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Insulin Restores the Neurochemical Effects of Nicotine in the Mesolimbic Pathway of Diabetic Rats

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Dedication

Dedicated to my beautiful friend and partner, Claudia Jane Woloshchuk and to my family:

Gerardo, Carmen, Narlin, Jerry, Hector Emanuel, Christian, Jorge, and Adrian.

Thank you for your never-ending love and support.

INSULIN RESTORES THE NEUROCHEMICAL EFFECTS OF NICOTINE IN THE MESOLIMBIC PATHWAY OF DIABETIC RATS

by

BRYAN CRUZ, M.A., B.A.

DISSERTATION

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Abstract

This study examined whether insulin modulates the neurochemical effects of nicotine in the mesolimbic pathway of diabetic rats. Rats received vehicle or streptozotocin (STZ) to induce hypoinsulinemia. A subset of STZ-treated rats was implanted with insulin pellets that normalized glucose levels. Two-weeks later, dialysis probes were implanted into the nucleus accumbens (NAc) and ipsilateral ventral tegmental area (VTA). The next day, dialysate samples were collected during baseline and then following systemic administration of nicotine. Samples were also collected following intra-VTA administration of the gamma-aminobutyric acid $(GABA)_A$ receptor antagonist, bicuculline. Dopamine, acetylcholine (ACh), GABA, and glutamate levels were assessed using liquid chromatography/mass spectrometry (LC/MS). The results revealed that vehicle-treated rats displayed a nicotine-induced increase in NAc dopamine. In contrast, STZtreated rats did not display any changes in NAc dopamine following nicotine administration, an effect that was likely related to a concomitant increase in GABA and decrease in glutamate levels in both the NAc and VTA. Intra-VTA administration of bicuculine produced an increase in NAc dopamine in vehicle-treated rats that was absent in STZ-treated rats. Vehicle-treated rats displayed a nicotine-induced increase in ACh levels in the NAc (but not VTA), an effect that was lower in the NAc of STZ-treated rats. Insulin supplementation normalized the neurochemical effects of nicotine in the NAc and VTA of STZ-treated rats, suggesting that insulin modulates the neurochemical effects of nicotine in the mesolimbic pathway of STZ-treated rats.

Keyword: tobacco use, diabetes, micro-dialysis, dopamine, amino acids, insulin

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Introduction

1.1 Tobacco use in a clinical setting

A major public health and economic concern is the high rates of tobacco use. According to the Centers 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, nearly 439,000 persons in the United States and 6 million people worldwide experience tobacco-related deaths every year (CDC, 2016; WHO, 2011). 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 heart and/or cardiovascular disease and various types of cancer (CDC, 2016).

Although there are over 4,800 chemicals in tobacco, 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 & Goldberg, 2006, 2009). The addictive properties of nicotine are believed to be one of the major motivating factors for continued smoking behavior despite its harmful consequences (Balfour, 2004; Sohn et al., 2003; Mansvelder & McGehee, 2002). Indeed, nicotine is readily self-administered by humans, dogs, rodents, and primates (Caille, 2012; Corrigall, 1999; Harvey et al., 2004; Katner et al., 2004; Le Foll & Goldberg, 2006; 2009; Risner et al., 1983). The recent rise in electronic nicotine delivery systems (i.e., ENDS) also speaks to the strong reinforcing effects of this drug (Yoong et al., 2019; Drope et al., 2017).

Clinical studies have revealed that patients with diabetes display greater susceptibility to tobacco use (Sattar el al., 2015; López et al., 2017). Specifically, persons with diabetes report higher tobacco use and lower cessation rates than non-diabetic smokers (Bishop et al., 2009). There

is also a greater incidence of negative affective states and depression during smoking abstinence among patients with diabetes (Haire-Joshu et al., 1994; Spangler et al., 2006). Unfortunately, tobacco use enhances the health complications associated with diabetes, including poorer glycemic control and an increased risk of cardiovascular problems and mortality (Lycett et al. 2015; Winhusen et al., 2019). Despite these problems, the underlying factors that motivate tobacco use in this vulnerable population remain unclear. Rodent models are needed to address important questions regarding the underlying mechanisms that promote tobacco use in patients with diabetes, as described below.

1.2 Rodent models of diabetes

Various rodent preparations have been used to study how the behavioral effects of nicotine are altered by diabetes. The most common rodent model of diabetes involves administration of streptozotocin (STZ), a drug that is toxic to the insulin-producing β-cells in the pancreas (Lee et al., 2010; Lenzen, 2008). STZ has high binding affinity for type 2 glucose transporters that are prevalent in the pancreas. Following STZ administration, DNA methylation induces cellular toxicity to the insulin-producing β-cells of the pancreas (Lenz, 2008). As a result, STZ produces a permanent and rapid increase in blood glucose levels within 48 hrs of administration (Arison et al., 1967; Lenzen, 2008; Cruz et al., 2019). The disruption of insulin systems and the resultant increase in glucose levels mimics the etiology of Type 1 and later stages of Type 2 diabetes (Akbarzadeh et al., 2007; Lee et al., 2010).

Prior work has assessed the rewarding effects of nicotine in rodent models of diabetes involving STZ. Our initial work revealed that STZ-treated rats display greater nicotine-induced conditioned place preference (CPP; Íbias et al., 2018; Pipkin et al., 2017) and intravenous self-administration (IVSA; O'Dell et al., 2013) as compared to vehicle-treated controls. Subsequent

studies revealed that the heightened rewarding effects of nicotine observed in STZ-treated rats are insulin mediated. This was based on our finding that STZ-treated rats that received insulin supplementation displayed similar nicotine-induced CPP (Íbias et al., 2018) and IVSA (Cruz et al., 2019) as compared to vehicle-treated controls. These behavioral studies suggested that insulin plays a key role in modulating the rewarding effects of nicotine. This work also raised important questions regarding the underlying mechanisms by which insulin systems modulate the behavioral effects of nicotine.

1.3 Mechanisms of nicotine in the mesolimbic pathway

Much work has established that the behavioral effects of nicotine are due to activation of nicotinic acetylcholine receptors (nAChRs) in the brain. nAChRs consist of pentameric membrane proteins of homomeric or heteromeric α or β receptor subunit complexes. In the presence of nicotine, the various α or β nAChR subunits leads to distinct channel activation and/or desensitization. It is well established that the rewarding effects of nicotine are due to the ability of this drug to enhance dopamine transmission in the mesolimbic pathway. This pathway originates in the ventral tegmental are (VTA) and terminates in several forebrain structures including the nucleus accumbens (NAc; Koob, 2000; Koob & Kreek, 2007; Mansvelder et al., 2003). **Figure 5** displays the mesolimbic pathway projections described here. Specifically, dopamine release in the NAc is controlled by excitatory and inhibitory mechanisms locally and via modulation of the dopamine cell bodies in the VTA. The control of dopamine release occurs via the amino acid neurotransmitters gamma aminobutyric acid (GABA) and glutamate, which innervate dopamine release in the mesolimbic pathway. Specifically, dopamine release in the NAc is suppressed by local GABAergic interneurons that modulate dopamine terminals from the VTA. Dopamine release in the NAc is enhanced via glutamatergic projections from the prefrontal cortex that

enhance dopamine release from terminals arising from the VTA. The principal role of GABA is to decrease neural excitability via opening of ion channels that allow the flow of negatively charged chloride ions into the cell, which results in cellular depolarization. GABAergic inhibition of dopamine transmission occurs through activation of $GABA_A$ and $GABA_B$ receptors located on dopamine cell bodies in the VTA (Criswell et al., 1993; Cruz et al., 2004; Labouèbe et al., 2007; Okada et al., 2004). On the other hand, glutamate enhances dopamine release via activation of ionotropic receptors that produce excitatory electrical responses in their targets a fraction of a millisecond after being stimulated. Glutamatergic stimulation of dopamine release occurs via activation of NMDA and AMPA ionotropic receptors located on VTA dopamine cell bodies (Choi et al., 2011; Johnson et al., 1992; Mao et al., 2011). Also, local dopamine release in the NAc is controlled via glutamatergic ionotropic receptors that are activated via glutamatergic inputs from the prefrontal cortex (Choi et al., 2011; Johnson et al., 1992; Mao et al., 2011).

With regard to how nicotine enhances dopamine release in the NAc, there are an array of different nAChRs expressed on the GABAergic and glutamatergic inputs to the VTA that regulate dopamine release in the mesolimbic pathway (Xu et al., 2006; Mansvelder & McGehee, 2002; Johnson et al., 1992; Kalivas et al., 1989; Kalivas et al., 1993; Mansvelder et al., 2003). Dopamine cell bodies in the VTA express α2-10 and β2-4 nAChRs subunits (Mansvelder et al., 2003). GABAergic interneurons express α4β2 and glutamate terminals in the VTA express α7 nAChRs. Following administration of nicotine, α4β2 sites on VTA GABA neurons quickly desensitize (Mansvelder et al., 2002). As a result, VTA dopamine neurons receive less inhibition from GABA due to the desensitization of α4β2 sites. This effect of nicotine produces a reduction in inhibitory tone that results in enhanced activation of dopamine neurons. Nicotine also binds to α7 nAChRs on glutamate terminals, and these receptors quickly sensitize following nicotine administration

(Wooltorton et al., 2003). As a result, nicotine produces a shift in 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.

1.4 Prior work that provided the foundation for this Dissertation

Previous studies that were conducted for my Master's thesis established that the enhanced rewarding effects of nicotine in STZ-treated rats is insulin mediated. Specifically, we demonstrated that STZ-treated rats that received insulin supplementation displayed "normal" levels of nicotine intake similar to control rats (Cruz et al., 2019). The latter report also revealed that insulin-associated metabolic peptides including insulin, leptin, amylin, and glucagon peptide-like 1 (GLP-1) are disrupted in STZ-treated rats. Importantly, we observed that these metabolic peptide changes were normalized to control levels in STZ-treated rats that received insulin supplementation. Work in my Master's thesis also revealed that STZ administration lowered insulin receptor substrate-2 (IRS-2) and insulin growth factor-like 1 receptor β (IGF-1Rβ) in the NAc (but not the VTA), an effect that was normalized in the NAc to control levels following insulin supplementation. Together, this work suggested that a lack of insulin modulates the neurochemical effects of nicotine in a region-specific manner. However, the mechanisms by which insulin modulates dopamine release in the mesolimbic pathway remain unclear.

1.5 Dissertation goals and hypotheses

The overall goal of this dissertation was to assess the role of insulin in modulating the neurochemical effects of nicotine in the mesolimbic pathway of diabetic rats. A sub-goal was to

assess whether insulin modulates the neurochemical effects of nicotine via amino acid regulation of dopamine release in the mesolimbic pathway.

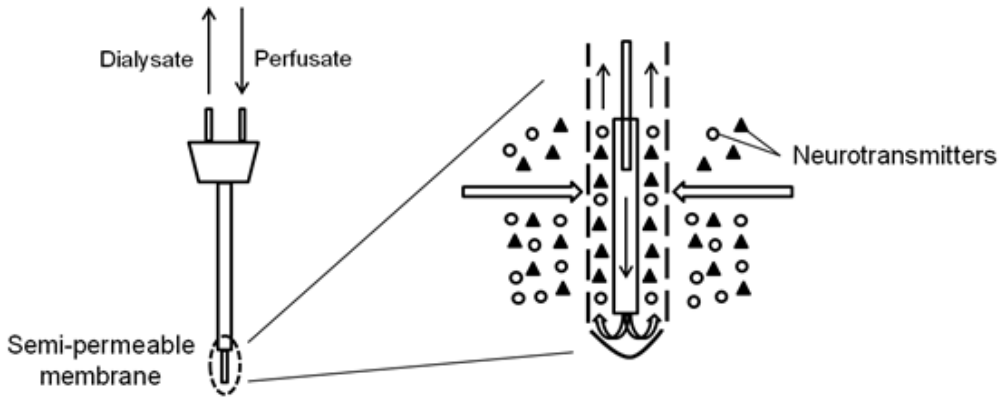
Aim 1 assessed whether insulin modulates nicotine-induced dopamine release in the mesolimbic pathway of STZ-treated rats. We first compared dopamine release following nicotine administration in the NAc and VTA of vehicle- and STZ-treated rats. To examine the role of insulin, a group of STZ-treated rats were treated with insulin immediately after STZ administration. Two-weeks later, dialysate levels of dopamine were assessed in the NAc and VTA during baseline and following systemic administration of nicotine. It was ***hypothesized*** that dopamine release would be suppressed in the VTA and NAc of STZ-treated rats as compared to controls. Also, it was ***hypothesized*** that insulin supplementation would restore the suppression of nicotine-induced dopamine release in both the NAc and VTA of STZ-treated rats. The ***rationale*** for our hypotheses is based on previous work showing that the increase in nicotine-induced dopamine release in the NAc is absent in STZ-treated rats (O'Dell et al., 2013). The latter study also revealed that STZ-treated rats display more dopamine transporters (DAT) in the NAc, which may reflect greater dopamine clearance in the NAc of STZ-treated rats.

Aim 2 assessed whether insulin modulates nicotine-induced dopamine release in the mesolimbic pathway via amino acids and cholinergic systems. We compared GABA, glutamate, and ACh levels following nicotine administration in the NAc and VTA of vehicle- and STZ-treated rats. To examine the role of insulin, a group of STZ-treated rats were also treated with insulin immediately after STZ administration. Two-weeks later, dialysate levels of GABA, glutamate, and ACh were assessed in the NAc and VTA of these groups during baseline and following systemic administration of nicotine. Our sample analysis was obtained from the same rats from **Aim 1**. It was ***hypothesized*** that the suppression of dopamine release would be due to an increase in

inhibitory GABA release and a decrease in excitatory glutamate release in the NAc and VTA of STZ-treated rats. It was also ***hypothesized*** that insulin supplementation would restore the neurochemical effects of nicotine via amino acid control of dopamine release in the mesolimbic pathway (Koob, 2000; Koob and Kreek, 2007; Scofield et al., 2016; Kalivas, 2009). With regard to ACh, it was ***hypothesized*** that ACh release would be reduced in the NAc and VTA of STZ-treated rats following nicotine administration, an effect that we expected to also be restored by insulin supplementation. The ***rationale*** for **Aim 2** was based on previous work showing intra-NAc administration of insulin enhances local dopamine and glutamate release (Labouèbe et al., 2013; Oginsky et al., 2019). Another report revealed that systemic administration of insulin increased dopamine release in NAc via activation of insulin receptors on striatal ACh interneurons (Stouffer et al., 2015). Studying the manner in which insulin regulates mesolimbic dopamine transmission is important towards understanding how a lack of insulin signaling alters the behavioral effects of nicotine in STZ-treated rats.

1.6 Background information on neurochemical methods

Analyzing neurotransmitter content dynamics is an important method in neuroscience research. *In vivo* microdialysis procedures allow an experimenter to estimate changes in levels of neurotransmitters in the synaptic space of a discrete brain region. Using a stereotactic apparatus, a probe is inserted into the brain region of interest.



The schematic above depicts a microdialysis probe, which consists of a semi-permeable membrane tip attached to the edge of a cannula (adapted from Chaurasia et al., 2007).

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 "clean" aCSF perfusate creates a concentration gradient across the microdialysis membrane allowing for small molecules to enter the membrane surface via diffusion properties. The dialysate is collected in a vial from a line attached to the outlet of the probe. The dialysate contains the neurotransmitters of interest to be analyzed later. The passage of molecules across the membrane tip is influenced by changes in the concentration of neurotransmitters in the brain, the flow rate of the perfusate, and the size of the pores on the membrane tip. Typically, the external diameter of the membrane is about 250-300 μm with a molecular weight limit to capture between 6,000-20,000 Daltons. In this Dissertation, dialysate samples were collected from both the NAc and VTA to estimate nicotine-induced changes in synaptic levels of dopamine, GABA, glutamate, and ACh.

Different methods have been used to measure neurotransmitter content in dialysate samples. These assays include high performance liquid chromatography (HPLC) electrochemical detection, HPLC-fluorescence detection, capillary electrophoresis-laser induced fluorescence, and immunoassays. A limitation with some of these methods is that they only measure one neurotransmitter at a time and they lack sensitivity for detecting small chaanges in neurotransmitter levels. 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) methods to test the hypothesis of **Aims 1 and 2**. The rationale for using LC/MS is related to the high level of

sensitivity of the MS system and its ability to assess an array of neurotransmitters in a single dialysate sample.

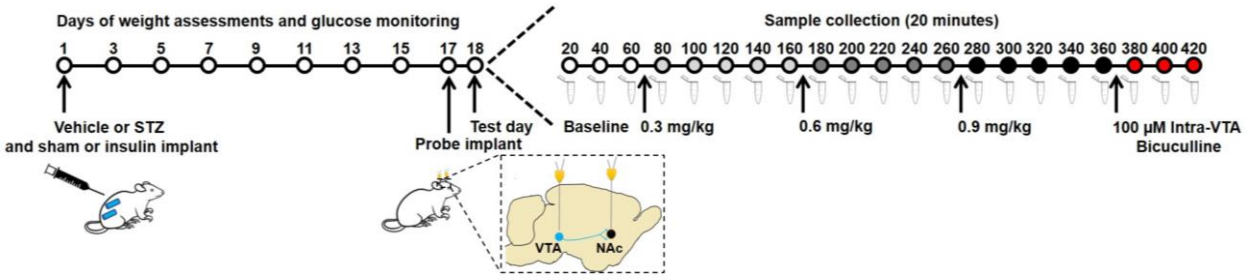
Chapter 2: Materials & Methods

2.1 Subjects

Adult male Wistar rats were obtained from an out-bred stock of rats (Envigo Inc; RRID:RGD_13508588) that were maintained on a regular 12-hr light/dark cycle (lights off at 6:00 PM and on at 6:00 AM) in a humidity- and temperature- controlled vivarium (22·C). The rats were single housed in standard ventilated hanging cages (41.5 cm long × 17 cm wide × 21 cm high). Starting between postnatal day (PND) 52-60, the rats were handled and weighed for at least 3-5 days. The rats weighed between 300-350 grams at the start of the experiments prior to STZ administration. The rats had *ad libitum* access to food and water throughout the study. 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-201902-01). Although our hypotheses were not pre-registered or our sample size predetermined by statistical test, the experimental procedures, sample size, and statistical approach used in this report were based on prior published works (Cruz et al., 2019; Carcoba et al., 2018; O'Dell et al., 2013). The experimental groups consisted of rats that were randomly selected from a fully out-bred line of animals. Simple randomization for treatment conditions occurred prior to the start of the experiment via a number labeling system for each rat. Then, the animals were arbitrarily assigned to different treatment groups. The experimenters were blinded to the subjects' treatment condition during dialysis testing and the sample preparation for the analysis of neurotransmitter content. Thus, blinding was achieved by only allowing experimenters to know the rat number, but not their treatment group assignment.

2.2 Procedural summary

The diagram below depicts the experimental timeline and dialysis testing procedures for this Dissertation.



This study employed dual probe dialysis procedures to compare nicotine-induced changes in neurotransmitter release in the NAc and VTA of vehicle- and STZ-treated rats. A group of rats were supplemented with insulin pellets immediately after STZ administration. Changes in plasma glucose levels and body weight were assessed throughout the study. Two-weeks after STZ administration, nicotine-induced changes in dopamine, GABA, glutamate, and ACh were assessed in dialysate collected from the NAc and VTA using LC/MS methods. At the end of testing, neurochemical changes were assessed following intra-VTA administration of the $GABA_A$ receptor antagonist, bicuculline. This manipulation was included as a neurochemical control that produces a robust increase in NAc dopamine levels and allowed us to assess group differences in GABAergic control of downstream changes in NAc dopamine levels (see O'Dell and Parsons, 2004). Indeed, previous work has demonstrated that intra-VTA administration of bicuculline produces strong ipsilateral turning, which provides evidence of correct probe placement in the small region of the VTA (Grubb et al 2002; Westerink et al 1996). The **schematic** below illustrates the group size for each neurotransmitter. The final analysis only included rats for which dopamine was assessed in both the VTA and NAc. For some amino acids, our analyses included fewer

samples than dopamine due to technical challenges associated with MS analysis of GABA and glutamate in rat brain dialysate (Carcoba et al., 2018).

Group sizes (n=number of analytes per neurotransmitter)

Groups:	Vehicle-treated		STZ-treated		STZ-treated+insulin	
Brain Region:	NAc (*n*)	VTA (*n*)	NAc (*n*)	VTA (*n*)	NAc (*n*)	VTA (*n*)
Dopamine	9	9	11	11	10	10
GABA	9	9	11	11	10	7
Glutamate	8	8	7	7	7	8
Acetylcholine	9	7	11	11	8	10

** The total number of rats in the study was N=30*

2.3 Diabetes induction and insulin supplementation

Rats received vehicle or STZ administration (45 mg/kg, s.c.). STZ was prepared immediately prior to administration and was dissolved in 0.1M sodium citrate buffer pH 6.0 (Sigma-Aldrich, St. Louis, MO, USA; catalogue number: S0130). The dose of STZ was selected based on previous work demonstrating that this concentration produces a reliable and long-lasting increase in glucose levels in adult male Wistar rats (Cruz et al., 2019; O'Dell et al., 2013). Immediately after vehicle or STZ administration, the rats were anesthetized with an isoflurane/oxygen vapor mixture (1-3%). They then received a sham surgery or were prepared subcutaneously with 2 insulin pellets based on the manufacturer specifications for the weight range of the rats in this study (Linplant® Toronto, ONT, CA; catalogue number: LHR-10BV). To minimize discomfort, the rats received an injection of the analgesic, flunixin prior to and the day after surgery (2.5 mg/kg; SC; catalogue number: NDC 11695-4012-1). The rats also received topical application of lidocaine and Neosporin® at the incision site (Henry Schein, Melville, NY, USA). Each pellet releases 2 units of insulin per 24 hrs for at least 60 days. Glucose levels were assessed every other day using a glucose meter calibrated for rodent blood (AlphaTRAK® Abbott

Park, IL, USA). A lancet was used to prick the tip of the tail to extract a small drop of blood that was placed on a test strip. STZ-treated rats had to display glucose levels within 250-650 mg/dL in order to proceed in the study (see **Figure 1**). Lastly, STZ-treated rats that received insulin displayed a healthy range of glucose levels that were lower than 250 mg/dL. Three rats were eliminated from the study that displayed glucose levels higher than 650 mg/dL.

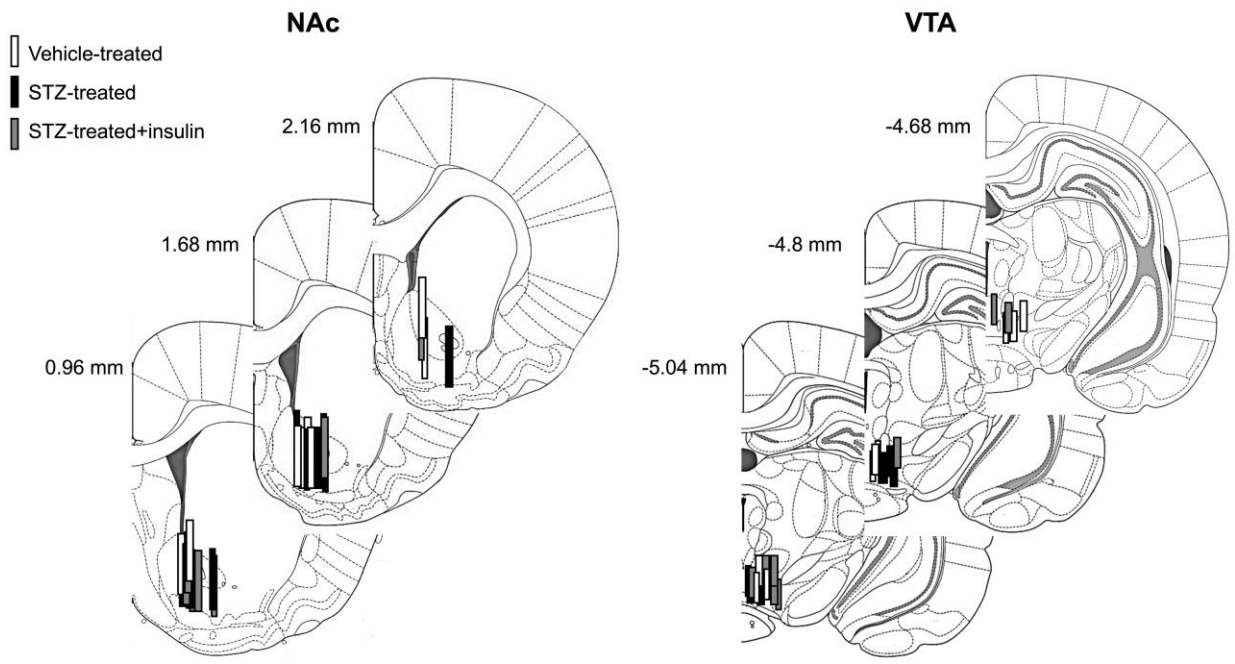
2.4 Dialysis testing

Two-weeks after vehicle or STZ administration, the rats were re-anesthetized with an isoflurane/oxygen vapor mixture (1-3%). They were then implanted with dialysis probes (Model CMA 11, Holliston, MA, USA; catalogue number: CMA8309581) in the NAc and VTA (1- or 2-mm membrane length, respectively). The probes were implanted using the following coordinates from bregma for the NAc (AP = +1.7, ML = ±1.4, DV = −8.1) and VTA (AP = −4.8, ML = ±0.8, DV = −8.5) using the rat brain atlas of Paxinos and Watson (2004). 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. Their probes were perfused with artificial cerebrospinal fluid (aCSF; 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) that was dissolved in HPLC-grade water and adjusted to a pH of 7.4. The probes were perfused with aCSF for at least 1 hr prior to probe placements at a rate of 1.0 µL/min. After placement, the probes were allowed to perfuse overnight with aCSF at a rate of 1.0 µL/min. The following day, the flow rate for the NAc was adjusted to 0.6 µL/min and the VTA probe was adjusted to 1.1 µL/min for 1 hr prior to dialysate collection. Baseline samples were then collected in 20-min intervals for 1 hr. Dialysate levels were then collected for 5 20-min sampling periods following administration of 3 doses of nicotine in increasing order (0.3 mg/kg, 0.6 mg/kg, then 0.9 mg/kg; SC). (-) Nicotine hydrogen ditartrate (NIDA, Research Triangle, Bethesda, MA, USA;

catalogue number: NICT-015) 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. For the last series of dialysate samples, bicuculline (Sigma-Aldrich, St. Louis, MO; catalogue number: B7686) was infused into the VTA probe (100 μM) and dialysate was collected for 3 additional 20-min sampling periods. The dialysate samples were immediately frozen on dry ice and then stored at −80 °C until assayed.

2.5 Probe placement verification

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 10 μm coronal sections. Our estimates of the probe placements were superimposed on digital images derived from the Paxinos and Watson atlas (2004). The schematic below illustrates our probe placements in the NAc and VTA of vehicle-treated (white probe tip), STZ-treated (black probe tip), and STZ-treated+insulin rats (gray probe tip). 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 the NAc (2 mm in length), and the right panel reflects placements in the VTA (1 mm in length). 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. This schematic illustrates that our probe placements were contained in the NAc and VTA.



2.6 Neurotransmitter detection methods

Stock solutions of each neurotransmitter were prepared in MS grade water and kept at −80 °C. The standard mixture was diluted from stock solutions using aCSF from the dialysis test day. Calibration curves were made using standard concentrations in a range of 5, 50, 100, 500, 1000 nM (GABA and glutamate) and 1, 5, 10, 25, 50 nM (dopamine and ACh). The internal standard preparation and sample derivatization procedures followed the methods described in Song et al (2012). Quantification of neurotransmitters in dialysate samples was performed by LC/MS analysis. 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. The peaks were visually inspected to detect peak and artifacts. Standard curves were used to calculate the concentration of all neurotransmitters in each sample.

2.7 Statistics

For the analyses of glucose levels and body weight, separate 2-way mixed-model repeated measures analysis of variance (ANOVA) were used with treatment group (vehicle, STZ-, and STZ-treated+insulin) as a between-subject factor and time (days) as a within-subject factor. For the neurochemical analyses, each neurotransmitter was analyzed separately in the NAc and VTA. Separate analyses were used to compare group differences in baseline and nicotine-induced changes across time. The first level of analysis compared baseline levels using 1-way ANOVAs with treatment group (vehicle-, STZ-, and STZ-treated+insulin) as a between subject factor. The second level of analysis compared changes across time using 2-way mixed-model repeated-measures ANOVAs with treatment group (vehicle-, STZ-, and STZ-treated+insulin) as a between subject factor and time (20-min sampling periods) as a within-subject factor. The third level of analysis compared group differences across time with the data collapsed across baseline versus post-nicotine samples. For the repeated measures analyses with more than 3 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 more accurate *F*-ratios. Where significant main or interaction effects were observed, post-hoc analyses were conducted using protected Fisher's least significant difference test ($p \leq 0.05$). 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 Baseline neurotransmitter levels

Table 1 illustrates baseline neurotransmitter levels (nM±SEM) in the NAc and VTA of vehicle-, STZ-, and STZ-treated+insulin rats. The analysis of the NAc revealed that there were no differences between treatment groups in baseline levels of dopamine [$F_{(2,27)}$=1.72, p=0.19], GABA [$F_{(2,27)}$=2.29, p=0.12], or glutamate [$F_{(2,19)}$=2.86, p=0.08]. There was a significant difference between treatment groups in baseline levels of ACh [$F_{(2,25)}$=4.45, p=0.02], with STZ-treated rats displaying lower baseline ACh levels than STZ-treated+insulin rats (#p<0.05). The analysis of the VTA revealed a significant difference across treatment groups in baseline levels of dopamine [$F_{(2,27)}$=7.09, p=0.003], GABA [$F_{(2,24)}$=14.60, p=0.001], and glutamate [$F_{(2,20)}$=8.81, p=0.02]. Specifically, STZ-treated rats displayed lower baseline levels of dopamine and glutamate and higher levels of GABA relative to vehicle- and STZ-treated+insulin rats (†p<0.05). However, there were no differences across treatment groups in baseline levels of ACh [$F_{(2,25)}$=0.80, p=0.45]. Within the VTA, insulin supplementation appeared to have restored the STZ-induced changes in baseline levels of dopamine, GABA, and glutamate, as there were no baseline differences in these neurotransmitters across vehicle- and STZ-treated+insulin rats.

3.2 Body weight and glucose levels

Figure 1 illustrates mean body weight (g±SEM) and glucose levels (dL±SEM) across days. The analysis revealed a significant group by day interaction for body weight [$F_{(8.38,113.21)}$=13.63, p=0.0001] and glucose levels [$F_{(10.21,137.95)}$=27.62, p=0.0001]. STZ-treated rats displayed lower body weight on days 9-17 relative to vehicle- and STZ-treated+insulin rats (†p<0.05). Also, STZ-treated rats displayed significantly higher glucose levels on days 3-17 relative to both vehicle- and

STZ-treated+insulin groups of rats (†p<0.05). There were no significant differences in body weight and glucose levels between vehicle- and STZ-treated+insulin groups.

3.3 Neurotransmitter levels in the NAc

Figure 2 illustrates mean neurotransmitter levels (nM±SEM) in the NAc across time (left panels) and collapsed across baseline and post-nicotine samples (right panels). The analysis of NAc dopamine levels across time revealed a treatment group by time interaction [$F_{(15.21,\ 205.42)}$=1.83, p=0.03], with STZ-treated rats displaying a significant decrease in dopamine levels during the 100-, 180-, 200-, 260-, 280-, 320-, and 360-min sampling periods relative to vehicle- and STZ-treated+insulin rats (†p<0.05). STZ-treated rats also displayed a significant decrease in dopamine levels during the 240- and 300-min sampling periods relative to vehicle-treated rats (*p<0.05). This decrease in dopamine was also significant during the 80-, 160- and 340-min sampling period relative to STZ-treated+insulin rats (#p<0.05). The analysis of dopamine levels in the right panel revealed an interaction between treatment group and sampling condition (baseline versus post-nicotine) [$F_{(2,27)}$=3.60, p=0.04], with nicotine producing an increase in dopamine levels relative to baseline in vehicle- and STZ-treated+insulin rats (βp<0.05). Also, STZ-treated rats displayed lower levels of dopamine relative to vehicle- and STZ-treated+insulin rats post-nicotine administration (†p<0.05).

Subsequent analyses of changes in neurotransmitter levels across time revealed that there were no interaction effects between treatment group and time for GABA [$F_{(10.46,\ 141.25)}$=0.68, p=0.75], glutamate [$F_{(14.74,\ 140.04)}$=0.94, p=0.51], and ACh [$F_{(20.84,260.52)}$=1.32, p=0.160]. There were significant main effects of treatment group in the analysis of GABA [$F_{(2,27)}$=3.87, p=0.03], glutamate [$F_{(2,19)}$=3.64, p=0.04], and ACh [$F_{(2,25)}$=9.35, p=0.001]. Specifically, STZ-treated rats displayed lower levels of glutamate and ACh, but higher levels of GABA relative to

both vehicle- and STZ-treated+insulin rats (†p<0.05). Separate analyses of neurotransmitter levels in the right panels revealed that there was no interaction between treatment group by sampling condition (baseline versus post-nicotine) for GABA [$F_{(2,27)}$=0.40, p=0.67] or glutamate [$F_{(2,19)}$=0.94, p=0.40]. However, there was a significant main effect of treatment group in the analysis of GABA [$F_{(2,27)}$= 4.21, p=0.02], an effect that was largely driven by the higher levels of GABA observed in STZ-treated versus vehicle- and STZ-treated+insulin rats (†p<0.05). Also, there was a main effect of treatment group in the analysis of glutamate [$F_{(2,19)}$= 3.56, p=0.04], with STZ-treated rats displaying lower levels of glutamate relative to vehicle- and STZ-treated+insulin rats (†p<0.05). Lastly, there was an interaction between treatment group by sampling condition (baseline versus post-nicotine) for ACh [$F_{(2,25)}$=3.41, p=0.04], with STZ-treated rats displaying lower ACh levels versus vehicle- and STZ-treated+insulin rats (†p<0.05). Moreover, vehicle-treated rats displayed higher ACh levels post-nicotine as compared to baseline (βp<0.05). Overall, there were no differences in the analysis of dopamine, GABA, glutamate, or ACh levels in the NAc of vehicle- and STZ-treated+insulin rats.

3.4 Neurotransmitter levels in the VTA

Figure 3 illustrates mean neurotransmitter levels (nM±SEM) in the VTA across time (left panels) and collapsed across baseline and post-nicotine samples (right panels). The analyses of dopamine levels across time revealed that there was no interaction between treatment group and time [$F_{(19.18,267.54)}$=0.92, p=0.56]. However, there was a significant main effect of treatment [$F_{(2,27)}$=20.17, p=0.0001], with STZ-treated rats displaying lower levels of dopamine relative to vehicle- and STZ-treated+insulin rats (†p<0.05). The analysis of dopamine levels in the right panel revealed an interaction between treatment group and sampling condition (baseline versus post-nicotine) [$F_{(2,27)}$= 3.48, p=0.04]. Nicotine produced an increase in dopamine levels relative to

baseline in STZ-treated+insulin rats (βp<0.05). Also, STZ-treated rats displayed lower levels of dopamine relative to vehicle- and STZ-treated+insulin rats during baseline and following nicotine administration (†p<0.05).

Subsequent analyses of changes in neurotransmitter levels across time revealed that there was no interaction between treatment group and time for GABA [$F_{(14.28,171.37)}$=1.34, p=0.27], glutamate [$F_{(6.53,65.31)}$=0.57, p=0.76], and ACh [$F_{(10.42,130.29)}$=0.46, p=0.92]. There were significant main effects of treatment group in the analysis of GABA [$F_{(2,24)}$=18.23, p=0.0001] and glutamate [$F_{(2,20)}$=4.26, p=0.02]. Specifically, STZ-treated rats displayed higher levels of GABA and lower levels of glutamate relative to vehicle- and STZ-treated+insulin rats (†p<0.05). The analysis of GABA in the right panel revealed an interaction between treatment group and sampling condition (baseline versus post-nicotine) for GABA [$F_{(2,24)}$=3.88, p=0.035]. Nicotine produced an increase in GABA levels relative to baseline in vehicle- and STZ-treated groups of rats (βp<0.05). Also, after nicotine administration, STZ-treated rats displayed higher levels of GABA relative to vehicle- and STZ-treated+insulin rats (†p<0.05). The analysis of glutamate levels in the right panel revealed that there was no interaction between treatment group and sampling condition (baseline versus post-nicotine) for glutamate [$F_{(2,20)}$=0.51, p=0.60]. In contrast, there was a main effect of treatment group in the analysis of glutamate [$F_{(2,20)}$=.8.94, p=0.002], with STZ-treated rats displaying lower levels of glutamate relative to vehicle- and STZ-treated+insulin rats (†p<0.05). The analyses of ACh revealed that there was no interaction between treatment group and sampling condition (baseline versus post-nicotine) [$F_{(2,25)}$=0.65, p=0.52] and no main effect of treatment group [$F_{(2,25)}$= 1.93, p=0.16]. Overall, there were no differences in the analysis of dopamine, GABA, glutamate, or ACh levels in the VTA of vehicle- and STZ-treated+insulin rats.

3.5 Dopamine levels following intra-VTA bicuculline

Figure 4 illustrates mean dopamine levels (nM±SEM) in the NAc following intra-VTA administration of bicuculline. The analysis revealed a significant interaction between treatment and time [$F_{(7.09, 95.84)}$=7.49, p=0.0001], with STZ-treated rats displaying lower levels of dopamine at the 20-, and 40- min sampling periods relative to both vehicle- and STZ-treated+insulin rats (†p<0.05). Also, STZ-treated rats displayed lower dopamine levels at the 60-min sampling period relative to STZ-treated+insulin rats (#p<0.05).

Chapter 4: Discussion

4.1 Summary

The STZ-treated rats in the present study displayed some of the hallmark symptoms of diabetes, including an increase in glucose levels and a decrease in body weight. The STZ-induced changes in glucose levels and body weight were restored to control levels following insulin supplementation. **Figure 5** depicts a summary of the neurochemical effects of nicotine in vehicle- and STZ-treated rats. Briefly, vehicle-treated rats displayed an increase in NAc dopamine levels following nicotine administration, an effect that was absent in STZ-treated rats. The absence of nicotine-induced increases in NAc dopamine in STZ-treated rats appeared to be modulated via amino acids. Specifically, across all sampling conditions, STZ-treated rats displayed overall higher GABA and lower glutamate levels in the NAc and VTA. Lastly, vehicle-treated rats displayed a nicotine-induced increase in NAc ACh levels, an effect that was absent in STZ-treated rats. All of the neurochemical effects produced by STZ were restored to control levels following insulin supplementation, with the exception of ACh levels in the VTA.

4.2 Baseline changes

An important consideration is that STZ altered the neurochemical environment prior to nicotine administration in a region-specific manner. Specifically, in the NAc, the reduction in insulin produced by STZ did not alter baseline measures of any of the measured neurotransmitters relative to vehicle-treated controls. In contrast, STZ-treated rats displayed lower baseline levels of dopamine and glutamate and higher levels of GABA in the VTA as compared to vehicle-treated controls. These results suggest that a lack of insulin, produced by STZ, results in a tonic reduction in excitatory glutamate and an increase in inhibitory GABA release in the VTA. The observed

alterations in baseline levels in the cell body region may have led to the suppression of phasic release of dopamine in the NAc following nicotine administration.

4.3 Nicotine effects

The largest neurochemical effect of nicotine was observed in the NAc, with control rats displaying a 2-fold increase in NAc dopamine levels. This nicotine-induced increase in NAc dopamine was blunted in STZ-treated rats, consistent with previous work (O'Dell et al., 2013). The present findings expand upon work in the latter study by showing that STZ also reduces nicotine-induced dopamine release in the cell body region of the VTA. Consistent with our findings, work in other laboratories has revealed that STZ administration reduced dopamine synthesis in rat striatal tissue (Trulson and Himmel, 1983; Bitar et al., 1986). Also, STZ administration has been shown to lower dopamine levels in dialysate samples collected from the hippocampus (Yamato et al., 2004) and tissue levels in the hypothalamus (Bitar et al., 1986; Chu et al., 1986). Together, these studies provide converging lines of evidence that a reduction in insulin levels produced by STZ reduces dopamine transmission in the mesolimbic pathway, possibly via amino acid regulation of dopamine release as described below.

Prior work has established that the amino acid neurotransmitters GABA and glutamate regulate dopamine release in the NAc (Qi et al., 2016; Hjelmstad, 2004; Kalivas et al., 1993; Melchior et al., 2015). Following nicotine administration, the present study revealed that STZ-treated rats displayed higher GABA and lower glutamate levels in both the NAc and VTA. Based on this finding, we suggest that a lack of insulin produced by STZ might have suppressed nicotine-induced dopamine release in the NAc via reduced excitatory and greater inhibitory regulation of dopamine in the cell body and terminal region of the NAc. Our assertion with regard to reduced glutamatergic control is supported by previous studies in the hippocampus showing that STZ

suppresses AMPA-mediated excitatory currents (Sasaki-Hamada et al., 2012), glutamate receptor expression (Viswaprakash et al., 2015), and extracellular glutamate levels (Reisi et al., 2009). Also, our assertion with regard to greater GABAergic inhibition may be supported by the finding that intra-VTA blockade of $GABA_A$ receptors completely suppressed dopamine transmission in the NAc of STZ-treated rats.

The present study also revealed that vehicle-treated rats displayed an increase in ACh release in the NAc following nicotine administration, and this effect was absent in STZ-treated rats. Interestingly, STZ administration did not alter ACh levels in the VTA, suggesting that neurochemical changes produced by a lack of insulin are primarily focused in the NAc. A previous report found that intraventricular administration of STZ increased choline acetyltransferase activity, an effect that was associated with greater clearance of extracellular ACh levels (Naik et al., 2016). Based on the latter report, it is possible that STZ produced an increase in choline acetyltransferase levels, and this may have blunted the ability of nicotine to increase ACh levels in the NAc.

4.4 Insulin restoration

The present study compared baseline measures and the neurochemical effects of nicotine in rats that received insulin immediately after STZ administration. The results revealed that insulin prevented the neurochemical changes produced by STZ during baseline and following nicotine administration. The precise mechanisms by which insulin restores the neurochemical effects of nicotine in STZ-treated rats remain unclear. One possibility is that the restorative effects of insulin occur via second messenger signaling pathways. This suggestion is based on a previous report demonstrating that STZ administration results in lower insulin receptor substrate-2 (IRS-2) and insulin growth factor-1 receptor β (IGF-1Rβ) expression in the NAc, and these effects were

restored to control levels following insulin supplementation (Cruz et al., 2019). Prior work has linked IRS-2 expression to dopamine cell integrity in the VTA, as a down-regulation of IRS-2 in the VTA has been shown to reduce the size of dopamine cell bodies in the VTA (Russo et al., 2007). Another possibility is that insulin restores dopamine release in the NAc indirectly via activation of insulin receptors on cholinergic interneurons. This is based on a prior report showing that insulin enhances striatal dopamine release via activation of insulin receptors located on cholinergic interneurons in the NAc (Stouffer et al., 2015). Future studies are needed to more fully understand the mechanisms by which insulin modulates the neurochemical effects of nicotine in the mesolimbic pathway of STZ-treated rats.

4.5 Final considerations

The studies in this report involved measurements of different neurotransmitter systems collected from different brain regions. Future studies will need to examine the causal relationships between insulin and the amino acid systems that regulate dopamine transmission in the mesolimbic pathway. Future studies will also need to incorporate other brain regions, such as the medial habenula where there is high expression of a diabetes-related gene (TCF7L2) that is closely associated with nicotine intake in rodents (Duncan et al., 2019). Also, the present study normalized insulin to physiological levels immediately after STZ administration using a protocol that has been established in rodents (see Hu et al., 1993). The temporal overlap in STZ administration with insulin restoration in the present study may not reflect the typical onset of diabetes with delayed clinical interventions involving insulin therapy and/or smoking cessation medications. Thus, future studies are needed to examine whether our observed neurochemical effects persist in STZ-treated rats that are supplemented with insulin, as might occur in a clinical situation. This work might also assess whether smoking cessation medications that enhance dopamine transmission reduce

nicotine intake in rodent models of diabetes. This work will be important towards understanding the underlying mechanisms that promote greater nicotine use in persons with diabetes.

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Table 2. Baseline neurotransmitter levels (nM±SEM) in all groups

Groups:	Vehicle-treated	STZ-treated	STZ-treated+insulin
NAc:			
Dopamine	3.50±0.38	2.46±0.41	2.80±0.35
GABA	46.46±7.24	129.95±38.49	84.03±20.81
Glutamate	4716.49±857.85	2061.95±570.09	4134.20±964.71
Acetylcholine	19.39±3.29	13.46±2.37#	23.02±2.12
VTA:			
Dopamine	3.68±0.59	1.76±0.22†	3.41±0.36
GABA	33.98±3.96	85.63±1.76†	55.27±15.18
Glutamate	3007.02±311.65	1087.61±184.11†	3922.43±709.25
Acetylcholine	15.40±2.57	13.72±3.11	10.54±1.81

(#) indicates different from STZ-treated+insulin rats ($p \leq 0.05$)
(†) indicates different from vehicle- and STZ-treated+isulin rats ($p \leq 0.05$)

Table 1.

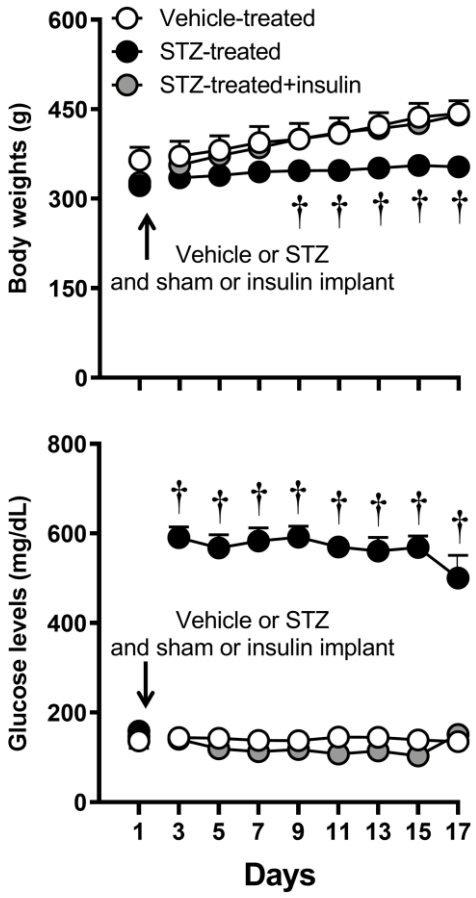

Figure 1. Mean (±SEM) body weight (top panel) and glucose levels (bottom panel) in vehicle-treated (open circles), STZ-treated (black circles), and STZ-treated+insulin rats (gray circles). The daggers (†) denotes a difference from vehicle-treated and STZ-treated+insulin rats ($p \leq 0.05$).

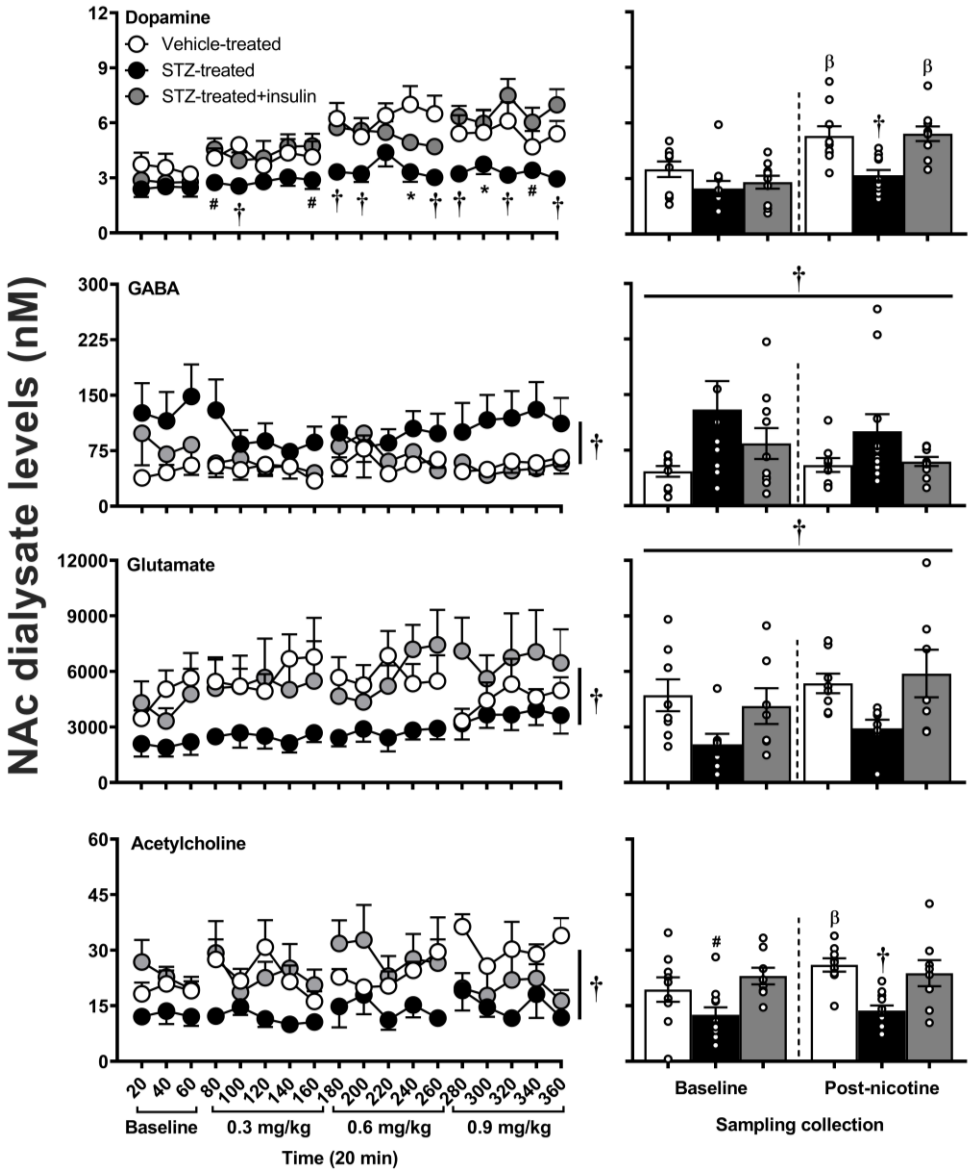

Figure 2. NAc dialysate levels of dopamine, GABA, glutamate, and ACh levels during baseline and following nicotine administration. The panels on the left reflect each 20-min sampling period, and the panels on the right reflect sampling conditions (baseline versus nicotine) in vehicle-treated (open circles), STZ-treated (black circles), and STZ-treated+insulin (gray circles) rats. Data are expressed in nM concentrations (±SEM). The asterisk (*) denotes a significant difference from vehicle-treated rats, the number sign (#) denotes significant difference from STZ-treated+insulin rats, the (β) denotes a significant difference from baseline within-group, and the daggers (†) denote a significant difference from both vehicle-treated and STZ-treated+insulin rats ($p \leq 0.05$).

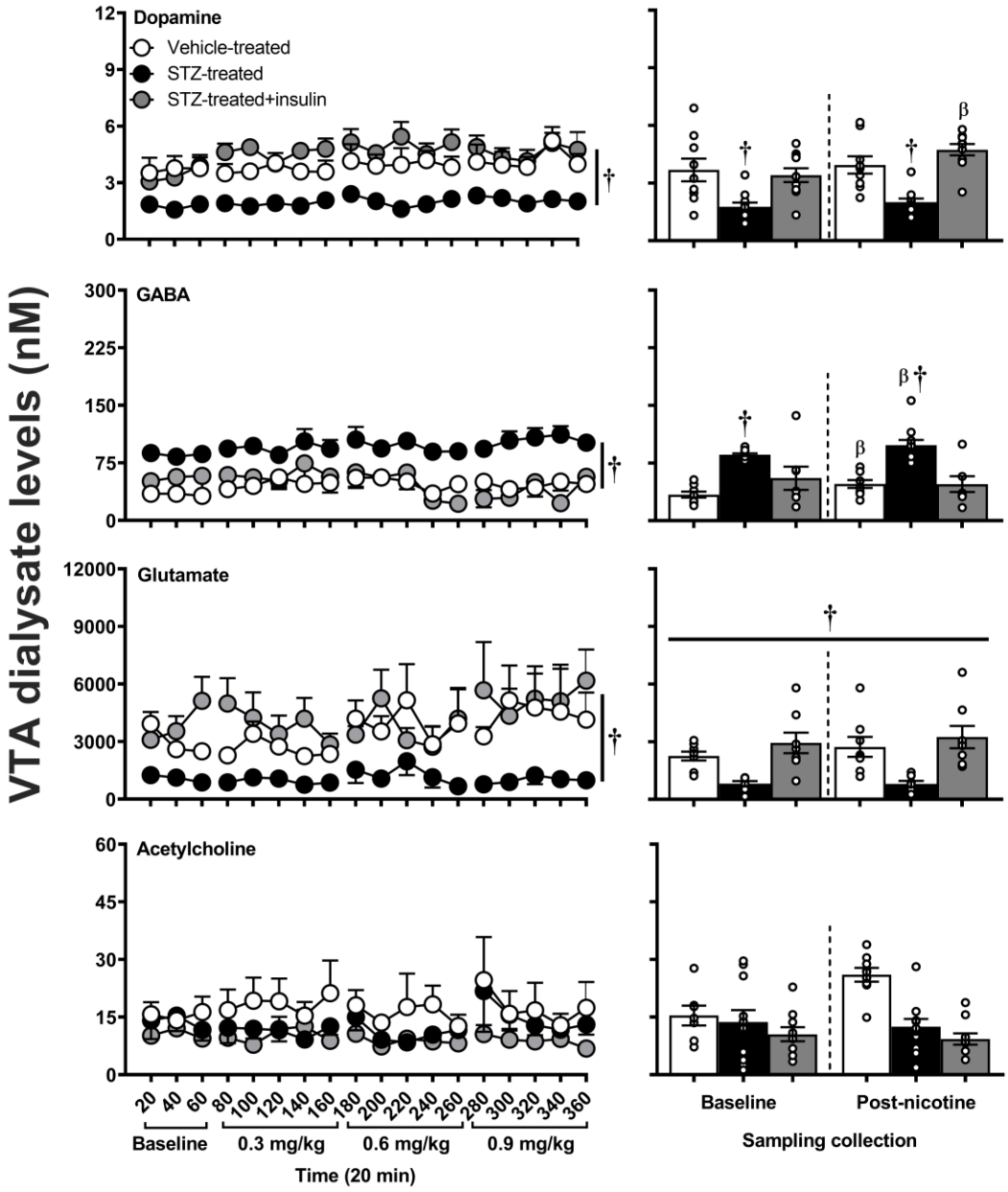

Figure 3. VTA dialysate levels of dopamine, GABA, glutamate, and ACh levels during baseline and following nicotine administration. The panels on the left reflect each 20-min sampling period, and the panels on the right reflect sampling conditions (baseline versus nicotine) in vehicle-treated (open circles), STZ-treated (black circles), and STZ-treated+insulin (gray circles) rats. Data are expressed in nM concentrations (±SEM). The (β) denotes a significant difference from baseline within-group, and the dagger (†) denotes a significant difference from both vehicle-treated and STZ-treated+insulin rats ($p \leq 0.05$).

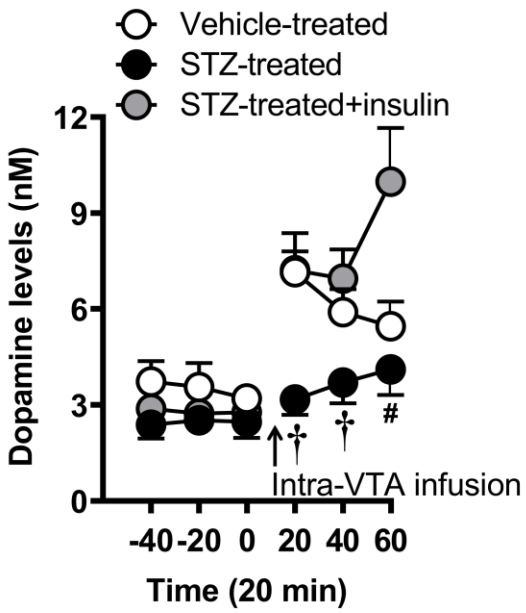

Figure 4. NAc dopamine levels during baseline and following intra-VTA infusion of bicuculline (100 μM) in vehicle-treated (open circles), STZ-treated (black circles), and STZ-treated+insulin (gray circles) rats. Data are expressed in nanomolar (nM) concentrations (±SEM). The number sign (#) denotes significant difference from STZ-treated+insulin rats, and the dagger (†) denotes a significant difference from both vehicle-treated and STZ-treated+insulin rats ($p \leq 0.05$).

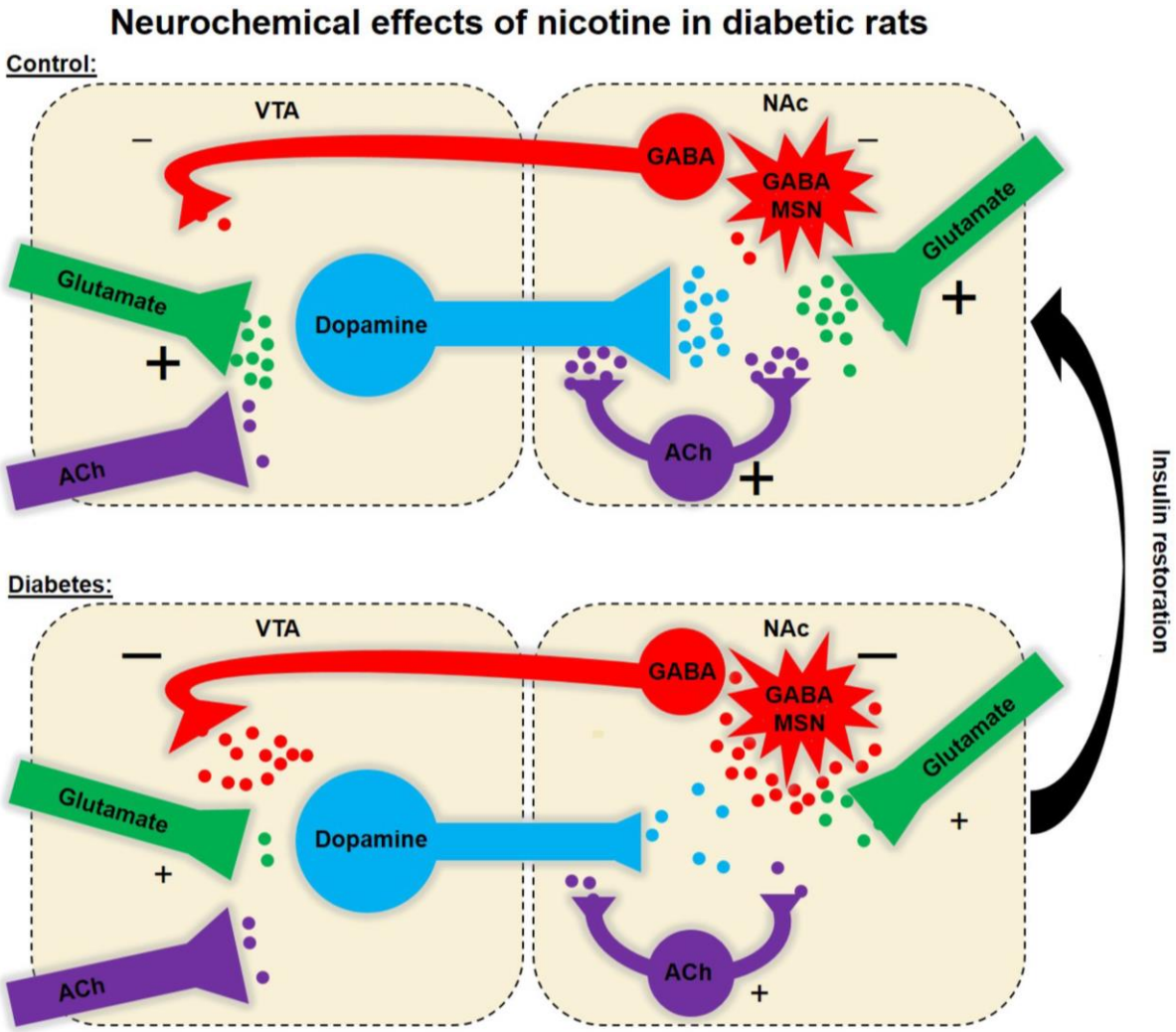

Figure 5. The image summarizes the neurochemical effects of nicotine in the mesolimbic pathway of vehicle- and STZ-treated rats. In STZ-treated rats, nicotine-induced increases in NAc dopamine are blunted as compared to vehicle-treated rats. It is hypothesized that the latter effect is mediated via greater GABA inhibition (larger minus sign) and reduced glutamate excitation (smaller plus sign) in STZ-treated rats. Importantly, STZ-induced neurochemical changes were restored to vehicle-treated control levels following insulin supplementation (Abbreviations: acetylcholine; ACh; gamma-amino butyric acid; GABA; medium spiny neuron; MSN).

Curriculum Vitae

Bryan Cruz was born to Gerardo and Carmen in Los Angeles, California on December 25th, 1991. Bryan graduated from Patriot High School in Mira Loma, California in 2010, and thereafter, entered the California State University of San Bernardino (CSUSB). In 2015, he completed his Bachelor's degree in Biological Psychology from CSUSB. Bryan became interested in Behavioral Neuroscience research and began conducting studies in the laboratory of Dr. Sergio D. Iñiguez on the effects stress on motivated behaviors for drugs of abuse in rodent models. Afterwards, he entered the Behavioral Neuroscience graduate program at The University of Texas at El Paso (UTEP) to continue his research work. He joined the laboratory of Dr. Laura E. O'Dell who studies the underlying biological factors that promote tobacco use. Her laboratory combines various approaches to address the public health problem of tobacco addiction. Bryan graduated with his Master's degree under the guidance of Dr. O'Dell in December, 2018. He has 4 oral and 26 poster abstracts accepted at numerous scientific conferences. He received the *National Award of Excellence in Research* by a graduate student from the National Hispanic Science Network and the *Graduate Excellence Fellowship* from UTEP. Bryan has published 2 first-author articles and co-authored 7 publications and 1 book chapter on the topic of drug addiction. While pursuing his graduate degree, Bryan has instructed 3 courses including Introduction to Psychology and General Experimental Psychology. His long-term plan is to pursue a research-oriented career at an academic institution. He plans to continue his research work on investigating the neurobiology of drug addiction. Bryan will start his post-doctoral research position at The Scripps Research Institute in La Jolla, California in the laboratory of Dr. Marissa Roberto.

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