in females. The relatively higher excitatory glutamate input in males appeared to have contributed to the trend towards sex differences in nicotine-induced dopamine release in the NAc. This suggestion is based on the males displaying higher dopamine levels following nicotine administration than females. We speculate that CRF overexpression enhances glutamatergic transmission in males in a manner that "primes" the NAc for stronger neurochemical effects of nicotine as compared to females. In male rats, prior work has shown that antagonists for both ionotropic and metabotropic glutamate receptors block nicotine-induced dopamine release in the NAc (Kosowski et al., 2004). In striatal cells, previous work has shown that amino acid systems modulate dopamine release (Chiodo et al., 1986; Kitai et al., 1976). Indeed, Hjelmstad (2004) demonstrated that chronic exposure of striatal cells to dopamine decreased GABAergic inhibition, and this resulted in a greater excitation of these

neurons. Together, these data suggest that CRF overexpression in males increases excitatory transmission in the NAc in a manner that "primes" this brain region for greater nicotine-induced dopamine release that is larger relative to females. Below we provide a hypothesized mechanism for the manner in which viral overexpression of CRF may have produced sex-dependent effects in our neurochemical measures. The ANCOVA analysis revealed there were no significant CRF-induced changes in dopamine, GABA, or glutamate levels in the study. This may due to the relatively low sample size of female and male rats in the study. We acknowledge the low sample size in males may have contributed to a lack of statistical power in our results as explained further below.

4.4 Hypothesized mechanisms

Our proposed *mechanistic hypothesis* is that sex differences in the behavioral and neurochemical effects of nicotine are modulated via inhibitory (GABA) and excitatory (glutamate) amino acids that regulate dopamine release in the NAc (see **Figure 6**). Our proposed circuitry focuses on the *shell of the NAc* based on the distribution of CRF receptors in this region and the anatomical continuity of the shell with the extended amygdala, which is believed to modulate drug addiction (Balfour, 2009; Koob, 2010). We offer that CRF overexpression alters nAChR and CRF1 receptors that modulate sex differences in amino acid control of dopamine release in the NAc. First, prior work has shown that females display higher plasma CRF levels in response to a stressor and increased activation of CRF receptors, and a weaker negative feedback inhibition of the HPA axis (Bangasser et al., 2010; Kokras et al., 2019). My previous work also revealed that overexpression of CRF produced a larger downregulation of CRF1 receptors in the NAc of male versus female rats (Uribe et al., 2020). We propose that males display a larger down regulation of CRF1 receptors on glutamatergic terminals in the NAc. It is expected that this effect might result

in an enhancement of excitatory glutamate release, which would then enhance dopamine release produced by nicotine administration in the NAc of male versus female rats.

Our hypotheses regarding CRF1 receptors is in line with prior work showing that male rats display greater internalization of CRF1 receptors than females. Specifically, male rats display greater expression of the CRF1 internalizing protein, β-arrestin2, as compared to females (Bangasser et al., 2010; Valentino et al., 2013). Indeed, our prior work revealed that CRF overexpression in the NAc produced a greater increase in β-arrestin2 levels in males versus females (Uribe et al., 2020). Thus, we posit that males would display a large downregulation of CRF1 receptors which would result in a reduction in the ability of CRF to reduce glutamatergic input to the NAc in males versus females. In females, our hypothesis that CRF receptors produce greater effects than in males is consistent with prior work. For example, Weathington et al (2014) found that female rats display a greater increase in CRF binding to CRF1 receptors than males, suggesting that ovarian hormones enhance CRF1 receptor-mediated effects. For example, the highest levels of CRF are observed during proestrus when estradiol levels are highest (Bohler et al., 1990). Direct activation of estrogen receptor-β (ERβ) increases CRF mRNA expression in vitro (Zhu and Zhou, 2008). Also, the E2 gene sequence promotes CRF gene transcription (Vamvakopoulos and Chrousos, 1993). Together, these data suggest that CRF1 receptors may modulate sex differences in the neurochemical effects of nicotine via changes in glutamate release in the NAc. It is noteworthy that male rats only displayed an increase in glutamate following administration of the high dose of nicotine. This may be related to activation of α7-type nAChRs that are located on glutamatergic terminals that project to the NAc from the prefrontal cortex (Marchi et al., 2002; Pirttimaki et al., 2013). Indeed, previous work has shown α7 nAChRs are preferentially activated under conditions involving higher nicotine concentrations (Lester and

Dani, 1995; Wooltorton et al., 2003). Thus, the possibility that greater expression of a7 receptors that may also enhance glutamate release in the NAc.

4.5 Limitations and future directions

A major limitation of this dissertation relates to the relatively lower sample size in male versus female rats. Specifically, the sample size for our neurochemical measures was larger in females (n = 8) versus males (n = 4). We recognize that our low sample size in males may have contributed to a lack of statistical power in our results. Importantly, we did find that overexpression of CRF produced significant effects in glutamate transmission in the NAc of males, and our analyses for dopamine and GABA revealed strong trends that will be addressed with more animals once the current pandemic-related restrictions are lifted. We have collected additional female (n =1) and male (n = 2) rats that will be analyzed prior to the publication of my dissertation work.

Another limitation of this dissertation work is that we did not track the estrous cycle of the female rats. This information would have been informative based on recent work showing that ovarian hormones alter the behavioral effects of nicotine. Specifically, estradiol enhances the rewarding effect of nicotine, whereas progesterone reduces the behavioral effects of nicotine withdrawal (Flores et al., 2016; 2019). Differences in dopamine levels has been partly attributed to the estrous cycle and relatively higher levels of estradiol as greater DA release has been observed in females in proestrus (Xiao et al., 1994). In addition, OVX female rats display a reduction in dopamine release in the striatum and, following estradiol supplementation, is restored to intact female levels (Becker, 1990). Interestingly, castration of males does not reduce DA concentration when compared to intact males (Xiao et al., 1994; Castner et al., 1993). This study is the first step towards evaluating CRF modulation of sex differences in the neurochemical effects of nicotine in the NAc. Future studies are needed to more closely examine the manner in which fluctuations in ovarian hormones influence the contribution of stress systems in the neurochemical effects of

nicotine. For example, one might consider examining how overexpression of CRF alters nicotine-induced neurochemical effects in OVX female rats supplemented with estradiol and/or progesterone.

A final limitation is that we only focused on neurochemical effects of nicotine in the NAc, and one might expect that this brain region does not modulate sex differences in isolation. Indeed, ongoing work in our laboratory is showing that sex differences in nicotine withdrawal are mediated by amino acids in the interpeduncular nucleus (IPN). The present work is a first step in a long line of work examining the underlying neural mechanisms that modulate sex differences produced by nicotine and withdrawal from this drug. There may also be other neurotransmitter systems, such as acetylcholine (ACh) that modulate sex differences in the neurochemical effects of nicotine in the NAc. This suggestion is based on the work of Lemos et al (2019) who found that CRF1 receptor activation increases the firing of cholinergic interneurons in the NAc. The present work laid the groundwork for future studies aimed at uncovering the underlying mechanisms that promote sex differences produced by nicotine.

4.6 Conclusions and significance

The current study found that CRF systems in the NAc modulate the neurochemical effects of nicotine, with males displaying greater excitatory regulation of the NAc, a key structure that modulates the motivational properties of drug abuse. In contrast, CRF overexpression seemed to dampen the neurochemical effects of nicotine on dopamine transmission in the NAc of females. Our prior work revealed that overexpression of CRF in the NAc *increased* the reinforcing effects of nicotine to a larger extent in female versus male rats (Uribe et al., 2020). Thus, we expected that overexpression of CRF in the NAc of females would result in greater (not reduced) nicotine-induced dopamine release in the NAc. It is possible; however, that the strong reinforcing effects of nicotine in females is not modulated via dopamine release in the NAc. It is also possible that

female rats may have to self-administer higher amounts of nicotine to compensate for reduced dopamine release in the NAc. Indeed, parallel work in our laboratory has shown that diabetic rats display greater nicotine self-administration, but reduced nicotine-induced dopamine release in the NAc (O'Dell and Nazarian, 2015). Thus, enhanced drug intake may be related to an overcompensation of reduced dopamine transmission, a hypothesis that has been posited to explain compulsive overeating behavior (Yoshida et al., 1992). Future studies are needed to better understand how amino acid systems modulate greater vulnerability to nicotine use in vulnerable populations, such as females that are more susceptible to nicotine use than males in a clinical setting. This work is important toward the development of more effective cessation strategies that target stress systems that may help reduce health disparities produced by nicotine in women.

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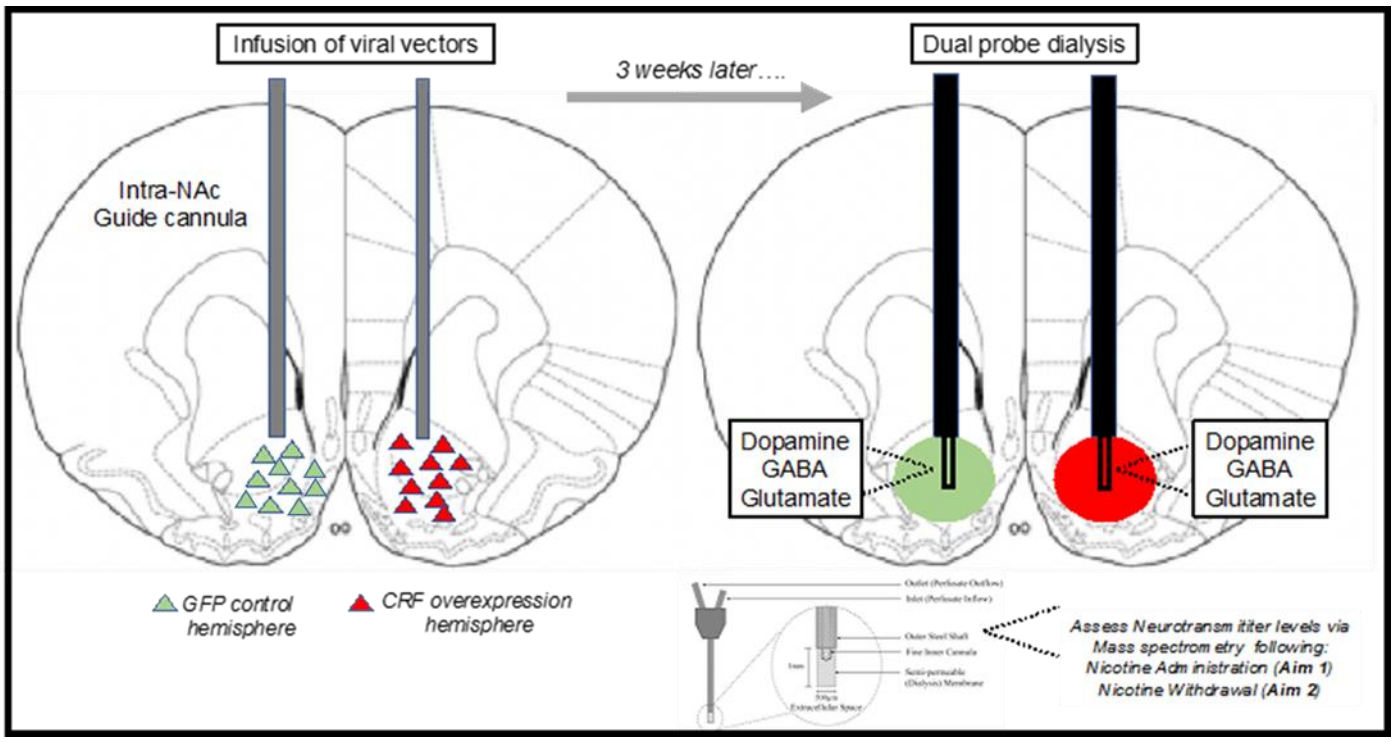

<u>Figure 1</u>: The schematic illustrates intra-NAc infusion of the AAV2/5-CRF in one hemisphere and infusion of the control virus, AAV2/5-GFP, in the contralateral hemisphere in female and male rats. Three weeks later, two dialysis probes with semi-permeable membrane into the NAc of both hemispheres.

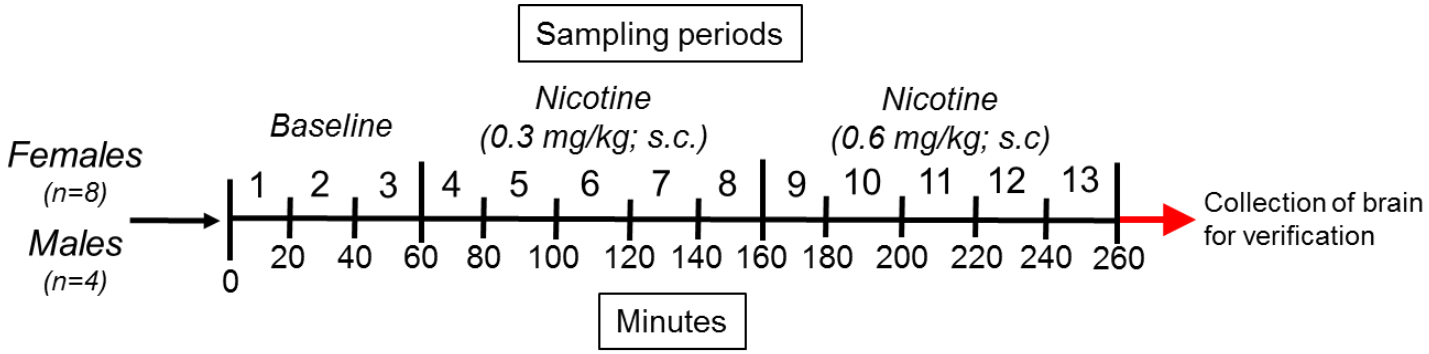

Figure 2: The schematic illustrates the dialysis regimen and timeline during collection day. Dialysate samples were collected in 20-minute sample periods for baseline and following injections of two doses of nicotine.

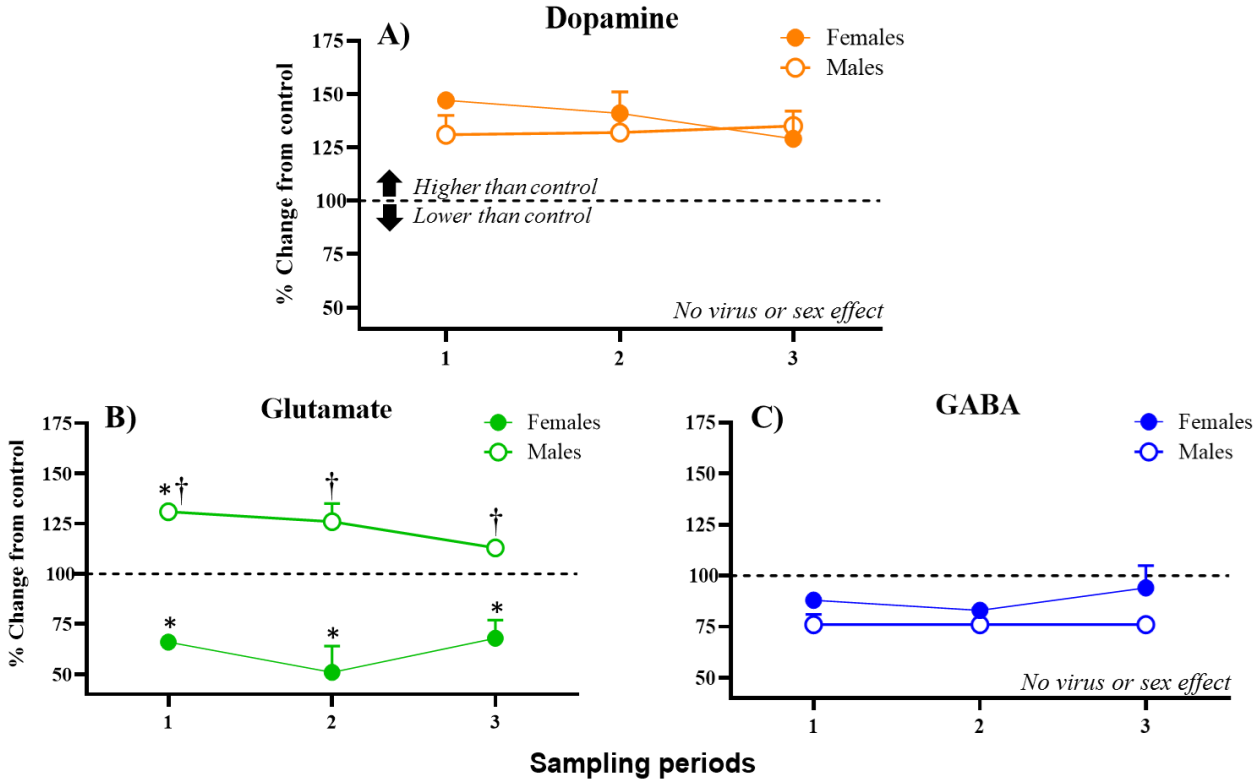

Figure 3: NAc levels of dopamine, glutamate, and GABA during baseline. The top panel (A) reflects dopamine, the bottom left panel (B) reflects glutamate, and the bottom right panel (C) reflects GABA in CRF overexpressing females (open circles) and males (open circles). Data are expressed as percent (%) change (CRF value/control value * 100) with values above and below the dotted line representing an increase or decrease in dialysate levels, respectively. The asterisk (*) denotes a significant difference from respective GFP control group and the daggers (†) denote a significant difference from CRF overexpressing female rats ($p \leq 0.05$).

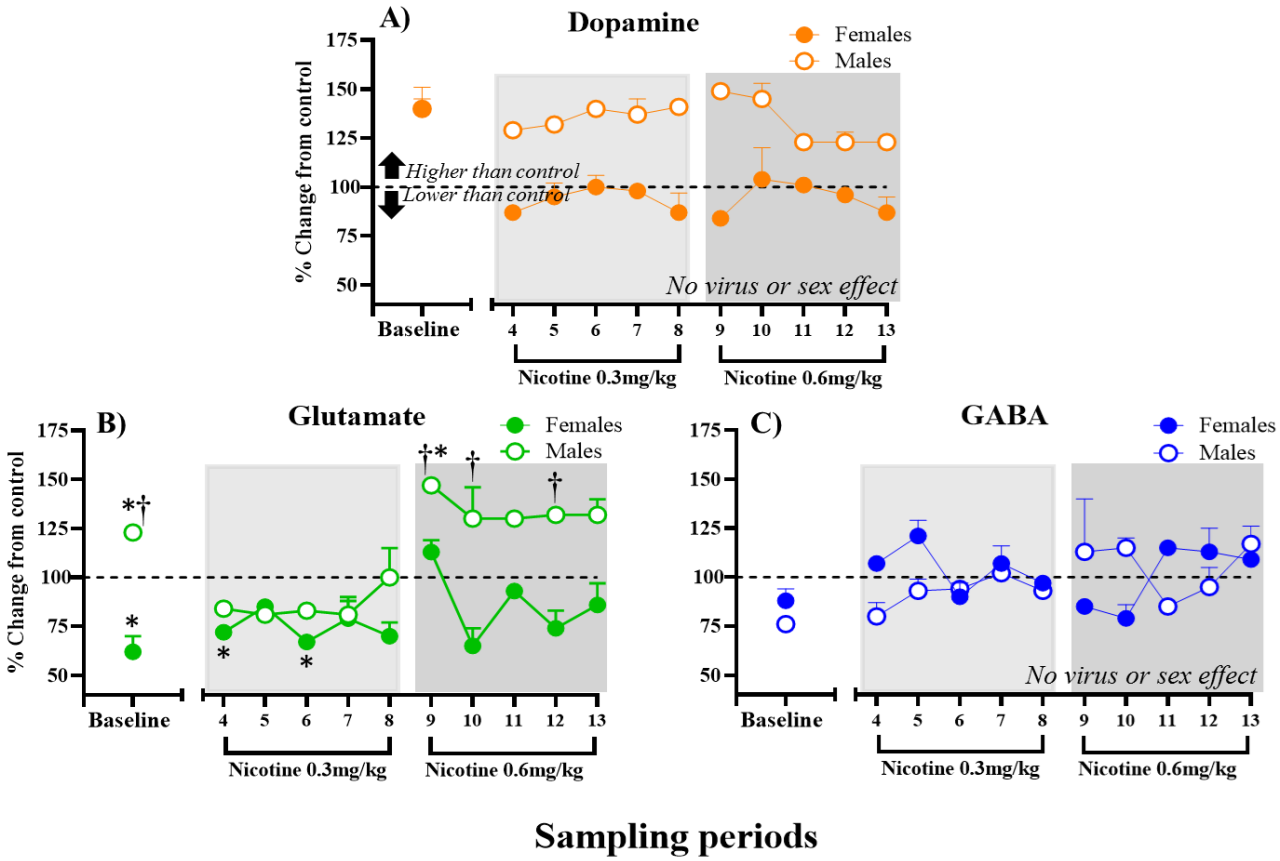

Figure 4: NAc levels of dopamine, glutamate, and GABA during baseline and following nicotine administration. The top panel (A) reflects dopamine, the bottom left panel (B) reflects glutamate, and the bottom right panel (C) reflects GABA in CRF overexpressing females (open circles) and males (open circles). Data are expressed as percent (%) change (CRF value/control value * 100) with values above and below the dotted line representing an increase or decrease in dialysate levels, respectively. The asterisk (*) denotes a significant difference from respective control group and the daggers (†) denote a significant difference from CRF overexpressing female rats ($p \leq 0.05$).

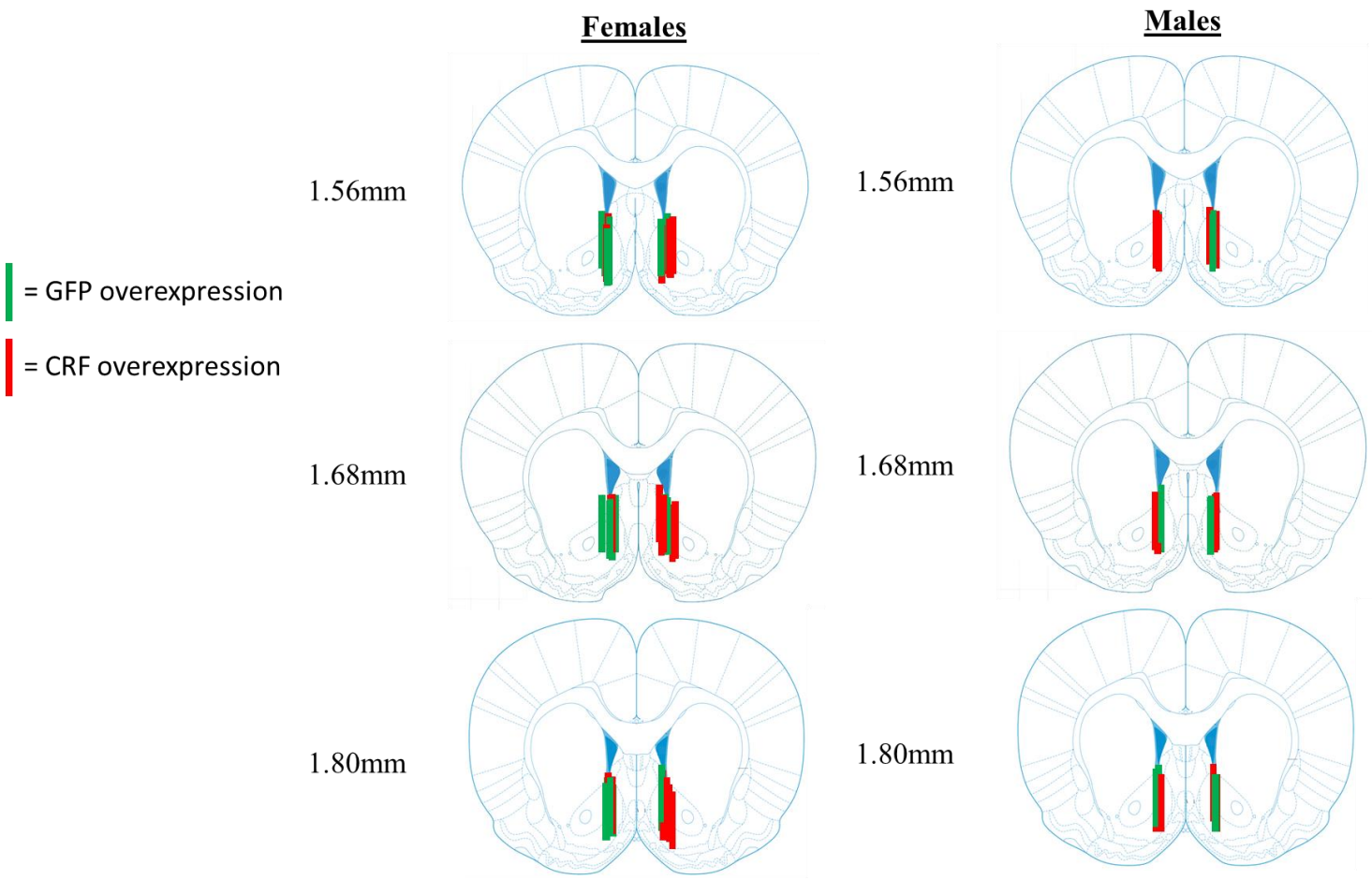

Figure 5: The schematic illustrates microdialysis probe placements in the NAc of both hemispheres in female and male rats. Probe placements were localized to the NAc shell in both hemispheres.

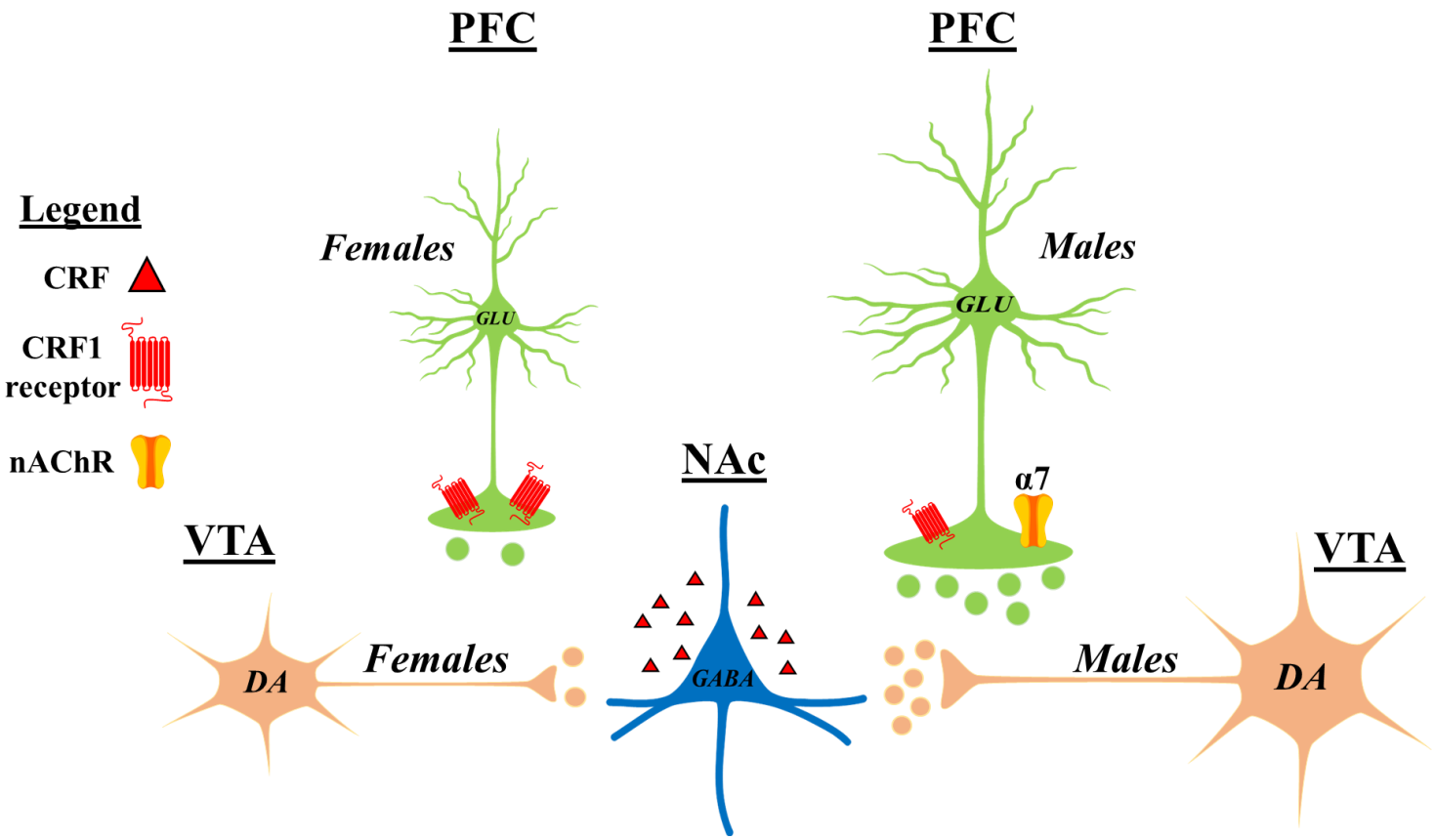

Figure 6: The image summarizes the neurochemical effects of CRF overexpression in the mesocorticolimbic pathway of female and male rats. CRF overexpression increased glutamate and dopamine release in male rats. In contrast, CRF decreases glutamate and dopamine release in females. It is hypothesized that the latter effect is mediated via greater expression of CRF1 receptor on excitatory terminals in female rats. Importantly, reduced expression of CRF1 receptor and activation of α7-type receptors on PFC terminals increase glutamatergic release, enhancing nicotine-induced dopamine transmission in male rats. (Abbreviations: GLU, glutamate; GABA, γ-amino butyric acid; DA, dopamine; PFC, prefrontal cortex; VTA, ventral tegmental area; NAc, nucleus accumbens; CRF, corticotrophin-releasing factor; CRF1, corticotrophin-releasing factor 1 receptor; nAChR, nicotinic acetylcholine receptor).

Curriculum Vitae

Kevin P. Uribe was born to Luis Enrique and Maria Nelly Uribe in Queens, NY. He graduated from Information Technology High School in Long Island City, NY in May 2010 and entered The City College of New York (CCNY) in Manhattan, NYC, August 2010. During his undergraduate career, Kevin entered into a pre-medical program. His goal was to go to medical school and become a Sports Medicine practitioner. However, through a summer program at the Albert Einstein School of Medicine, he changed his trajectory and became interested in research. Through the MARC program, Kevin entered and began working in the laboratory of Dr. Kaliris Y. Salas-Ramirez. He became interested in investigating how the gonadal hormones, estrogen and testosterone, make individuals more vulnerable to the psychostimulant effects and become addicted to cocaine. Kevin applied and entered into the combined dual degree B.S./M.S. program, where he would earn his Bachelors and Master of Science in five years. In 2015, Kevin joined the laboratory of Dr. Laura E. O'Dell at the University of Texas at El Paso (UTEP) where he continued his investigation of sex differences and how hormones modulate the addictive properties of drugs. During his PhD, Kevin applied for his independent funding and received the Diversity Supplement and a F31-NRSA fellowship from NIDA. Kevin has published 1 first-author article, 1 shared-first author publication, and is co-author on 3 publications examining nicotine reward and withdrawal in animal models of addiction.

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