2018-01-01

Insulin Modulates The Strong Reinforcing Effects Of Nicotine And Changes In Insulin Biomarkers In A Rodent Model Of Diabetes

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INSULIN MODULATES THE STRONG REINFORCING EFFECTS OF NICOTINE AND CHANGES IN INSULIN BIOMARKERS IN A RODENT MODEL OF DIABETES

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Dedication

Dedicated to the ones etched in my heart, Gerardo Cruz Velázquez, Maria del Carmen Rios Geronimo, Narlin Carlota Cruz, and Jerry Josue Cruz.

Thank you for your never-ending love and support.

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by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF ARTS

Department of Psychology

THE UNIVERSITY OF TEXAS AT EL PASO

December 2018

Acknowledgements

More than anything else, I would like to thank my mentor, Dr. Laura E. O'Dell for her perpetual support and guidance in my endeavors. I am grateful to be training under your wing, and for giving me the chance to learn about the scientific process. I would also like to give a special thank you to Dr. Sergio D. Iñiguez for his insightful comments on this study. Especially for his continuous support and guidance, and for setting a premise for me during undergraduate to pursue graduate school. I would like to say thank you to Dr. Katherine M. Serafine for her support and critical feedback of this project. Additionally, I would like to thank Dr. Charles T. Spencer for his insightful comments of this work, and for his instruction on the Multiplex Assay. Thank you for sharing your laboratory space and time. I would also like to thank Dr. Joseph Pipkin who helped optimized the insulin regimen parameters, and Dr. Oscar Torres for teaching me the protocols to assess insulin-signaling. An immense thank you to my laboratory colleagues and friends: Dr. Luis Carcoba, Rodolfo Flores, Kevin Uribe, Evangelina Espinoza, Melissa Ibarra, Francisco Flores, and Israel Garcia. Many thanks for their countless help, encouragement, and laughs throughout this time in graduate school. I could not have asked for a better group of people to work with. Lovingly, I would like to thank my parents, Gerardo Cruz and Carmen Rios, and my siblings, Narlin Cruz and Jerry Cruz, for their endless love and support for my life goals. Many thanks to my best friends, Jorge Abraham Rodriguez and Adrian Macias for their constant encouragement. Lastly and lovingly, I would like to thank Claudia Jane Woloshchuk for her immense patience, support, commitment, and for always be there for me throughout this time. All of their continuous reinforcement is the spark for my enthusiasm.

Abstract

This study examined whether the strong reinforcing effects of nicotine and changes in insulin biomarkers observed in diabetic rats are modulated via insulin. A model of diabetes was employed involving administration of streptozotocin (STZ), which produces hypoinsulinemia in rats. The present study included vehicle- or STZ-treated rats that received sham surgery or an insulin pellet. Two-weeks later, the rats were given extended access to intravenous self-administration (IVSA) of saline or nicotine. Concomitant changes in food intake, water responses, and body weight were assessed during 12 days of IVSA. After the last session, plasma levels of insulin, leptin, amylin, and glucagon-like peptide 1 (GLP-1) were assessed using Luminex® technology. In a separate cohort, phosphorylated insulin receptor substrate-2 (pIRS-2) and insulin growth factor-1 receptor β (IGF-1Rβ) were assessed in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of vehicle- or STZ-treated rats that received sham surgery or an insulin pellet. STZ-treated rats displayed an increase in glucose levels, a decrease in body weight, and an increase in nicotine, food, and water intake relative to controls. STZ-treated rats also displayed a decrease in plasma insulin and leptin levels and an increase in amylin and GLP-1 levels relative to controls. Importantly, all of the STZ-induced changes in behavior and insulin biomarkers were prevented by insulin supplementation. STZ-treated rats also displayed a decrease in pIRS-2 and IGF-1Rβ in the NAc (but not VTA), an effect that was also prevented by insulin. These data suggest that insulin systems in the NAc modulate the strong reinforcing effects of nicotine in male diabetic rats.

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Chapter 1: Introduction

1.1 Tobacco use is a public health concern

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, 2014; 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, 2014).

1.2 Tobacco use in persons with diabetes

There is growing evidence that patients with diabetes display a higher propensity for tobacco use than the general population. For example, patients with Type 1 diabetes report higher rates of current smoking (12.3%) than non-diabetic smokers (8.6%; Bishop et al., 2009). Also, patients with diabetes report lower quit rates and display a greater concern for weight gain if they quit smoking as compared to non-diabetic smokers (Gill, Morgan, & MacFarlane, 2005). Patients with diabetes also experience more intense negative affective states and depression symptoms during smoking abstinence (Haire-Joshu et al., 1994; Spangler et al., 2006). Unfortunately, patients with diabetes that smoke also experience greater mortality rates and health complications, such as disrupted glucose homeostasis (Scemama et al., 2006; Tonstad, 2009). Despite the magnitude of the problem associated with diabetes and smoking, the underlying mechanisms that promote tobacco use among patients with diabetes remain unknown. Given that the etiological origin of diabetes involves a lack of insulin signaling, the possibility

exists that a disruption in insulin systems results in greater rewarding effects of nicotine, the main psychoactive compound that motivates tobacco use.

1.3 Rodent model of diabetes

Diabetes causes metabolic complications and an elevation in blood glucose levels via a decrease in insulin production from β-cells in the pancreas (Type 1 diabetes) or development of insulin resistance (Type 2 diabetes). Prior work in our laboratory has examined the behavioral effects of nicotine in a rodent model of diabetes involving administration of streptozotocin (STZ) (Pipkin et al., 2017; O'Dell et al., 2014). STZ is taken up by glucose transporters, and this drug displays the highest affinity for type 2 transporters that are prevalent in the pancreas. When STZ is administered to rodents, DNA methylation induces cellular toxicity to the insulin-producing β-cells of the pancreas (Lenz, 2008). The disruption of insulin release from the pancreas mimics the etiology of Type 1 diabetes and later stages of Type 2 diabetes.

1.4 Previous work leading to my Master's Thesis

Previous work in our laboratory has revealed that STZ-treated rats display greater nicotine intake compared to healthy controls in a rodent model involving 23-hour access to intravenous self-administration (IVSA; O'Dell et al., 2014). Consistent with the latter finding, a subsequent report revealed that STZ-treated rats display a more robust conditioned place preference (CPP) produced by nicotine as compared to healthy control rats (Pipkin et al., 2017). These studies suggest that a lack of insulin leads to an increase in the rewarding effects of nicotine. Previous work has also shown that insulin-associated metabolic proteins, including amylin, GLP-1, leptin, and insulin cross the blood-brain barrier and modulate motivated behavior (Ferrario et al., 2016; Narayaman, Guarnieri, & DiLeone, 2010; Figlewicz et al., 2003; Mietlicki-Baase et al., 2015). To examine the role of insulin in modulating the behavioral and biochemical

effects of nicotine, the present study assessed changes in an array of insulin-related biomarkers following chronic nicotine IVSA in diabetic rats that received insulin supplementation.

1.5 Potential brain mechanisms

It is well known that the rewarding effects of nicotine are modulated via dopamine transmission in the mesolimbic pathway, which originates in the VTA and terminates in several forebrain structures including the NAc (Koob, 2000; Koob & Kreek, 2007; Mansvelder et al., 2003). It is well established that nicotine produces behavioral effects via 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. There are an array of different nAChRs located on the excitatory and inhibitory inputs to the VTA that regulate dopamine release in the mesolimbic pathway. Three major cell types exist in the VTA that express nAChRs, including including dopamine terminals, gamma-aminobutyric acid (GABA) interneurons and glutamate inputs from the prefrontal cortex (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). Glutamate terminals in the VTA express α7 nAChRs and GABAergic interneurons express α4β2 sites. 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 activiation 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.

Previous studies have revealed that there is a large distribution of insulin receptors in the NAc and the VTA that modulate motivated behavior (Figlewicz et al., 2003; Havrankova, Roth, & Brownstein, 1978; Unger, Livingston, & Moss, 1991). There are insulin receptors on cholinergic interneurons in the NAc, providing a mechanism by which changes in insulin signaling may influence the behavioral effects of nicotine (Stouffer et al., 2015). Within the NAc, STZ-treated rats display a down-regulation of pIRS-2 and an up-regulation of protein kinase B, an insulin-related marker coupled to pIRS-2 receptors (O'Dell & Nazarian, 2016). It is presently unclear whether STZ-treated rats display a change in insulin-signaling proteins in the NAc and/or VTA that promotes the rewarding effects of nicotine in diabetic rats.

1.6 Master's thesis experiments

The present study examined the role of insulin in modulating the strong rewarding effects of nicotine previously observed in STZ-treated rats. It was hypothesized that insulin supplementation would reduce the strong reinforcing effects of nicotine to control levels in STZ-treated rats. This hypothesis was based on the recent finding that STZ-treated rats display an increase in CPP produced by nicotine that was reduced to control levels following insulin supplementation (Íbias, O'Dell, & Nazarian, 2018). Also, an intracranial self-stimulation study revealed that STZ-treated rats display a decrease in brain reward function that was reduced to control levels following insulin supplementation (Ho et al., 2012). It was also expected that STZ-

treated rats would display a decrease in insulin signaling in the NAc given a previous study showing that STZ-treated rats displayed a reduction in pIRS-2 in the NAc (O'Dell & Nazarian, 2016).

Aim 1*:* Determine whether insulin modulates the strong reinforcing effects of nicotine and changes in biomarkers of metabolic syndrome in a rodent model of diabetes.

Aim 2*:* Determine whether insulin normalizes changes in insulin biomarkers in the NAc and VTA in a rodent model of diabetes.

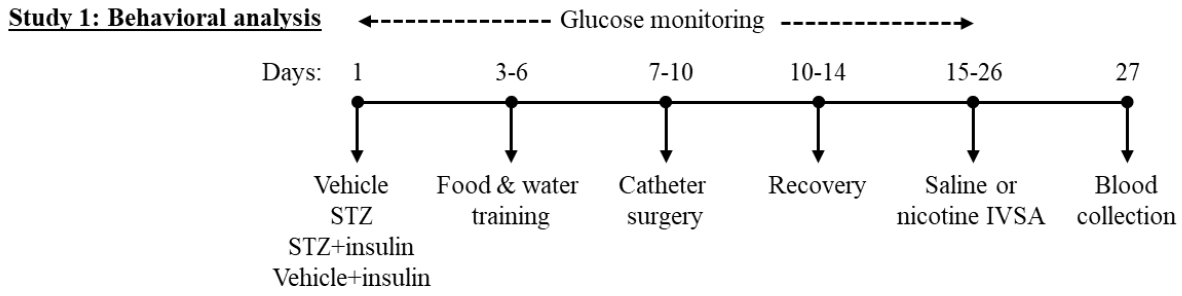
Chapter 2: Methods and Materials

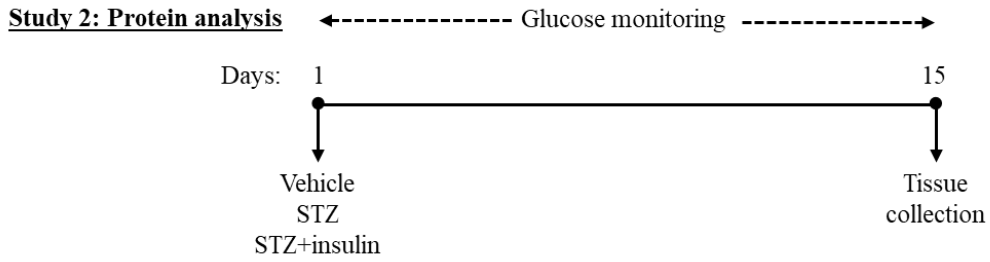
2.1 Subjects

Adult male rats were derived from an out-bred stock of Wistar rats that were maintained on a 12-hour light/dark cycle (lights off at 6:00 PM and on at 6:00 AM) in a humidity- and temperature- controlled vivarium (22° C). Between postnatal day 52-60, the rats were handled and weighed for 3-5 days prior to the start of the experiments. The animals had *ad libitum* access to food and water prior to IVSA testing, and they were allowed to operant respond for food and water without limitations during the IVSA sessions. All procedures were approved by the UTEP Institutional Animal Care and Use Committee.

2.2 Overall experimental design

Below is a summary diagram of the overall experimental timeline.





This report consists of 2 studies that were conducted in separate cohorts of rats. Study 1 employed extended IVSA procedures to examine whether STZ-treated rats display an increase in

nicotine intake that is reduced to control levels following insulin supplementation. Specifically, Study 1 compared nicotine IVSA in vehicle-treated (*n*=17), STZ-treated (*n*=16), and STZ-treated + insulin (*n*=13) rats. To examine the role of insulin on nicotine IVSA in the absence of STZ, a separate group of vehicle-treated rats received insulin supplementation (*n*=8). This study also included vehicle-treated rats that received saline IVSA (*n*=10). Changes in food intake, water responses, and body weight were assessed each day of IVSA. Twenty-four hours after the last IVSA session, metabolic biomarkers were assessed in plasma collected from a subset of rats from Study 1. Study 2 employed protein analysis procedures to assess changes in insulin biomarkers in the NAc and VTA of STZ-treated rats that received insulin supplementation. Specifically, Study 2 compared insulin-signaling proteins in nicotine naïve vehicle-treated (*n*=6), STZ-treated (*n*=6), or STZ-treated + insulin (*n*=5-6) rats. Brain tissue was collected 2 weeks after STZ administration to examine protein markers at a time point that corresponded to the onset of nicotine IVSA in Study 1.

2.3 Diabetes induction and insulin supplementation

Rats received vehicle or STZ administration (45 mg/kg, s.c.). STZ was dissolved in citrate buffer (0.1 M citric acid and 0.1 M sodium citrate; Sigma-Aldrich, St. Louis, MO, USA). The dose of STZ was selected based on previous work demonstrating that this concentration produces a rapid and reliable increase in glucose levels 3-5 days after STZ administration (Pipkin et al., 2017; O'Dell et al., 2014). Immediately after vehicle or STZ administration, the rats were anesthetized with an isoflurane/oxygen vapor mixture (1-3%) and received a sham surgery or implanted subcutaneously with 2 insulin pellets based on the manufacturer specifications for the weight (Linplant® Toronto, ONT, CA). According to the manufacturer, each pellet releases 2 U/24 hours, consistent with physiological levels in humans. Also, the pellets are purported to

release insulin for at least 60 days, which exceeds our experimental timeline. Indeed, Table 1 illustrates that the insulin pellets maintain control levels of glucose in STZ-treated rats throughout IVSA testing. Plasma glucose levels were assessed approximately every 2-3 days using a glucose meter calibrated for rodent plasma (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 glucose test strip. For both studies, the rats were tested 2 weeks after vehicle or STZ administration and insulin supplementation.

2.4 Study 1 methods

Study 1 examined the effects of insulin on the reinforcing effects of nicotine and changes in plasma metabolic biomarkers in vehicle- and STZ-treated rats. The rats were tested in operant chambers that were housed inside sound-attenuated ventilated cubicles (Med Associates, St. Albans, VT, USA). During IVSA testing, the rats lived in a dedicated operant chamber for 23 hours per day. The rats were removed daily from the chambers for 1 hour (11:00 AM-12:00 PM) to clean the chambers, retrieve the data, and replenish the food and water levels. During that 1-hour period, the rats were placed into a home cage in the same testing room, and they were given *ad libitum* access to food and water.

Prior to IVSA testing, the rats were trained to perform operant responses for food and water for 4-5 days on a fixed ratio (FR) 1 schedule of reinforcement. The total number of food and water operant responses were recorded daily for each rat. The rats were allowed to nose-poke in a food receptacle that delivered palatable chow from a pellet dispenser (45 mg/kg; Bio-Serv, Frenchtown, NJ, USA). The rats also performed nose-poke responses in a separate hole located in the opposite side of the food pellet dispenser and levers. Each nose poke dispensed 0.1

mL of water into an adjacent dipper cup via a syringe pump. All rats reached stable levels of responding for food and water the initial 1-2 days of food and water training.

The rats were then returned to their home cage and allowed to free feed the day before the catheterization surgery. The rats were anesthetized using an isoflurane/oxygen vapor mixture (1-3%) and were then prepared with IV catheters into the jugular vein, as previously described O'Dell et al., 2014; Flores et al., 2016; Natividad et al., 2013). After surgery, the rats were allowed to recover for 4 days in their home cage where they were given ad libitum access to food and water. The catheters were flushed daily (0.3-0.5 mL) with an antibiotic solution containing Cefazolin® dissolved in saline and heparin (30 USP units/mL).

Two-weeks after STZ administration, the rats were given extended access to saline or nicotine by introducing 2 novel levers (active and inactive) on the first IVSA session. The rationale for the delay in testing after STZ was based on a prior study showing that 2 weeks after STZ administration, diabetic rats display a profound and dose-dependent increase in nicotine-induced CPP (Pipkin et al., 2017) and IVSA (O'Dell et al., 2014). Presses on the active lever delivered 0.1 mLs of saline or nicotine via a syringe pump. Responses on the active lever delivered nicotine on a FR-1 schedule of reinforcement. Each response on the active lever illuminated a 28 V cue light above the lever at the onset of the 1 second infusion, and the cue light was terminated after a 20 second time-out period. Presses on the active lever during the time-out period had no scheduled consequences. (−) Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline and adjusted to 7.4 pH. The nicotine solutions were prepared every day and adjusted to the rats' body weight from the previous day. The doses of nicotine were selected based on previous work using 23-hour access to nicotine IVSA (O'Dell et al., 2007; O'Dell & Koob, 2007), The latter studies revealed dose-

dependent differences in nicotine IVSA that remain stable across 40 days. Also, previous work revealed that nicotine intake is increased following intermittent periods of drug abstinence, referred to as the "nicotine deprivation effect." Thus, in the present study each dose of nicotine was available for 4 consecutive days with 3 intervening days of forced abstinence prior to initiating the next higher dose of nicotine. Three animals were removed from our final analysis that were leaking from the exit port of the IV catheter, 1 rat was removed due to a faulty active lever, and 8 STZ-treated rats were eliminated that displayed glucose levels greater than 700 mg/dl or were too sick to continue behavioral testing.

Twenty-four hours after the last IVSA session, the rats were sacrificed and trunk blood was collected to assess changes in plasma metabolic biomarkers. The blood was centrifuged for 15 minutes at 6,900 rpm at 4°C. The plasma was extracted and stored at -80°C until assayed using commercially available MilliPlex® kits (Millipore Sigma, MA, USA) specific for insulin, leptin, amylin, and GLP-1. The plates were analyzed on a MAGPIX® system using xPONENT® software. The MAGPIX® system was calibrated before each assay using calibration and performance verification kits (Luminex Corporation Inc. Austin, TX, USA).

2.5 Study 2 methods

Study 2 assessed changes in insulin-signaling proteins in the NAc and VTA of vehicle- and STZ-treated rats that received insulin supplementation. This study was conducted in nicotine naïve rats in order to assess the effects of insulin in the absence of any confounding effects produced by chronic nicotine exposure. Two-weeks after vehicle or STZ administration, the rats were sacrificed and the NAc and VTA were dissected from vehicle-, STZ-, and STZ-treated+insulin rats. The tissue was flash frozen and stored at -80°C until assayed. The tissue was homogenized in lysis buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1% Igepal

(Sigma-Aldrich, St. Louis, MO, USA) dissolved in a protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN, USA), as previously described (Ramos et al., 2017). The tissue homogenates were then centrifuged at 4°C for 5 minutes at 14,000 rpm, and the resultant supernatant was collected. Protein concentrations (25 ug) were quantified from the cytosol using a Bio-Rad Protein Assay Standard II Kit with bovine serum albumin as the standard solution (Bio-Rad Laboratories, Hercules, CA, USA). Insulin-signaling proteins were separated via SDS-PAGE then transferred to nitrocellulose membranes (pIRS-2) or PVDF (IGF-1Rβ). Nitrocellulose membranes and PVDF were blocked in 5% non-fat dry milk dissolved 1X-tris-buffered saline containing tween 20 (1X-TBST; Cell-Signaling Technology Inc. Danvers, MA, USA). Primary antibodies specific for pIRS-2 (186kDa; 1:2000) and IGF-1Rβ (95kDa; 1:2000) were incubated in 5% non-fat dry milk dissolved in 1X-TBST and probed overnight at 4°C. Subsequently, membranes were repeatedly washed in 1X-TBST and re-probed for corresponding HRP-conjugated secondary antibodies specific for anti-mouse IgG (1:10000) and anti-rabbit IgG (1:10000). Membranes were imaged on a ChemiDoc^{TM} XRS+ in chemiluminescent reagents for protein intensity. All blots were normalized to α-tubulin (52kDa; 1:6000) and analyzed using ImageJ software provided by NIH.

2.6 Statistics

Three-way mixed-measures analysis of variance (ANOVA) were used to analyze intake, infusions, active lever presses, and inactive lever presses (Figure 1) as well as food intake, water responses, and changes in body weight (Figure 2). For the behavioral analyses, treatment was the between-subject factor and dose (saline or escalating doses of nicotine) and day were within-subject factors. For the behavioral results, test for assumptions of multiple sphericity were applied using the Huynh-Feldt correction factor. As a result, appropriate corrections were applied

to the degrees of freedom resulting in more conservative *F*-ratios for the behavioral measures. For the glucose analysis of Study 1, a 2-way mixed-measures ANOVA was used with treatment as a between-subject factor and time as a within subject factor (prior to IVSA versus after the last day of IVSA). For the glucose analysis of Study 2, a 1-way ANOVA was used with treatment as a between-subject factor (Table 1). For each metabolic biomarker, separate 1-way ANOVAs were used with treatment as a between-subject factor (Figure 3). For the insulin-signaling proteins, 2-way ANOVA was used with treatment and brain region (NAc or VTA) as between-subject factors. Post-hoc analyses compared group differences following significant interaction effects collapsed across day or dose depending on which main effects were observed using Fisher's LSD test, ($p \leq 0.05$).

Chapter 3: Results

3.1 Analysis of glucose levels

Table 1 illustrates mean (±SEM) plasma glucose levels. For Study 1, the initial analysis of glucose levels revealed that there was no interaction between treatment and time [$F_{(4,59)}$=0.54, p=0.71]. However, this analysis revealed a main effect of treatment [$F_{(4,59)}$=183.2, $p\leq$0.0001], with STZ-treated rats displaying higher glucose levels as compared to all other groups both prior to and at the end of IVSA (†$p\leq$0.01). Vehicle-treated rats that received insulin displayed lower glucose levels than their respective vehicle-treated rats that pressed for nicotine (*$p\leq$0.01), suggesting that the insulin regimen produced a degree of hypoglycemia in healthy animals. For Study 2, the analysis revealed a main effect of treatment [$F_{(2,14)}$=137.2, $p\leq$0.0001], with STZ-treated rats displaying higher glucose levels than all other groups (†$p\leq$0.01). Importantly, in both Study 1 and 2, STZ-treated rats that received insulin displayed similar glucose levels relative to their respective vehicle-treated controls.

3.2 Analysis of saline or nicotine IVSA

Figure 1 illustrates mean (±SEM) intake, infusions, active lever presses, and inactive lever presses. The initial analysis of daily intake (panel A) revealed that there was no interaction between treatment, dose, and day [$F_{(17.1,252.4)}$=1.12, p=0.32]. However, this analysis revealed a significant interaction between treatment and dose [$F_{(7.6,113.5)}$=4.23, $p\leq$0.0001]. Thus, the panel on the right reflects intake collapsed across day. Post hoc analyses revealed that all groups that pressed for nicotine displayed higher intake as compared to vehicle-treated rats that pressed for saline (*$p\leq$0.01). Importantly, STZ-treated rats displayed higher levels of intake at each dose of nicotine as compared to all other groups (†$p\leq$0.05). There were no significant differences in nicotine intake between vehicle- and STZ-treated+insulin groups.

The analysis of infusions (panel B) revealed that there was no interaction between treatment, dose, and day [$F_{(20.0,296.1)}$=1.15, p=0.30]. However, there was a main effect of treatment [$F_{(4,59)}$=17.34, $p\leq$0.0001]. Thus, the panel on the right reflects infusions collapsed across dose and day. Post hoc analyses revealed that all groups that pressed for nicotine displayed more infusions as compared to vehicle-treated rats that pressed for saline (*$p\leq$0.01). Importantly, STZ-treated rats displayed more nicotine infusions as compared to all other groups (†$p\leq$0.05). There were no differences in the number of nicotine infusions between vehicle- and STZ-treated+insulin groups.

The analysis of active lever presses (panel C) revealed that there was no interaction between treatment, dose, and day [$F_{(18.1,267.6)}$=0.48, p=0.97]. However, there was a main effect of treatment [$F_{(4,59)}$=20.45, $p\leq$0.01]. Post hoc analyses revealed that both vehicle-treated groups that pressed for nicotine displayed more active lever presses as compared to the vehicle-treated group that pressed for saline (*$p\leq$0.01). Importantly, STZ-treated rats displayed more active lever presses for nicotine as compared to all other groups (†$p\leq$0.05). There were no differences in active lever presses between vehicle- and STZ-treated+insulin groups. The analysis of inactive lever presses (panel D) revealed that there was no interaction between treatment, dose, and day [$F_{(18.1,268.0)}$=0.57, p=0.92]. There were also no main effects of treatment [$F_{(4,59)}$=1.63, p=0.18] or dose [$F_{(1.6,98.8)}$=0.75, p=0.69].

3.3 Analysis of food, water, and weight

Figure 2 illustrates mean (±SEM) food intake, water responses, and weight change. The analysis of food intake (panel A) revealed that there was no interaction between treatment, dose, and day [$F_{(21.1,312.6)}$=0.61, p=0.91]. However, there was a main effect of treatment [$F_{(4,59)}$=14.56, $p\leq$0.01]. Post hoc analyses revealed that vehicle- and STZ-treated+insulin groups that pressed

for nicotine displayed less food intake as compared to vehicle-treated controls that pressed for saline (*$p \leq 0.01$), an effect that illustrates the food suppressant effects of nicotine. Importantly, STZ-treated rats displayed higher levels of food intake as compared to all other groups (†$p \leq 0.05$). There were no differences in food intake between vehicle- and STZ-treated+insulin groups. The analysis of water responses (panel B) revealed that there was no interaction between treatment, dose, and day [$F_{(11.5,170.7)}=1.04$, $p=0.41$]. However, there was a main effect of treatment [$F_{(4,59)}=6.31$, $p \leq 0.0001$]. Post hoc analyses revealed that STZ-treated rats displayed more water responses than all other groups (†$p \leq 0.05$). There were no differences in water intake between vehicle- and STZ-treated+insulin groups.

Weight change was calculated by subtracting daily body weight from values collected on the first day of IVSA, such that positive values reflect an increase and negative values reflect a decrease in body weight from the first day of IVSA. The analysis of weight change (panel C) revealed that there was no interaction between treatment, dose, and day [$F_{(21.0,311.0)}=1.15$, $p=0.29$]. However, there was a main effect of treatment [$F_{(4,59)}=16.84$, $p \leq 0.0001$]. Post hoc analyses revealed that vehicle-treated rats that pressed for saline displayed greater increases in body weight as compared to vehicle-, STZ-, and STZ-treated+insulin rats that pressed for nicotine (*$p \leq 0.01$), an effect that illustrates the weight suppressant effects of nicotine. Importantly, STZ-treated rats displayed lower body weights relative to all other groups (†$p \leq 0.05$). There were no differences between vehicle- and STZ-treated+insulin groups.

3.4 Analysis of insulin biomarkers

Figure 3 illustrates mean plasma levels (±SEM) of insulin, leptin, amylin, and GLP-1. The analysis of insulin (panel A) revealed a main effect of treatment [$F_{(4,43)}=8.62$, $p \leq 0.0001$]. Post hoc analysis revealed that STZ-treated rats displayed lower levels of insulin as compared to

all other groups (†$p \leq 0.0001$). There was no difference between the vehicle- and STZ-treated + insulin groups. The analysis of leptin (panel B) revealed a main effect of treatment [$F_{(4,43)}=11.62$, $p \leq 0.0001$]. STZ-treated rats displayed lower levels of leptin as compared to all other groups (†$p \leq 0.0001$). There were no differences in leptin levels between vehicle- and STZ-treated+insulin groups. The analysis of amylin (panel C) revealed a main effect of treatment [$F_{(4,43)}=5.43$, $p \leq 0.001$]. STZ-treated rats displayed higher levels of amylin than their respective vehicle-treated controls that pressed for nicotine (#$p \leq 0.0001$), an effect that illustrates STZ-induced enhancement of amylin levels. Also, vehicle-treated rats that received insulin displayed higher amylin levels than vehicle-treated rats that pressed for nicotine (#$p \leq 0.0001$), suggesting that our insulin regimen increased amylin levels. There were no differences in amylin levels between vehicle- and STZ-treated+insulin rats. The analysis of GLP-1 (panel D) revealed a main effect of treatment [$F_{(4,43)}=3.83$, $p \leq 0.01$]. STZ-treated rats displayed higher levels of GLP-1 than vehicle-treated rats that pressed for nicotine (#$p \leq 0.01$), an effect that illustrates STZ-induced increases in GLP-1 levels. Also, vehicle-treated rats that received insulin displayed an increase in GLP-1 levels as compared to vehicle-treated rats that pressed for nicotine (#$p \leq 0.0001$), suggesting that insulin enhanced GLP-1 levels alone. There were no differences in GLP-1 levels between vehicle- and STZ-treated+insulin rats.

3.5 Analysis of insulin-signaling proteins

Figure 4 illustrates mean protein levels (±SEM) of pIRS-2 and IGF-1Rβ in the NAc and VTA. The analysis of pIRS-2 revealed a significant interaction between treatment and brain region [$F_{(2,28)}=16.46$, $p \leq 0.0001$]. In the NAc (panel A), post-hoc analysis revealed that STZ-treated rats displayed lower levels of pIRS-2 as compared to all other groups (†$p \leq 0.0001$). There was no difference in pIRS-2 levels between vehicle- and STZ-treated+insulin rats. In the VTA

(panel B), post-hoc analysis revealed that both groups of STZ- and STZ-treated+insulin rats displayed higher levels of pIRS-2 as compared to vehicle-treated controls (*$p \leq 0.0001$). Also, STZ-treated+insulin rats displayed higher levels of pIRS-2 as compared to STZ-treated rats (#$p \leq 0.0001$), suggesting that insulin produced a further enhancement of pIRS-2 levels in the VTA.

The analysis of IGF-1Rβ revealed a significant interaction between treatment and brain region [$F_{(2,29)}=5.31$, $p \leq 0.01$]. In the NAc (panel C), post-hoc analysis revealed that STZ-treated rats displayed lower levels of IGF-1Rβ as compared to all other groups (†$p \leq 0.0001$). There was no difference in IGF-1Rβ levels between vehicle- and STZ-treated+insulin rats. In the VTA (panel D), post-hoc analysis revealed that STZ-treated+insulin rats displayed higher levels of IGF-1Rβ as compared to vehicle-treated controls (*$p \leq 0.0001$).

Chapter 4: Discussion

4.1 Summary

The present study revealed that STZ-treated rats displayed an increase in plasma glucose levels, body weight, food intake, and water responses. The pattern of changes in these measures was prevented in STZ-treated rats that also received insulin, consistent with another report using the same insulin supplementation procedure (Sevak et al., 2007). The finding that insulin prevented STZ-induced increases in glucose levels, food intake, and water responses to vehicle-treated control levels provides verification of the induction and reversal of hypoinsulinemia, a hallmark physiological effect of diabetes. The major finding of Study 1 was that STZ-treated rats displayed greater reinforcing effects of nicotine relative to vehicle-treated rats, and this effect was reduced to control levels in STZ-treated rats that also received insulin. STZ-treated rats also displayed a decrease in insulin and leptin and an increase in amylin and GLP-1 plasma levels, and the pattern of these changes was reduced to control levels in STZ-treated rats that received insulin. STZ-treated rats also displayed a decrease in pIRS-2 and IGF-1Rβ levels in the NAc, and this pattern of changes was also reduced to control levels in STZ-treated rats that received insulin supplementation. Also, STZ-treated rats that received insulin supplementation displayed an increase in pIRS-2 and IGF-1Rβ levels in the VTA, suggesting that an enhancement of insulin systems in the VTA may have promoted the behavioral effects observed in this group.

4.2 Reinforcing effects of nicotine

The present study revealed that STZ administration enhanced nicotine IVSA across a range of doses as compared to vehicle-treated rats, consistent with previous research from our laboratory using extended access to nicotine IVSA procedures (O'Dell et al., 2014). The notion that STZ enhances the rewarding effects of nicotine is consistent with previous work showing

that STZ enhances CPP produced by nicotine (Pipkin et al., 2017), an effect that is normalized to control levels following insulin supplementation (Íbias et al., 2018). Previous research has also revealed that insulin-resistant rats that were fed a high fat diet (HFD) displayed greater CPP produced by nicotine as compared to non-insulin resistant rats that received the HFD regimen (Richardson et al., 2014). Together, these findings suggest that a lack of insulin signaling enhances the reinforcing effects of nicotine.

4.3 Insulin biomarkers

STZ administration altered plasma metabolic biomarkers, and the pattern of changes in these biomarkers was reduced to control levels following insulin supplementation. Specifically, STZ-treated rats displayed a decrease in insulin and leptin, consistent with a previous report showing that STZ administration blunted insulin and leptin release (Havel et al., 1998). Given the role of insulin and leptin in regulating satiety and consummatory behaviors, the reduction in insulin and leptin likely contributed to the increase in food intake and water responses observed in STZ-treated rats (Figlewicz & Sipols, 2010; Stice et al., 2012). STZ-treated rats also displayed an increase in amylin and GLP-1 levels as compared to vehicle-treated rats. The direction of these changes is consistent with the anorectic effects of amylin, as administration of this metabolic biomarker has been shown to induce weight loss in rodents (Mietlicki-Baase et al., 2015). Moreover, long-term administration of amylin or an amylin agonist induces weight loss (Mietlicki-Baase et al., 2015; Lutz, 2012). With regard to GLP-1, this hormone stimulates the synthesis and secretion of insulin (Lee & Jun, 2014) and elevated levels of GLP-1 are associated with weight loss (Keleidari et al., 2018; Koliaki & Doupis, 2011). Plasma levels of GLP-1 also increase following food consumption, and the release of GLP-1 prevents pancreatic β-cell death

(Keleidari et al., 2018). Thus, it is possible that STZ-treated rats displayed higher levels of GLP-1 in response to lower levels of plasma insulin.

4.4 Insulin in the mesolimbic system

The changes in insulin-signaling proteins were focused in the NAc, a terminal region of the mesolimbic reward pathway. Specifically, STZ-treated rats displayed a down-regulation of NAc pIRS-2 and IGF-1Rβ levels that were returned to control levels in STZ-treated rats that received insulin. Consistent with previous findings, STZ-treated rats displayed a decrease in pIRS-2 levels in the NAc relative to controls (O'Dell & Nazarian, 2016). Previous work has also shown that STZ administration decreases IRS-2 signaling in the hippocampus (Li et al., 2002; Gomes et al., 2009) and hypothalamus (Gelling et al., 2006), an effect that was returned to control levels following viral-mediated over-expression of IRS-2 levels. Interestingly, the levels of pIRS-2 and IGF-1Rβ were increased in the VTA of STZ-treated rats that received insulin relative to vehicle-treated controls, suggesting that the insulin supplementation regimen stimulated pIRS-2 and IGF-1Rβ signaling in the VTA. Together, these findings suggest that the strong reinforcing effects of nicotine observed in STZ-treated rats are modulated via insulin systems in the NAc.

A critical common denominator in metabolic signaling downstream of the actions of insulin, leptin and GLP-1 are their effects on blood glucose levels and the resulting decrease in glucose levels upon insulin treatment. Glucose has been shown to have direct effects on increasing and decreasing mesolimbic dopamine neuronal activity in a concentration dependent manner (Levin, 2000). Glucose is also capable of producing rewarding effects independent of its sweet taste, as evident by CPP evoked following intragastric glucose administration (Ren et al., 2010). In fact, recent work in our laboratory has shown that the strong rewarding effects of

nicotine observed in STZ-treated rats can be attenuated by direct normalization of blood glucose levels with dapagliflozin, a sodium-glucose type 2 cotransporter inhibitor (Íbias et al., 2018) Importantly, a prior study revealed that administration of a GLP-1 receptor agonist (Exendin-4) reduces nicotine-induced CPP, locomotor activity, and increases in NAc dopamine release compared to controls (Egecioglu, Engel, & Jerlhag, 2013). Also, administration of a GLP-1 agonist into the interpeduncular nucleus reduces nicotine IVSA as compared to controls (Tuesta et al., 2017). Also, viral-mediated gene activation of GLP-1 neurons in the nucleus tractus solitarius has been shown to reduce nicotine IVSA as compared to controls (Tuesta et al., 2017). The latter study also demonstrated that viral-mediated knockdown of GLP-1 receptors in the medial habenula promotes nicotine IVSA compared to controls. The latter findings along with the present results suggest that the rewarding effect of nicotine are enhanced in diabetic rodents via a complex interplay of different metabolic factors.

It has been hypothesized that the high propensity of tobacco use among patients with diabetes is modulated via reduced dopamine transmission in the mesolimbic pathway [see 20]. Indeed, dampened dopaminergic systems may promote other compulsive behaviors, such as overeating (Blum et al., 2000, 2008; Fineberg et al., 2010). It is possible that the lack of insulin produced by diabetes suppresses dopamine systems in a manner that promotes tobacco use in an attempt to increase dopamine transmission. In line with this suggestion, STZ-treated rats display higher brain reward thresholds than healthy controls using intracranial self-stimulation procedures, an indication of a reward deficiency syndrome (Ho et al., 2012). The latter effect was normalized to control levels following insulin supplementation. The manner in which a disruption in insulin signaling produced by diabetes alters dopamine systems is unclear. However, recent evidence suggests that insulin facilitates dopamine transmission via cholinergic

interneurons in the NAc (Stouffer et al., 2015). The latter study demonstrated that insulin administration enhances dopamine release in NAc indirectly via activation of insulin receptors expressed on cholinergic interneurons. Thus, it is possible that diabetes reduces insulin receptor activation in striatal cholinergic interneurons, thereby attenuating dopamine release in the NAc. Indeed, STZ-treated rats display a reduction in nicotine-induced dopamine release in the NAc (O'Dell et al., 2014). STZ-treated rats also displayed an up-regulation of dopamine transporters (DAT) in the NAc, an effect that is likely related to greater clearance of extracellular dopamine in STZ-treated rats. STZ-treated rats also displayed a larger down-regulation of dopamine D1-receptors in the NAc as compared to controls, an effect that is likely due to chronic low levels of dopamine observed in STZ-treated rats (O'Dell et al., 2014).

4.5 Other drugs of abuse

Prior studies have produced mixed results with regard to the rewarding effects of drugs of abuse other than nicotine in STZ-treated rats. For example, STZ-treated rats display similar levels of cocaine IVSA as compared to vehicle-treated rats (Galaci et al., 2003). However, STZ-treated rats display a reduction in the magnitude of cocaine-induced CPP relative to vehicle-treated rats (Kamei & Ohsawa, 1997). STZ-treated rats display less amphetamine IVSA relative to vehicle-treated rats (Galici et al., 2003; Sevak et al., 2008). The latter study also found no differences in the magnitude of amphetamine-induced CPP in vehicle- versus STZ-treated rats (Sevak et al., 2008). STZ-treated rats display a larger magnitude of CPP produced by methamphetamine (Bayat & Haghparast, 2015; Kamei & Ohsawa, 1996) and morphine (Kamei, Ohsawa, & Suzuki, 1997; Samandari et al., 2013). Previous work has also shown that STZ-treated rats display greater sensitivity to the behavioral effects (locomotor activity, catalepsy, and yawning) of dopamine D2/D3 receptor agonists (Sevak et al., 2007; Sevak, Koek, & France,

2005). This increase in dopamine receptor sensitivity produced by STZ was reduced to control levels following insulin supplementation (Sevak et al., 2007). The latter finding is consistent with the present finding that the pattern of changes in behavior and biological markers induced by STZ are returned to control levels following insulin supplementation.

4.6 Reduced aversive effects of nicotine

In addition to nicotine reward, the short-term effects of nicotine also include aversive effects, particularly at high doses of this drug. Previous studies have shown that rats display CPA for an environment that was previously paired with a high dose of nicotine (Torres et al., 2008). Also, the aversive effects nicotine have been studied using conditioned taste aversion (CTA) procedures, where rats avoid a flavor previously paired with a high dose of nicotine. Previous work has revealed that the aversive effects of nicotine are lower in STZ-treated versus health control rats (Pipkin et al., 2017). Specifically, the latter report found that STZ-treated rats display reduced CPA following administration of a high dose of nicotine as compared to healthy controls. The present finding suggests that STZ-treated rats display strong rewarding effects of nicotine that are insulin mediated. Future studies are needed to examine whether the lack of aversive effects of nicotine is insulin mediated, and whether reduced aversive effects of nicotine promotes tobacco use in persons with diabetes.

4.7 Other considerations

The possibility exists that STZ produces an array of biological consequences that may have influenced our IVSA results. With regard to body weight, the concentration of nicotine was adjusted daily for each rat in order to account for the reduction in body weight across our experimental timeline with diabetic rodents. With regard to fluid loss produced by excessive urination, a previous study revealed that STZ-treated rats displayed lower levels of saline versus

nicotine IVSA (O'Dell et al., 2014). Thus, it does not appear that STZ-treated rats self-administer more nicotine in an attempt to increase fluid levels. Another potential consideration is that STZ-treated rats display tactile allodynia and cataract formation (Morrow, 2004; Wei et al., 2003), and these effects of STZ could disrupt IVSA behavior. However, the emergence of these side effects of STZ occurs at a later time point that exceeds our 12-day period of IVSA. Furthermore, the present study found that STZ-treated rats display robust increases in operant responding for nicotine, food, and water that do not appear to be altered by the emergence of any negative effects of STZ. Another important consideration is that vehicle-treated rats that received insulin displayed a hypoglycemic state below vehicle-treated controls. This effect of insulin might explain the increases in food intake, weight change, GLP-1, and amylin levels that were observed in insulin-treated rats. Indeed, GLP-1 levels have been shown to increase in procedures that enhance food consumption in rodents (Keleidari et al., 2018). Also, administration of amylin has been shown to produce a hypoglycemic state in healthy mice, suggesting that hypoglycemia may produce elevated amylin levels in rodents (Guerreiro et al., 2013). Lastly, the possibility exists that STZ-treated rats may display an altered metabolic rate of nicotine that could influence our nicotine IVSA results. However, a previous study in our laboratory revealed that STZ-treated rats display similar plasma levels of the nicotine metabolic cotinine as compared to control rats (O'Dell et al., 2014).

4.8 Clinical implications

There are several clinical implications to consider from this report. First, these results suggest that a lack of insulin signaling promotes the strong rewarding effects of nicotine in patients with diabetes. There are converging lines of evidence suggesting that suppressed insulin signaling impairs dopamine transmission in both humans and rodent models (O'Dell et al., 2014;

Carvaggio et al., 2015;Trulson & Himmel, 1983; Lackovic et al., 1990; Kono & Takada, 1994; Crandall & Fernstorm, 1983; Bitar et al., 1986). Thus, pharmacological interventions that normalize insulin signaling and dopamine deficits may reduce tobacco use in patients with diabetes. As a result, maintaining proper glycemic control may be critical for diabetic patients seeking to reduce drug use and/or smoking behavior. Another rodent study revealed that IRS-2 knockout mice demonstrate impaired insulin signaling and glucose tolerance (Withers et al., 1998). Thus, pharmacotherapies that facilitate IRS-2 signaling should be considered as potential tools to reduce the strong rewarding effects of nicotine that may promote tobacco use in patients with diabetes. Future studies are needed to assess the role of glucose in modulating the rewarding effects of nicotine, especially given the finding that dapagliflozin (Farxiga®), a sodium-glucose transport inhibitor normalized CPP produced by nicotine in STZ-treated rats (Íbias et al., 2018). Also, a recent study revealed that administration of glucophage (Metformin®), a medication used to treat Type 2 diabetes reduced anxiety-like behaviors produce by nicotine withdrawal in mice (Brynildsen et al., 2018). It is recognized that different biological consequences may accompany the absence of insulin versus the insensitivity to insulin. These distinct etiological origins of Type 1 versus Type 2 diabetes may require different smoking cessation remedies in patients who suffer from different types of diabetes. Future studies are needed to assess the neurobiological substrates by which diabetes enhances tobacco use vulnerability in patients with diabetes.

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Table 1. Glucose levels (mg/dL±SEM) across experimental groups that received vehicle or STZ

Study 1: Behavioral analysis					Study 2: Protein analysis			
Groups:	**IVSA of:**	***n***	**Glucose levels prior to IVSA**	**Glucose levels at the end of IVSA**	**Groups:**	**NAc (*n*)**	**VTA (*n*)**	**Glucose levels**
Vehicle-treated	Saline	10	116.2±6.5	136.0±3.7	Vehicle-treated	6	6	137.6±5.0
Vehicle-treated	Nicotine	17	123.7±2.8	148.9±10.4				
STZ-treated	Nicotine	13	515.5±17.1†	505.3±28.4†	STZ-treated	6	6	570.0±23.8†
STZ-treated+insulin	Nicotine	16	116.6±21.8	127.5±25.3	STZ-treated+insulin	5	6	178.5±22.6
Vehicle-treated+insulin	Nicotine	8	77.7±5.9*	86.7±14.5				

*The values reflect mean glucose (mg/dL±SEM) levels that were taken prior to IVSA and at the end of IVSA. †Indicates different from all other groups, and *indicates different from vehicle-treated rats (p≤0.05).*

Table 1.

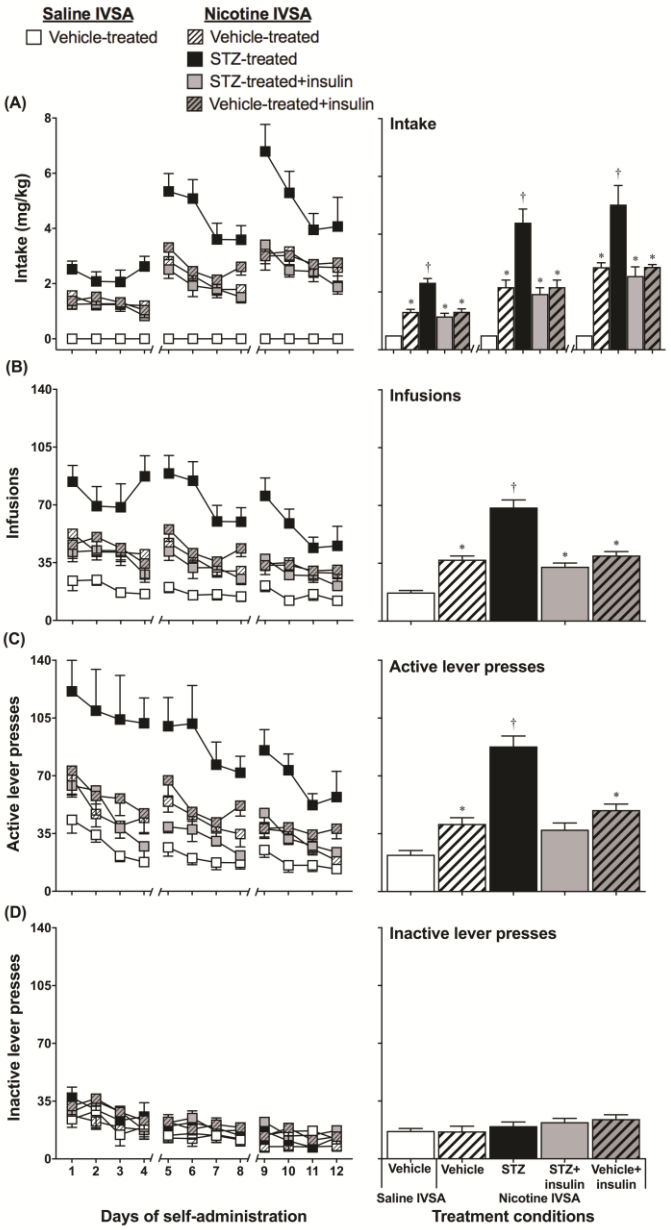

Figure 1. Mean (±SEM) nicotine intake (A), nicotine infusions (B), active lever presses (C), and inactive lever presses (D) during each day (left panels) and averaged across days (right panels). The group sizes were as follows: vehicle-treated/Saline IVSA (*n*=10), vehicle-treated/Nicotine IVSA (*n*=17), STZ-treated/Nicotine IVSA (*n*=16), STZ-treated+insulin/Nicotine IVSA (*n*=13), and vehicle-treated+insulin/Nicotine IVSA (*n*=8). The asterisks (*) denote a significant difference from vehicle-treated/Saline IVSA rats, and the daggers (†) denote a difference from all other groups ($p \leq 0.05$).

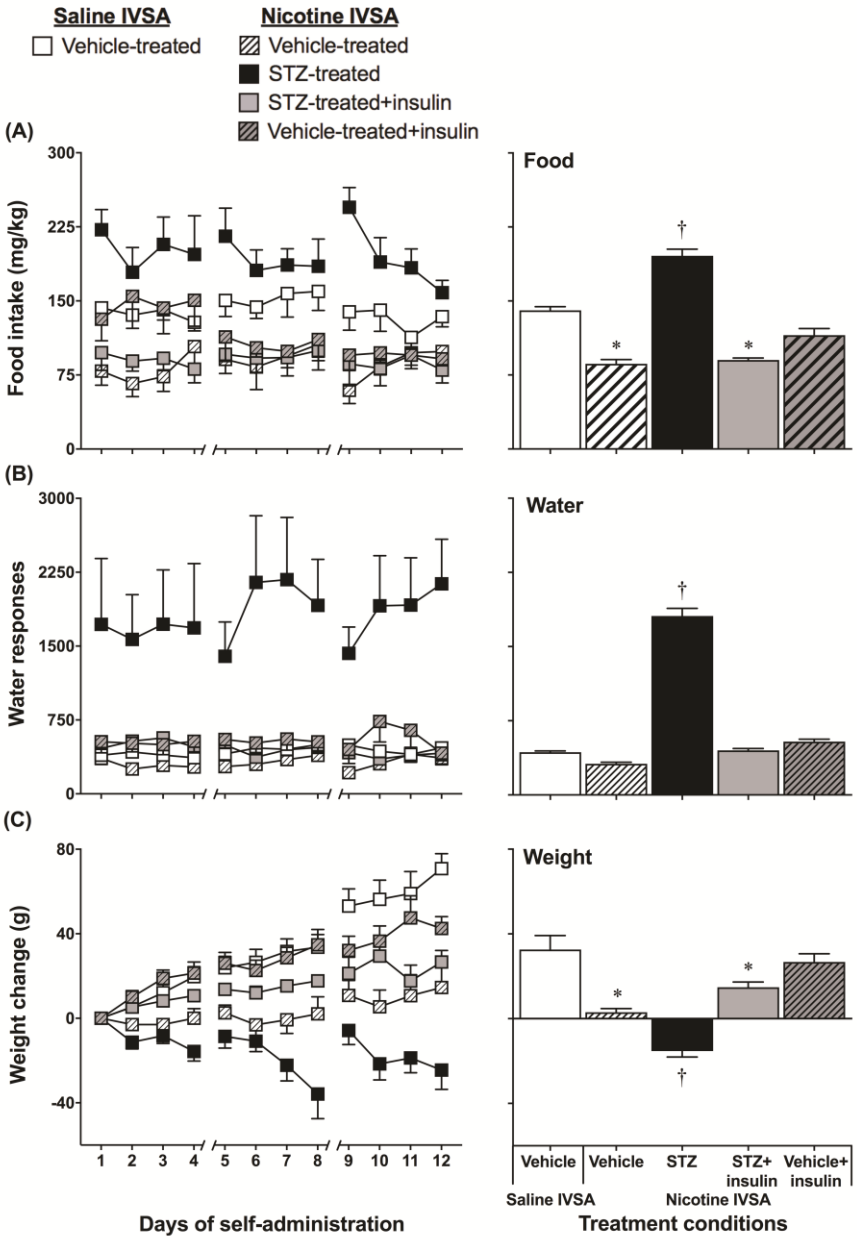

Figure 2. Mean (±SEM) food intake (A), water responses (B), and weight change (D) during each day (left panels) and averaged across days (right panels). The group sizes were as follows: vehicle-treated/Saline IVSA (*n*=10), vehicle-treated/Nicotine IVSA (*n*=17), STZ-treated/Nicotine IVSA (*n*=16), STZ-treated+insulin/Nicotine IVSA (*n*=13), and vehicle-treated+insulin/Nicotine IVSA (*n*=8). The asterisks (*) denote a significant difference from vehicle-treated/Saline IVSA rats, and the daggers (†) denote a difference from all other groups ($p \leq 0.05$).

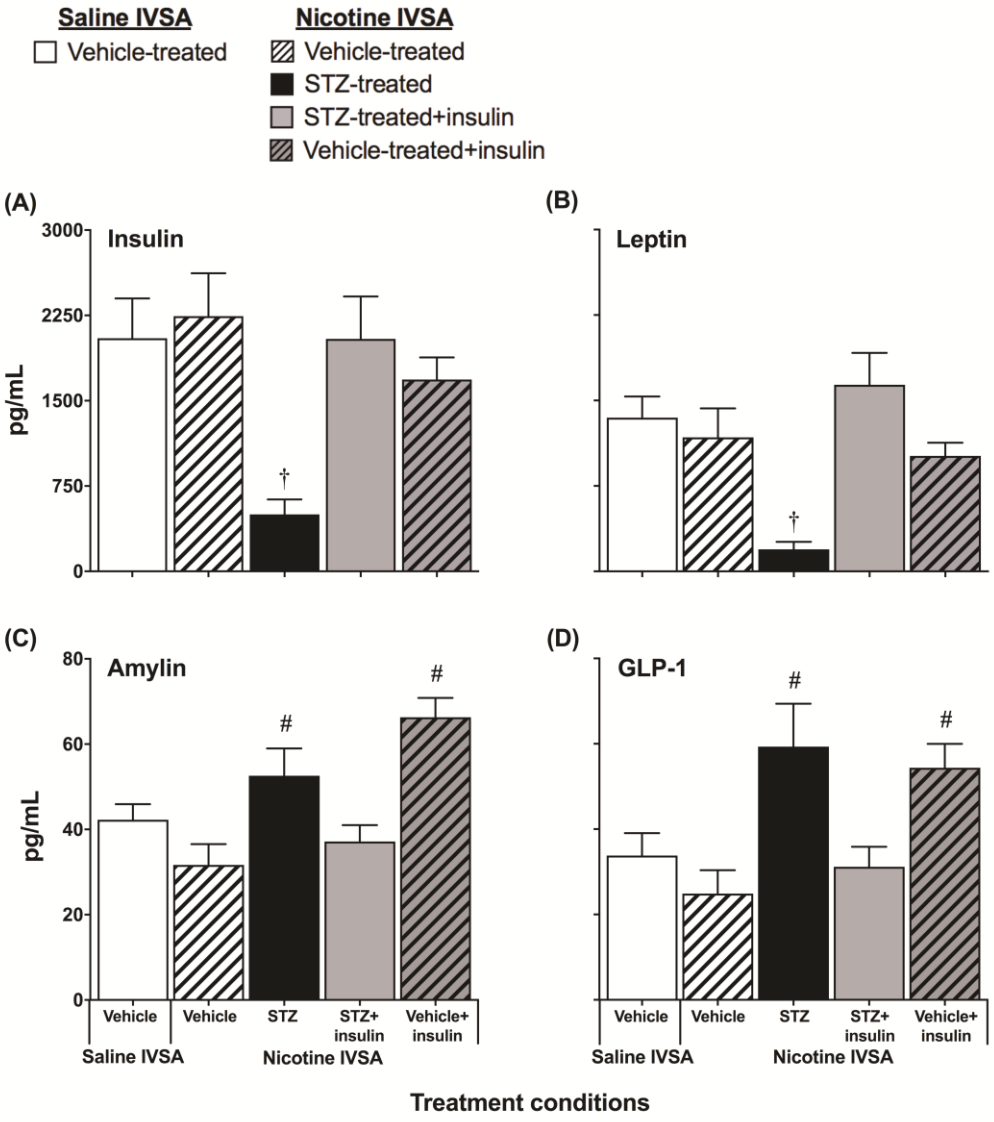

Figure 3. Mean (±SEM) plasma levels of insulin (A), leptin (B), amylin (C), and GLP-1 (D) collected after the last IVSA session. The daggers (†) denote a significant difference from all other groups ($p \leq 0.05$), and the number signs (#) denote a difference from vehicle-treated rats that pressed for nicotine.

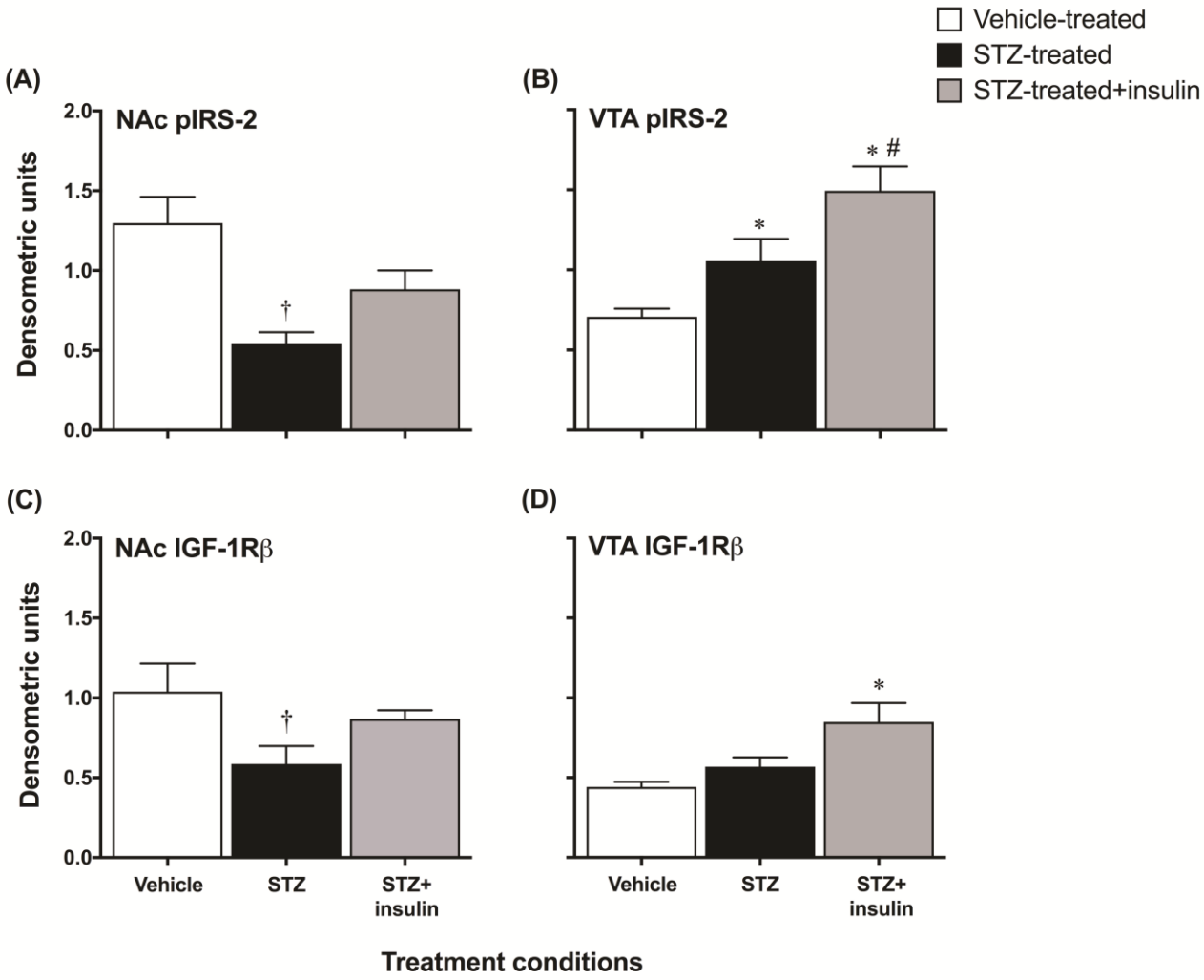

Figure 4. Mean (±SEM) protein levels of pIRS-2 (A and B) and IGF-1Rβ (C and D) in the NAc (left panels) and VTA (right panels). The group sizes were as follows: vehicle-treated (*n*=6), STZ-treated (*n*=6), and STZ-treated+insulin (*n*=5). The asterisks (*) denote a significant difference from vehicle-treated rats, and the daggers (†) denote a difference from all other groups ($p \leq 0.05$).

Vita

Bryan Cruz was born in Los Angeles, California to Gerardo Cruz Velázquez and Maria del Carmen Rios de Geronimo from Reforma de Pineda in Oaxaca, México. As a first generation Mexican-American, Bryan graduated from Patriot High School in Riverside, California in 2010. Later that year, he attended California State University of San Bernardino, where he earned his Bachelor's Degree in Biological Psychology. He received a minor in Spanish Literature from The Universidad de Valladolid in Valladolid, Spain. As an undergraduate, he conducted research under the mentorship of Dr. Sergio D. Iñiguez where he examined the effects of psychotropic medications on emotional stimuli across development in rodent models. Following this experience, he became interested in studying the underlying factors that promote drug use in vulnerable populations using rodent models. He pursued a summer research program in 2014, where he examined memory consolidation using fish as a model system at Hunter College in Manhattan, New York. In the Fall of 2015, he enrolled in the Social, Cognition, and Neuroscience graduate program at The University of Texas at El Paso. He joined the laboratory of Dr. Laura E. O'Dell who studies the underlying factors that promote tobacco use in vulnerable populations. Her laboratory uses various approaches to address the problem of tobacco use from a behavioral, molecular, and neurochemical perspective. His first graduate study investigated the role of insulin in modulating the strong reinforcing effects of nicotine in a rodent model of diabetes. Bryan has presented at numerous scientific conferences. He has first-authored 1 and co-authored 4 publications and 1 Book Chapter in peer-reviewed journals such as *Neuropsychopharmacology, Psychopharmacology, Scientific Reports, Stress, and Physiology & Behavior*.

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