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Acute nicotine intake increases feeding behavior through decreasing glucagon signaling in dependent male and female rats

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Abstract

Chronic use of nicotine is known to dysregulate metabolic signaling through altering circulating levels of feeding-related hormones, contributing to the onset of disorders like type 2 diabetes. However, little is known about the acute effects of nicotine on hormonal signaling. We previously identified an acute increase in food intake following acute nicotine, and we sought to determine whether this behavior was due to a change in hormone levels. We first identified that acute nicotine injection produces an increase in feeding behavior in dependent rats, but not nondependent rats. We confirmed that chronic nicotine use increases circulating levels of insulin, leptin, and ghrelin, and these correlate with rats' body weight and food intake. Acute nicotine injection in dependent animals decreased circulating GLP-1 and glucagon levels, and administration of glucagon prior to acute nicotine injection prevented the acute increase in feeding behavior. Thus, acute nicotine injection increases feeding behavior in dependent rats by decreasing glucagon signaling.

Keywords

addiction; rodent; glucagon; GLP-1; insulin; leptin; ghrelin; meal; nicotine dependence

Introduction

Cessation of nicotine use is difficult for most smokers, with fewer than ten percent of smokers successfully quitting. A commonly cited reason that hinders quitting is the increased weight gain and cravings that smokers report following abstinence. Nicotine use has canonically been thought to decrease food intake and increase satiety signaling; however, this dogma may not hold true at a short timescale. We previously reported that acute nicotine intake produced an acute increase in feeding behavior in nicotine-dependent rats[1]. While we observe this paradoxical effect of nicotine intake on feeding behavior, the

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mechanism by which acute nicotine produces this increased feeding is unknown. As nicotine is known to alter feeding behavior and energy metabolism[2,3], it is important to understand how nicotine may interact with these feeding hormones.

Nicotine is mainly studied for its actions within the central nervous system, but nicotine also acts on nicotinic acetylcholine receptors on cardiac and skeletal muscle cells as well as parasympathetic nerve cells to increase activity and produce physiologically stimulating effects[4-6]. Outside of the brain, tissues like white adipose tissue and organs like the pancreas, stomach, and small intestine secrete a wide variety of peptides in response to the body's physiological state to drive hunger and satiety behaviors. These chemicals enter the circulation and often cross the blood-brain barrier to activate neuronal signaling pathways that control food intake. A summary of some key feeding-related hormones and their functions can be found in Table 1 below. Chronic nicotine use has been shown to decrease food intake, body weight gain, and increase energy expenditure, so it may be hypothesized that there would be an increase in circulating hormones that increase satiety – leptin, insulin, glucagon, GLP-1, PYY – and a decrease in ghrelin which promotes hunger and feeding. Yet studies measuring the effect of nicotine on these feeding hormones have yielded mixed results. Leptin levels have been shown to either increase or decrease following nicotine use, making it unclear whether chronic nicotine use increases leptin sensitivity or resistance[7-12]. Ghrelin levels have been shown to increase after acute and chronic nicotine use[13-15]. Chronic nicotine use has been linked to the propensity for metabolic disorders like type 2 diabetes, and this can be partially due to the induction of insulin resistance following chronic nicotine use; however, many studies show a positive effect of nicotine administration on improving insulin sensitivity in diabetic subjects[16-18]. Another contribution of smoking to type 2 diabetes risk is its ability to reduce plasma glucose levels, potentially due to elevated glucagon in the fasted state[19].

However, little is still known about the acute effect of nicotine on hormonal changes. The goal of this study is to determine the contribution of feeding-related hormones toward producing this acute increased feeding behavior following nicotine intake. Having previously identified this behavioral phenomenon in rats made dependent to nicotine by intravenous self-administration, we first wanted to determine whether we could replicate the acute increase in feeding behavior in a passive model of nicotine dependence. Extended access to nicotine self-administration is a model with high face validity and is ideal for exploring changes in nicotine-taking behaviors. However, there is a greater amount of variability in nicotine intake across animals, so it is desirable to standardize nicotine exposure across animals in order to investigate factors that may contribute to this behavioral phenomenon. We developed a model consisting of subcutaneous injections of nicotine administered before and in combination with osmotic minipumps delivering a low dose of nicotine that still elicits dependence in rats[35]. We hypothesized that using this model, acute nicotine administration would increase pro-feeding hormone levels and decrease pro-satiety hormone levels, thus contributing to increased feeding behavior.

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Methods

Animals

Adult male and female Wistar rats (8-10 weeks at the start of the study; Charles River, Hollister, CA, USA) were used for the experiments. The rats were group-housed and maintained on a 12 h/12 h light/dark cycle (lights off at 10:00 AM) with *ad libitum* access to food (45 mg grain-based tablets, TestDiet, St. Louis, MO, USA) and tap water. Body weights of all rats were recorded daily throughout the study. All of the animal procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

Nicotine hydrogen tartrate (Glentham, USA) was dissolved in 0.9% sterile saline and pH was adjusted to 7.0. For osmotic minipump delivery, nicotine solution was prepared according to rats' body weight, loaded into osmotic minipumps (Alzet 2ML4, Braintree Scientific, USA) and delivered at a rate of 1 mg/kg/day (free base). Subcutaneous injections of nicotine were administered at a dose of either 0.09 mg/kg/300 μL/injection or 0.18 mg/kg/600 μL/injection dissolved in 0.9% saline. All hormones were purchased from Bachem (Torrance, CA) and administered via subcutaneous injection at the following doses: 5 μg/kg glucagon, 10 μg/kg peptide YY (PYY) (3-36), 20 μg/kg glucagon-like peptide 1 (GLP-1) (7-36 amide).

Experiment 1: Passive model of nicotine exposure

Adult male and female Wistar rats (N = 10 M, 10 F) were first trained in operant chambers to nosepoke for food and water on a fixed-ratio (FR1) schedule of responding in 21 h sessions to establish baseline food and water intake. Sham injections of 0.9% saline were administered subcutaneously during this period to habituate rats to injections. Rats were then given injections of 0 mg/kg nicotine (saline, vehicle), 0.09 mg/kg nicotine (to match a reinforcing dose of 3 intravenous infusions within the self-administration model[36]), or 0.18 mg/kg nicotine (to provide a higher dose due to the pharmacokinetics of subcutaneous delivery). All rats were given all treatments in a pseudo-randomized order, where equal numbers of males and females were administered each treatment, but the treatment order was randomized so not all animals received the same treatment on the same day. Animals were given a 24 h washout period between each treatment. All rats received their first injection at the start of the dark cycle, were placed in operant chambers, and the 21 h food and water intake session was started. Three additional injections of the same dose were given during the dark cycle 2.5 h apart; the half-life of nicotine administered subcutaneously is approximately 30 min, so this allowed for a clearance of > 95% nicotine from the previous injection before the next one was administered. Rats were briefly removed from the operant chambers, injected, and placed back in the chamber, resulting in a 10 s window where they were unable to press for food or water.

Following the above nondependent treatments, rats were surgically implanted with osmotic minipumps containing 1 mg/kg/day nicotine (free base). This dose was chosen as it is

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enough to elicit nicotine dependence after 2 weeks, but still a low enough dose to allow for the added effect of the subcutaneous injections of nicotine based on reports summarized by Matta *et al.* that show 1 mg/kg/day nicotine produces behavioral and physiological effects consistent with habitual nicotine users[35,37]. This dose has also been shown to increase withdrawal-like behaviors in rats following administration for 1-2 weeks[38,39]. After a 2-week period to establish nicotine dependence, rats were again given 4 injections of 0 mg/kg, 0.09 mg/kg, or 0.18 mg/kg nicotine throughout the dark cycle, and food and water intake events were recorded during the 21 h sessions. A 24 h washout period was given between each treatment. Minipumps remained on board during these injections so rats were nicotine-dependent during this period. Poststimulus time histograms (PSTH) were generated to examine the effect of nicotine injection on food intake. The average number of food intake events within each 10 s bin of the 20 min window following each injection was calculated, and then an average value for all 4 injections was obtained.

Experiment 2: Effect of nicotine dependence and acute nicotine injection on circulating hormone levels

A cohort of male and female rats (N = 6 M, 6 F) were placed into operant conditioning chambers and trained to self-administer food via lever press and water via nosepoke in 21 h sessions for 5-7 days or until stable baseline intake was reached. During this time, rats were habituated to injection with 2-3 subcutaneous injections of 0.9% saline prior to the start of the experiment. All rats were subcutaneously injected with either 0.18 mg/kg nicotine or 0.9% saline (vehicle). All blood collections were performed in a 1-2 h window between the end of the light cycle and start of the dark cycle; this time was consistent for all collections throughout the study. For the nondependent time points, 200 μL blood was collected via tail vein puncture in chilled EDTA-coated tubes at baseline (30 min prior to injection; 30-60 min prior to the start of the dark cycle) and 10 min post-nicotine or saline injection. Rats were fasted for 2 h prior to baseline blood collection and were not allowed access to food until all blood collections were complete. Following blood collection, rats were placed into operant chambers and food and water intake was recorded in 21 h sessions. Rats were then surgically implanted subcutaneously with osmotic minipumps containing 1 mg/kg/day nicotine. Rats were allowed to establish dependence to nicotine for 2 weeks, and injections were repeated with minipump on board to maintain nicotine dependence. 200 μL blood was again collected 30 min before injection (baseline, dependent) and 10 min post-injection (post-injection, dependent) time points. Following blood collection, rats were placed into operant chambers and food and water intake was recorded in 21 h sessions. For extended time course blood collection, blood collection was performed separately where 200 μL blood was collected at baseline, 10 min, 30 min and 60 min post-injection (4 separate blood collections); rats were not given access to food in operant chambers until all 4 blood collections were completed. All blood samples were kept on ice after collection, centrifuged at 4°C and 3000 rpm, and plasma was extracted and stored at −80°C until use.

Experiment 3: Effect of hormone administration on acute feeding behavior following nicotine injection

A second cohort of male and female rats (N = 6 M, 6 F) were placed into operant conditioning chambers and trained to self-administer food via lever press and water via

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nosepoke in 21 h sessions for 5-7 days or until stable baseline intake was reached. During this time, rats were habituated to injection with 2-3 subcutaneous injections of 0.9% saline prior to the start of the experiment. Rats were subcutaneously injected with either 5 μg/kg glucagon, 10 μg/kg PYY, or 20 μg/kg GLP-1. These doses were chosen based on literature reports using subcutaneous or intraperitoneal injections of these hormones (not long-lasting analogs or mimetics with a different pharmacokinetic profile) in rats that report physiological and behavioral effects[40-44]. 10 min post-glucagon or PYY injection, and 1 min post-GLP-1 injection, rats were injected with 0.18 mg/kg nicotine or 0.9% saline (vehicle). These timepoints were chosen in order to allow for presence of hormone in the system at the time of nicotine injection. As in the above experiment, 200 μL blood was collected via tail vein puncture in EDTA-coated at baseline (30 min prior to injection; 30-60 min prior to the start of the dark cycle), and 10 min post-nicotine or saline injection (nondependent). Rats were fasted for 2 h prior to baseline blood collection and were not allowed access to food until all blood collections were complete. Following blood collection, rats were placed into operant chambers and food and water intake was recorded in 21 h sessions. Rats were given a 24 h washout period in between hormone injections. Rats were then surgically implanted subcutaneously with osmotic minipumps containing 1 mg/kg/day nicotine. Rats were allowed to establish dependence to nicotine for 2 weeks, and injections were repeated with minipump on board to maintain nicotine dependence. 200 μL blood was again collected at baseline and post-injection time points. Following blood collection, rats were placed into operant chambers, and food and water intake was recorded in 21 h sessions. All blood samples were kept on ice after collection, centrifuged at 3000 rpm, and plasma was extracted and stored at −80°C until use. Food intake data were collected in 10 s bins throughout the 21 h operant session. Data were smoothed by taking the running average of the data 30 s before and after each data point, and the values between 6 and 9 minutes were chosen to focus on as this is the window in which we expect to see the increased feeding behavior following nicotine injection. These values were plotted as post-stimulus time histograms (PSTH).

Analysis of circulating hormone levels

Plasma samples were analyzed using a custom Mesoscale Discovery (MSD) U-PLEX plate assay (Mesoscale Discovery, Rockville, MD, USA). A general strength of the MSD analysis platform is the ability to quantify multiple analytes within the same sample, thus reducing variability between replicates. Analytes of interest were leptin, active ghrelin, insulin, glucagon, active GLP-1, and PYY. Prior to 200 μL blood collection, 2 μL 1X dipeptidyl peptidase IV (DPP-IV) inhibitor (MilliporeSigma, Burlington, MA, USA) and 6 μL 1X protease inhibitor cocktail containing AEBSF and aprotinin (P2714, Sigma Aldrich, St. Louis, MO, USA) were added to blood collection tubes to prevent degradation of active ghrelin and active GLP-1. Plates were first prepared with antibody linkers for analytes of interest such that all wells were calibrated to bind all analytes of interest. 25 μL of each thawed plasma sample (diluted 1:2 in MSD Metabolic Assay Working Solution) were added in duplicate wells to the plate, and incubated for 2 h while shaking. Plates were then washed 3x with wash buffer (1X PBS + 0.05% Tween-20), and detection antibody solution was added to the wells and incubated for 1 h while shaking. Plates were washed 3x with wash buffer and read buffer was added to wells immediately following last wash. Plates

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were read using MESO QuickPlex SQ 120MM plate reader (Mesoscale Discovery). Analyte concentrations in samples were calculated using MSD Discovery Workbench software.

Statistical analysis

All of the data was analyzed using Prism 9 software (GraphPad, San Diego, CA, USA). Outliers were removed prior to analysis using Grubb's outlier test. All data analyses combine males and females as we did not see a significant effect of sex (data not shown). Data were tested for normality using the D'Agostino & Pearson test, and as data passed the test of normality, parametric analyses were subsequently used. For repeated measures analysis, sphericity was assumed as data sets between groups had equal variances. Food and water intake following nicotine injections were analyzed using 1-way repeated measures analysis of variance (RM ANOVA) with Tukey's multiple comparisons *post hoc*. Baseline hormone levels in nondependent vs. dependent rats, hormone levels following subcutaneous injection of nicotine or saline, and hormone levels following subcutaneous injection of hormone were analyzed using paired *t*-tests. Correlational analyses of hormone levels vs. body weight, hormone levels vs. baseline food intake, and food intake post-injection vs. hormone level post-injection were performed using Pearson's correlational analysis. Analysis of circulating hormone time course following injection was performed using 1-way RM ANOVA with Tukey's multiple comparisons *post hoc*. PSTH of food intake following injection of hormone and nicotine or saline was analyzed using 2-way RM ANOVA with Sidak's multiple comparisons *post hoc*. Effect sizes for all significant data are reported by η^2 (ANOVA) or Hedges' g (t-tests) as guided in the detailed report by Lakens[45]. All data are expressed as mean ± SEM.

Results

Validation of acute feeding behavior using a passive model of nicotine administration

In nondependent rats, nicotine injection at either 0.09 mg/kg or 0.18 mg/kg did not elicit a significant increase in food intake compared to saline injection (Fig. 2A-B). However, following establishment of nicotine dependence with osmotic minipump, nicotine injection produced a significant increase in food intake (time x treatment interaction $p < 0.0001$, $F_{(238,6783)} = 1.388$, $\eta^2 = 0.0407$; main effect of time $p < 0.0001$, $F_{(238,6783)} = 3.074$, $\eta^2 = 0.0449$; Fig. 2C). Tukey's multiple comparisons *post hoc* revealed that 0.18 mg/kg nicotine significantly increased food intake between 5:40 and 7:40 min and 0.09 mg/kg nicotine significantly increased food intake between 6:20 and 7:50 min and again between 8:40 and 9:00 min. Analysis of nicotine dose using 1-way RM ANOVA with Tukey's *post hoc* showed that both doses of nicotine significantly increased food intake ($p = 0.0004$, $\eta^2 = 0.3708$) with no significant difference between doses (Fig. 2D). These results confirm that the acute increase in feeding behavior in nicotine-dependent rats is reproducible in a passive model of nicotine intake.

We similarly assessed the ability of the passive model to elicit increased drinking behavior following nicotine intake. In nondependent rats, nicotine injection at either 0.09 mg/kg or 0.18 mg/kg did not elicit a significant increase in water intake compared to saline injection (Fig. 3A-B). However, following establishment of nicotine dependence with

osmotic minipump, nicotine injection produced a significant increase in water intake (time x treatment interaction $p = 0.0298$, $F_{(238,6783)} = 1.184$, $\eta^2 = 0.0366$; main effect of time $p < 0.0001$, $F_{(238,6783)} = 6.099$, $\eta^2 = 0.0892$; Fig. 3C). Tukey's multiple comparisons *post hoc* revealed that 0.09 mg/kg nicotine significantly increased water intake between 5:40 and 6:00 min compared to both 0.18 mg/kg nicotine and saline and significantly decreased water intake between 1:50 and 2:00 min compared to the other groups. Analysis of nicotine dose using 1-way RM ANOVA revealed a significant difference between groups, but Tukey's *post hoc* analysis did not further elucidate a difference between groups, although there was a trend towards a difference between nicotine doses in water intake ($p = 0.0545$, 0.18 mg/kg *vs.* 0.09 mg/kg; Fig. 3D). These results also confirm that the acute increase in drinking behavior in nicotine-dependent rats is reproducible in a passive model of nicotine intake.

Nicotine dependence increases circulating insulin, leptin, and ghrelin levels and produces insulin tolerance

We first sought to understand the effects of chronic nicotine administration on changes in circulating hormone levels in rats. We hypothesized that chronic nicotine administration would increase circulating levels of leptin, insulin, glucagon, GLP-1, and PYY, coinciding with long-term nicotine use and decreasing overall feeding behavior and body weight gain. To test this hypothesis, we employed a within-subjects study where we sampled plasma from male and female rats before (nondependent) and with implantation of osmotic minipumps containing 1 mg/kg/day nicotine (dependent). Baseline samples were collected at nondependent and dependent timepoints prior to acute injections of nicotine or saline. We collected baseline samples collection in both experimental cohorts studying hormonal changes, therefore we combined the data from both cohorts for increased statistical power.

At baseline in dependent rats *vs.* nondependent rats, we observed a significant increase in insulin levels ($p = 0.0005$, $t = 4.121$, $g = 0.9641$, paired t-test; Fig. 4A). Leptin levels were also significantly increased at baseline ($p = 0.0109$, $t = 2.792$, $g = 0.6465$, paired t-test; Fig. 4B). Interestingly, we also observed a significant increase in circulating active ghrelin levels ($p = 0.0024$, $t = 3.455$, $g = 0.7792$, paired t-test; Fig. 4C). There was no significant difference in baseline circulating levels of active GLP-1, glucagon, or PYY between nondependent and dependent rats (Fig. 4D-F). Contrary to our hypothesis, these results show that chronic nicotine administration increases circulating levels of both satiety and hunger-related hormones.

As insulin and leptin levels are strongly tied to body weight and food intake, we sought to identify whether the increased levels of these hormones correlated with these measures. In dependent rats, circulating insulin levels at baseline strongly positively correlated with body weight ($R^2 = 0.6645$, $p = 0.0022$; Fig. 5A). However, at baseline there was only a weak inverse correlation between insulin levels and 24 h food intake (Fig. 5B). We observed a significant positive correlation between circulating leptin levels and body weight at baseline ($R^2 = 0.6123$, $p = 0.0044$; Fig. 5C). We also observed a significant inverse correlation between circulating leptin levels and 24 h food intake at baseline ($R^2 = 0.4814$, $p = 0.0179$; Fig. 5D). Thus, the data confirm that insulin and leptin levels are positively correlated with body weight and leptin is negatively correlated with food intake.

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Nicotine injection decreases active GLP-1 and glucagon levels in dependent rats

Next, we wanted to identify the effects of acute nicotine injection on circulating hormone levels in dependent rats to potentially elucidate the contributions of these hormones on increased feeding behavior. Since we hypothesized that acute nicotine would decrease pro-satiety hormones and increase pro-feeding hormones, we sought to determine whether these hormone levels could predict food intake at 30 min post-injection of nicotine. We expected to see an inverse correlation between the plasma levels of pro-satiety hormones and food intake, which would indicate a reduction in secretion of these hormones to subsequently increase feeding. There was no significant correlation between insulin, leptin, or ghrelin levels and food intake following injection of either saline or nicotine (Fig. 6A-C). Contrary to our hypothesis, we found that hormones that promote satiety and decrease feeding were positively correlated with food intake. Indeed, we observed a significant positive correlation between food intake following nicotine injection and active GLP-1 levels ($R^2 = 0.4868$, $p = 0.0249$; Fig. 6D), glucagon levels ($R^2 = 0.5106$, $p = 0.0202$; Fig. 6E), and PYY levels ($R^2 = 0.4125$, $p = 0.0453$; Fig. 6F) in dependent rats. These correlations were not present following saline injection. However, this positive correlation for the hormone levels 10 minutes post-nicotine injection does not make sense with the increased feeding behavior 30 min post-injection. This data suggests that nicotine is altering signaling of these hormones. Therefore, we wanted to further measure the circulating hormone levels at different timepoints to see if there were any changes that could explain the behavior.

To further understand the effects of nicotine injection on these three hormones, we sampled plasma at additional time points following injection in dependent rats to establish a time course of hormone level changes. Following saline injection, there was no significant difference in active GLP-1 levels across the time course (Fig. 7A). However, 1-way RM ANOVA revealed that nicotine injection significantly decreased active GLP-1 levels ($F_{(1.788,16.09)} = 9.219$, $p = 0.0027$, $\eta^2 = 0.5061$; Fig. 7B). Post hoc analysis using Tukey's multiple comparisons test identified a significant decrease in active GLP-1 levels at 10 min and 30 min post-injection ($p < 0.05$), but not 60 min post-injection, compared to baseline levels. Similarly, there was no significant effect of saline injection on glucagon levels across all sampled timepoints (Fig. 7C), but glucagon levels significantly decreased following acute injection of nicotine ($F_{(1.350,12.15)} = 8.597$, $p = 0.0084$, $\eta^2 = 0.4885$; Fig. 7D). Tukey's multiple comparisons post hoc revealed that glucagon levels were significantly decreased only at 10 min post-injection compared to baseline levels ($p < 0.05$). Conversely, saline injection produced a significant decrease in PYY levels ($F_{(2.403,21.62)} = 7.613$, $p = 0.0021$, $\eta^2 = 0.2747$; Fig. 7E); *post hoc* analysis revealed a significant decrease at all time points compared to baseline. Nicotine injection produced a similar decrease in PYY levels ($F_{(1.914,17.23)} = 12.20$, $p = 0.0006$, $\eta^2 = 0.5755$; Fig. 7F). Tukey's multiple comparisons post hoc showed that nicotine significantly decreased PYY levels at 10 min and 60 min post-injection compared to baseline levels. Because this effect was seen following both saline and nicotine injections, the decrease in PYY levels is not specific to acute nicotine injection. Thus, our results show that glucagon and active GLP-1 exhibit a decrease in circulating levels following acute nicotine injection.

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Glucagon administration prevents the acute increase in feeding behavior following nicotine injection

As acute nicotine injection decreased circulating glucagon and active GLP-1 levels, we hypothesized that this decrease in pro-satiety hormone levels might contribute to the increase in feeding behavior following acute nicotine injection in dependent rats. To test this hypothesis, we administered subcutaneous injections of these hormones prior to nicotine or saline injection and measured the subsequent short-term food intake. We validated the efficacy of the hormone injection and confirmed that following the injection of GLP-1, glucagon, or PYY, there was a significant increase in the plasma levels of these hormones (GLP-1 $p = 0.0035$, $t = 3.913$, $g = 2.217$; glucagon $p = 0.0142$, $t = 3.030$, $g = 1.108$; PYY $p = 0.0003$, $t = 5.684$, $g = 2.927$) (Fig. 8A, C, E).

To assess the effect of hormone injection on feeding behavior, a within-subjects study was performed, so that all rats were given injections of each hormone prior to nicotine or saline injection (N = 6/group). After nicotine or saline injection, rats were placed in operant chambers and food intake was recorded in 10 s bins for 20 minutes. Average food intake was analyzed using PSTH. Control data of food intake following nicotine or saline injection without additional hormone injection was also used for comparison, and we observed that acute nicotine injection did increase food intake (treatment $p = 0.05$). 2-way RM ANOVA of food intake following active GLP-1 injection showed no significant effect of hormone treatment on food intake following nicotine injection (Fig. 8B). Analysis of food intake following glucagon injection revealed a significant effect of glucagon administration on decreasing food intake following nicotine injection ($F_{(3,44)} = 5.513$, $p = 0.0027$, $\eta^2 = 0.1249$; Fig. 8D). 2-way RM ANOVA of PYY administration on food intake showed a trend towards the significant effect of hormone treatment ($F_{(3,44)} = 2.719$, $p = 0.0559$; Fig. 8F), but there was no clear effect of hormone administration on decreasing food intake following nicotine injection. These results show that glucagon administration prevented food intake following nicotine injection, suggesting that the decrease in glucagon levels following nicotine injection contributes to the increased feeding behavior phenomenon.

Discussion

This study aimed to examine the effects of acute and chronic nicotine exposure on circulating feeding hormones and identify their contribution to the increased feeding behavior following nicotine exposure. We developed a model of passive nicotine exposure in which we validated an acute increase in food intake following nicotine injection in dependent rats. We confirmed that long-term nicotine intake produces increased levels of circulating leptin, insulin, and ghrelin. Acute nicotine injection in dependent rats altered the relationship between GLP-1, glucagon, and PYY levels and short-term food intake, and decreased GLP-1 and glucagon levels. Administration of glucagon prevented the acute increase in feeding behavior following nicotine injection in dependent rats. These results show that glucagon may be decreased with acute nicotine injection and contribute to the acute increase in food intake produced by nicotine.

Establishment of nicotine dependence via 1 mg/kg/day nicotine for 2 weeks produced approximately a 30% increase in circulating leptin levels and a 50% increase in circulating

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insulin levels. Nicotine dependence also produced a 50% increase in circulating active ghrelin levels. We observed this increase across a large sample size, providing confidence in these results. Insulin and leptin are also adiposity signals, and we confirmed that circulating insulin and leptin levels positively correlated with body weight and negatively correlated with food intake, as expected. The increases in leptin, insulin, and ghrelin are consistent with previous literature and suggests that chronic nicotine use mediates peripheral regulation of feeding and energy metabolism[9,16,46,47]. Few studies have looked at the acute effects of nicotine on hormone levels in short timescales (minutes); notably, plasma ghrelin has been seen to increase after smoking[14]. While the hormones we studied have been shown to produce effects within minutes of secretion, most of these hormones peak in signaling over a longer period of time (minutes-hours)[48-50].

At baseline, we did not observe an effect of nicotine dependence on circulating levels of GLP-1, glucagon, or PYY. These hormones are secreted as a postprandial response, and since our rats were fasted prior to sample collection, we would expect no change in the circulating levels of these hormones. We did observe a significant decrease in glucagon and GLP-1 levels following acute nicotine injection in dependent rats. Active GLP-1 exhibited a persistent decrease in circulating levels up to 30 min post-nicotine injection. Active GLP-1 and glucagon have short lifespans (seconds-minutes), especially as active GLP-1 quickly gets degraded by DPP-IV[51,52]. This may explain inconsistency in our results, as it is harder to accurately capture the circulating hormone levels. PYY levels were decreased following both saline and nicotine injections; PYY is acutely affected by stress[53], which may explain our results despite habituation of rats to sham injections before the experiment.

We also saw that GLP-1, glucagon, and PYY levels and food intake were positively correlated following nicotine injection. We expected circulating levels of satiety-inducing hormones to inversely correlate with subsequent food intake, as these hormone levels increase postprandially to decrease feeding. The rats were fasted prior to the injections and blood collection to remove any confounding variable effects of individual food intake and related hormone secretion; however, this effect appears to be specific to the nicotine injection as we did not see the same correlation following saline injection, thus ruling out a general effect of feeding behavior. It is apparent that nicotine injection is altering signaling of these hormones; the decreased levels of hormone at 10 min post-injection may suggest a delayed release by nicotine, leading to an acute increase in feeding but an overall decrease in feeding.

Finally, we found that subcutaneous administration of glucagon prior to injection of nicotine in dependent rats prevented the acute increase in feeding behavior. We injected glucagon to allow for peak levels in the circulation at the time of nicotine injection[41], so that if nicotine was decreasing glucagon levels the effect would be masked. While we did inject GLP-1 immediately before injecting nicotine to avoid excessive degradation by DPP-IV, it is possible the lack of observed effect could be due to insufficient GLP-1 within the circulation. Studies examining the effects of GLP-1 administration on long-term changes in food intake and body weight use GLP-1 mimetics or GLP-1 receptor agonists that are not degraded by DPP-IV, such as liraglutide or exenatide, respectively[54]. However, GLP-1, glucagon, and PYY have all been studied as potential therapeutics for obesity

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treatments[55-59], and our results validate the potential of these compounds to restore the hormonal dysregulation of feeding due to nicotine.

We focused on nicotine's effects on the homeostatic peripheral signaling of leptin, ghrelin, insulin, glucagon, GLP-1, and PYY. However, an important consideration is that many of these hormones do have the ability to directly or indirectly affect central nervous system signaling. Insulin, leptin, ghrelin, and PYY can directly bind to neurons in orexigenic and anorexigenic regions. The NTS contains GLP-1 expressing neurons that project to multiple regions. Consequently, these regions can signal back to the periphery or produce additional signals that contribute to the circulating levels of hormones in the plasma. Given nicotine's rapid circulation and time of action within the brain, it is possible that hormone signaling may be altered centrally before it becomes altered peripherally. This may potentially explain why we did not see changes in peripheral hormone levels of leptin, insulin, ghrelin, and PYY following acute nicotine injection.

Conclusions

Overall, the results from this study have shown that nicotine alters peripheral signaling of feeding-related hormones, and these hormones contribute to the acute increase in food intake following acute nicotine in dependent rats. Further study is needed to characterize the effects of nicotine on these hormones more thoroughly and how peripheral *vs.* central signaling may contribute to feeding behaviors. Understanding the broad effects of nicotine on homeostatic regulation of feeding can provide greater insight into nicotine's mechanisms of action and potential avenues for therapeutic treatment.

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Experiment 1

Experiment 2

Experiment 3

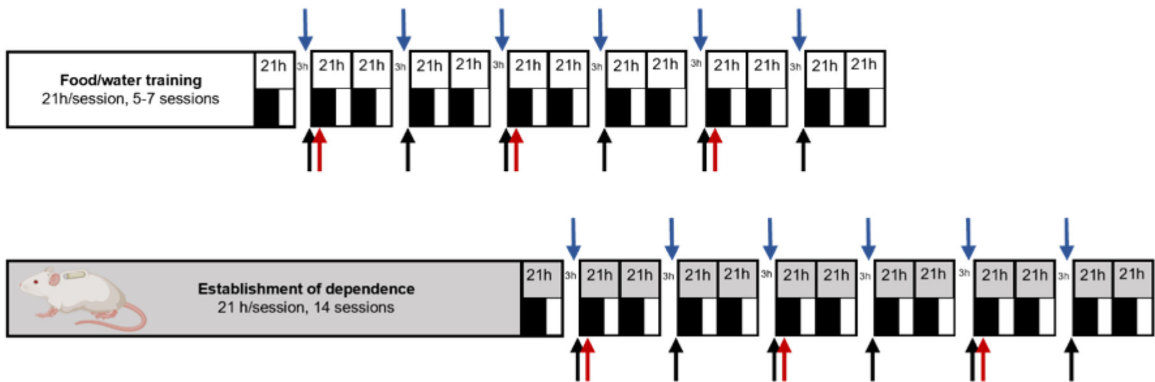

Figure 1.

Experimental timelines. Operant sessions (21 h) are shown with black boxes to represent dark cycle (12 h) and light boxes to represent light cycle (9 h). 3 h window in between operant sessions were used as needed to collect baseline blood (30-60 min before start of dark cycle) or inject hormone (1-10 min before saline or nicotine injection at start of dark cycle). Minipumps were implanted prior to session 1 start, and animals were left to establish dependence for 14 days before injections and blood collections. Experiment 1: Injections of saline or nicotine (black arrows). Injections were administered prior to minipump implantation and with minipump on board. Experiment 2: Blood collection (red arrows) at baseline and post-injection of nicotine or saline (black arrows). Injections were administered prior to minipump implantation and with minipump on board. Experiment 3: Hormone administration (blue arrows) prior to injection of nicotine or saline (black arrows) and either blood collection (red arrows) or measurement of food intake. Injections were administered prior to minipump implantation and with minipump on board.

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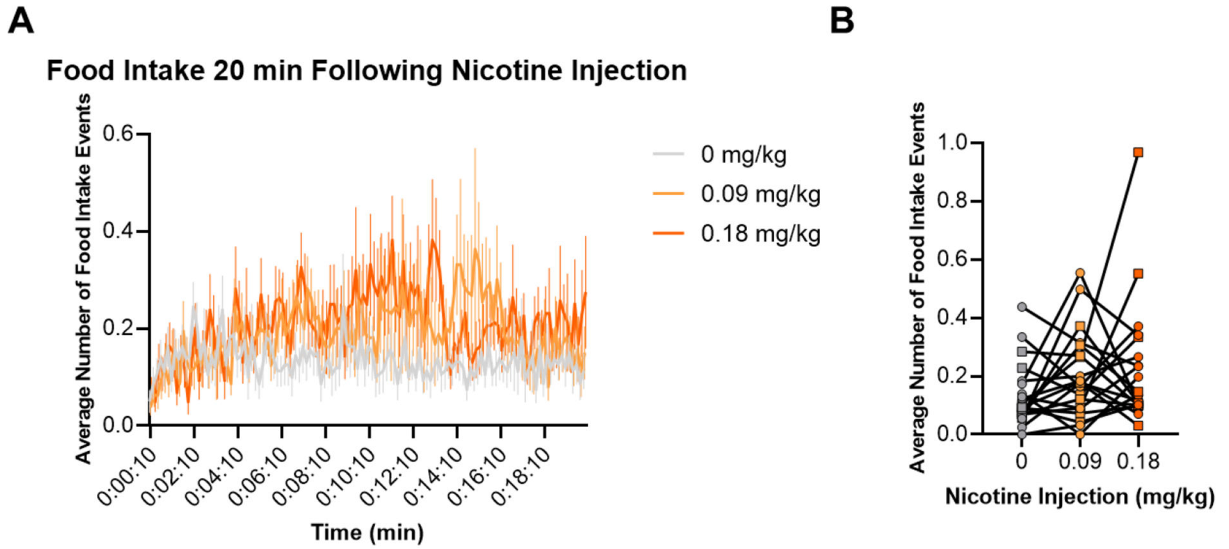

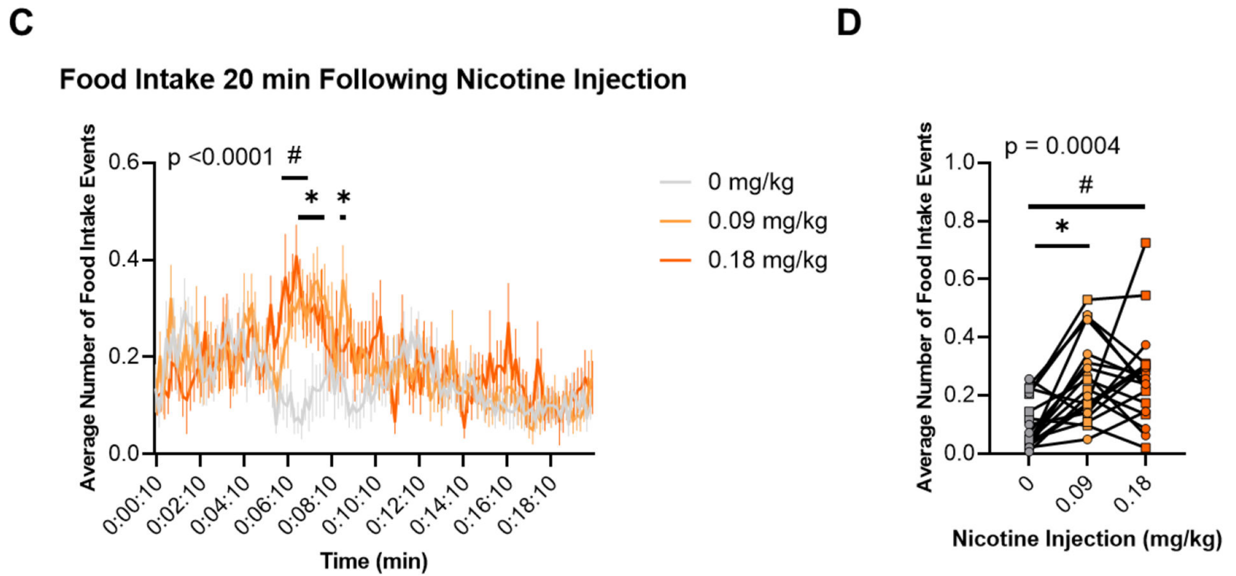

Figure 2.

Analysis of feeding behavior in nondependent (top) and dependent (bottom) rats using a passive model of chronic nicotine exposure. A) PSTH of average number of food intake events 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light orange), or 0.18 mg/kg nicotine (dark orange) in nondependent rats. B) Average number of food intake events per rat in 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light orange), or 0.18 mg/kg nicotine (dark orange). Squares represent females, circles represent males. C) PSTH of average number of food intake events 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light orange), or 0.18 mg/kg nicotine (dark orange) in dependent rats. $p < 0.0001$, 2-way RM ANOVA. D) Average number of food intake events per rat in 20 min following

injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light orange), or 0.18 mg/kg nicotine (dark orange). Squares represent females, circles represent males. $p = 0.0004$, 1-way RM ANOVA. *$p < 0.05$ for 0.09 mg/kg vs. 0 mg/kg, #$p < 0.05$ for 0.18 mg/kg vs. 0 mg/kg, Tukey's multiple comparisons post hoc. N = 20 per group.

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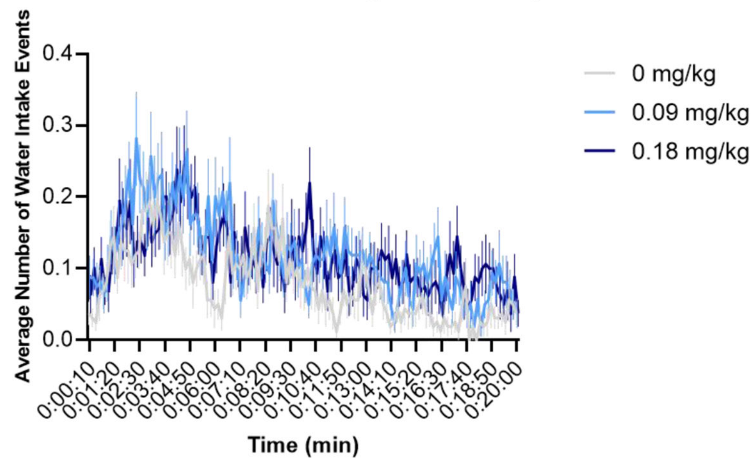
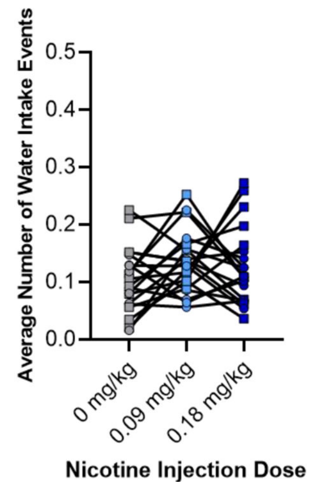
Nondependent

A

B

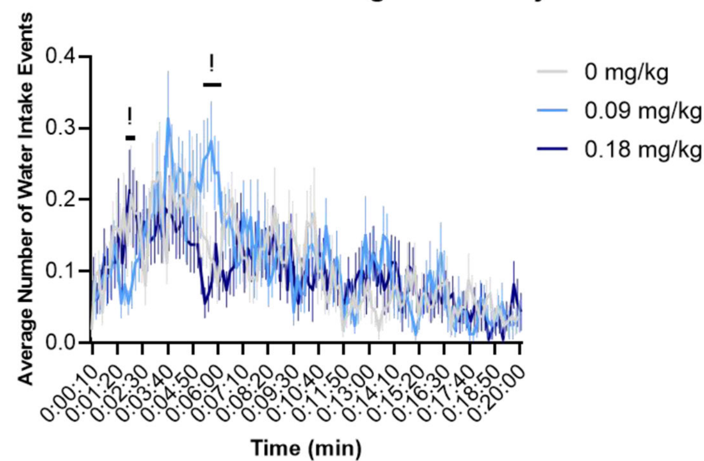




Dependent

C

D



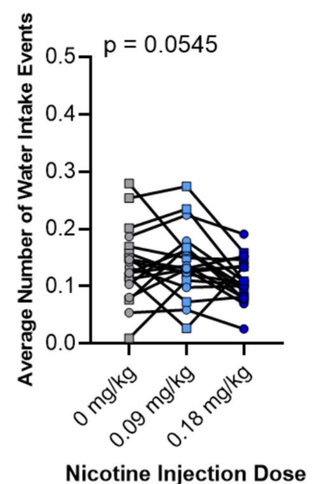


Figure 3.

Analysis of drinking behavior in nondependent (top) and dependent (bottom) rats using passive model of nicotine intake. A) PSTH of average number of water intake events 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light blue), or 0.18 mg/kg nicotine (dark blue) in nondependent rats. B) Average number of food intake events per rat in 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light blue), or 0.18 mg/kg nicotine (dark blue). Squares represent females, circles represent males. C) PSTH of average number of food intake events 20 min following injection of 0 mg/kg nicotine (saline, gray), 0.09 mg/kg nicotine (light blue), or 0.18 mg/kg nicotine (dark blue) in dependent rats. $p = 0.0298$, 2-way RM ANOVA. D) Average number of food intake events per rat in 20 min following injection of 0 mg/kg nicotine (saline, gray),

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0.09 mg/kg nicotine (light blue), or 0.18 mg/kg nicotine (dark blue). Squares represent females, circles represent males. $p = 0.0545$, 1-way RM ANOVA. !$p < 0.05$ for 0.09 mg/kg vs. 0.18 mg/kg, Tukey's multiple comparisons post hoc. N = 20 per group.

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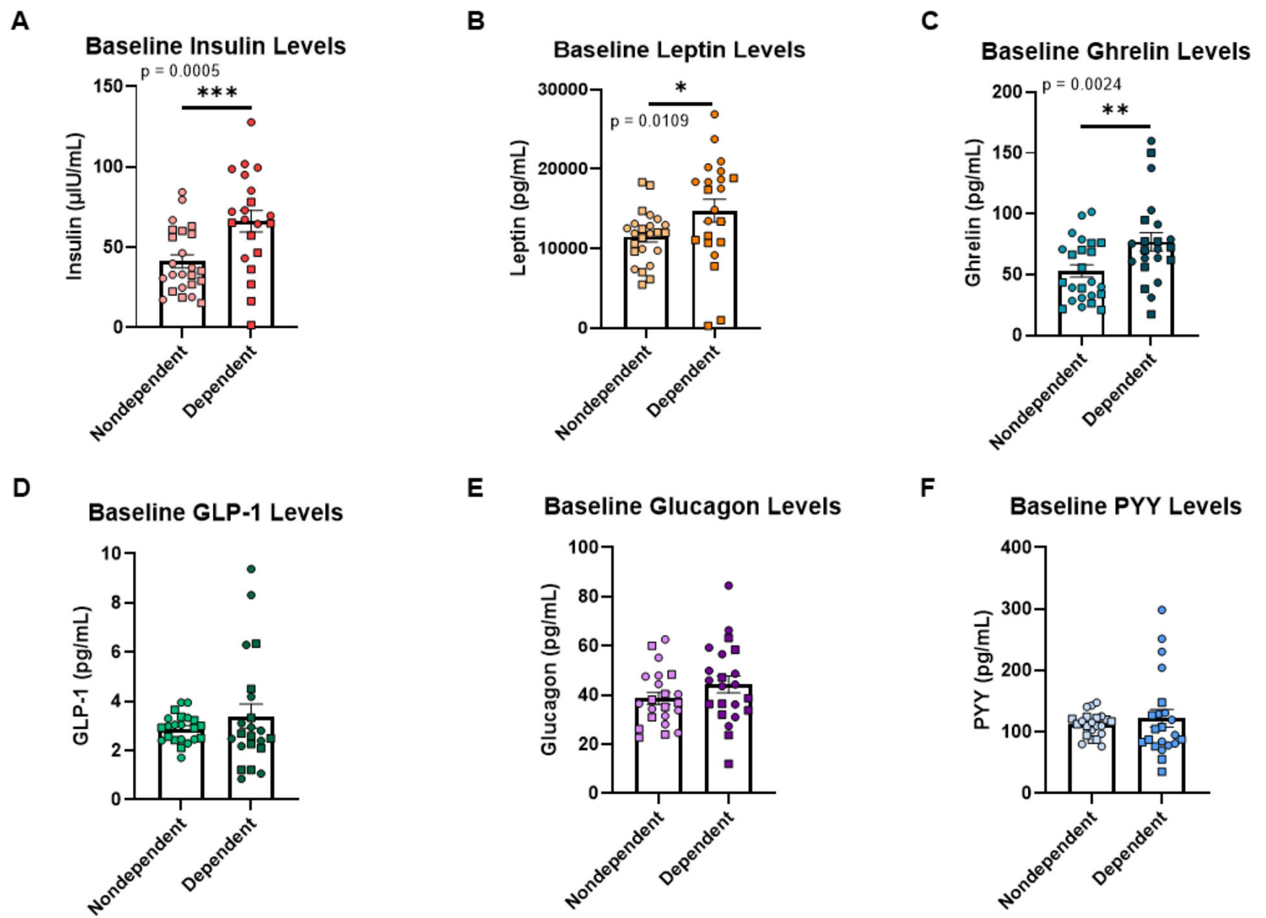

Figure 4.

Baseline plasma levels of hormones in nondependent *vs.* dependent rats. Squares represent females, circles represent males. A) Insulin levels in nondependent (left) and dependent (right) rats. p = 0.0005, paired t-test. B) Leptin levels in nondependent (left) and dependent (right) rats. p = 0.0109, paired t-test. C) Ghrelin levels in nondependent (left) and dependent (right) rats. p = 0.0024, paired t-test. D) GLP-1 levels in nondependent (left) and dependent (right) rats. E) Glucagon levels in nondependent (left) and dependent (right) rats. F) PYY levels in nondependent (left) and dependent (right) rats. N = 24 per group.

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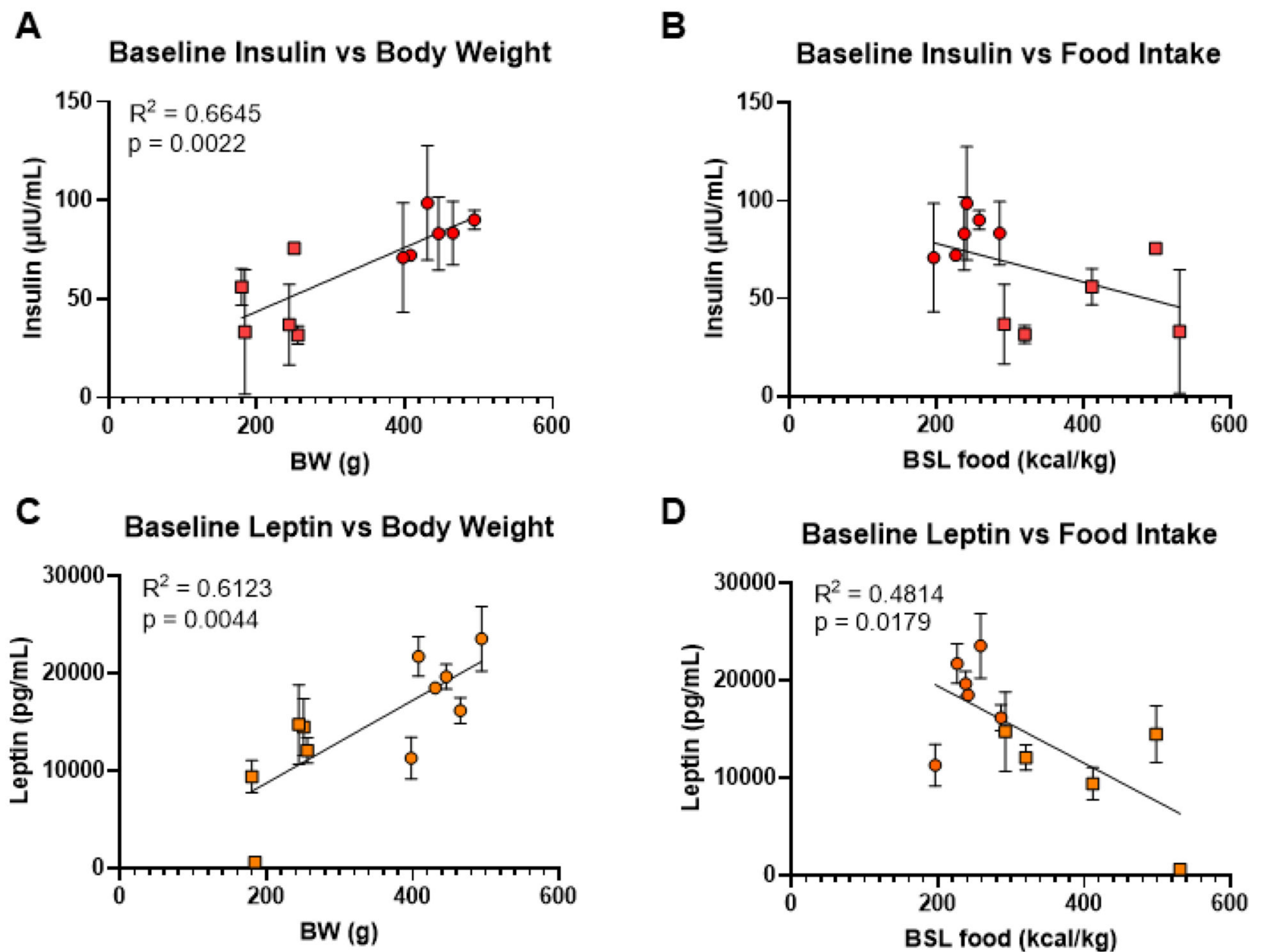

Figure 5.

Correlation of baseline insulin and leptin levels with baseline food intake and body weight in dependent rats. Squares represent females, circles represent males. A) Plasma insulin levels vs. body weight. $p = 0.0022$, $R^2 = 0.6645$, Pearson's correlational analysis. B) Plasma insulin levels vs. baseline 24 h food intake. $R^2 = 0.2206$, Pearson's correlational analysis. C) Plasma leptin levels vs. body weight. $p = 0.0044$, $R^2 = 0.6123$, Pearson's correlational analysis. D) Plasma leptin levels vs. baseline 24 h food intake. $p = 0.0179$, $R^2 = 0.4814$, Pearson's correlational analysis. N = 12 (6 M, 6 F).

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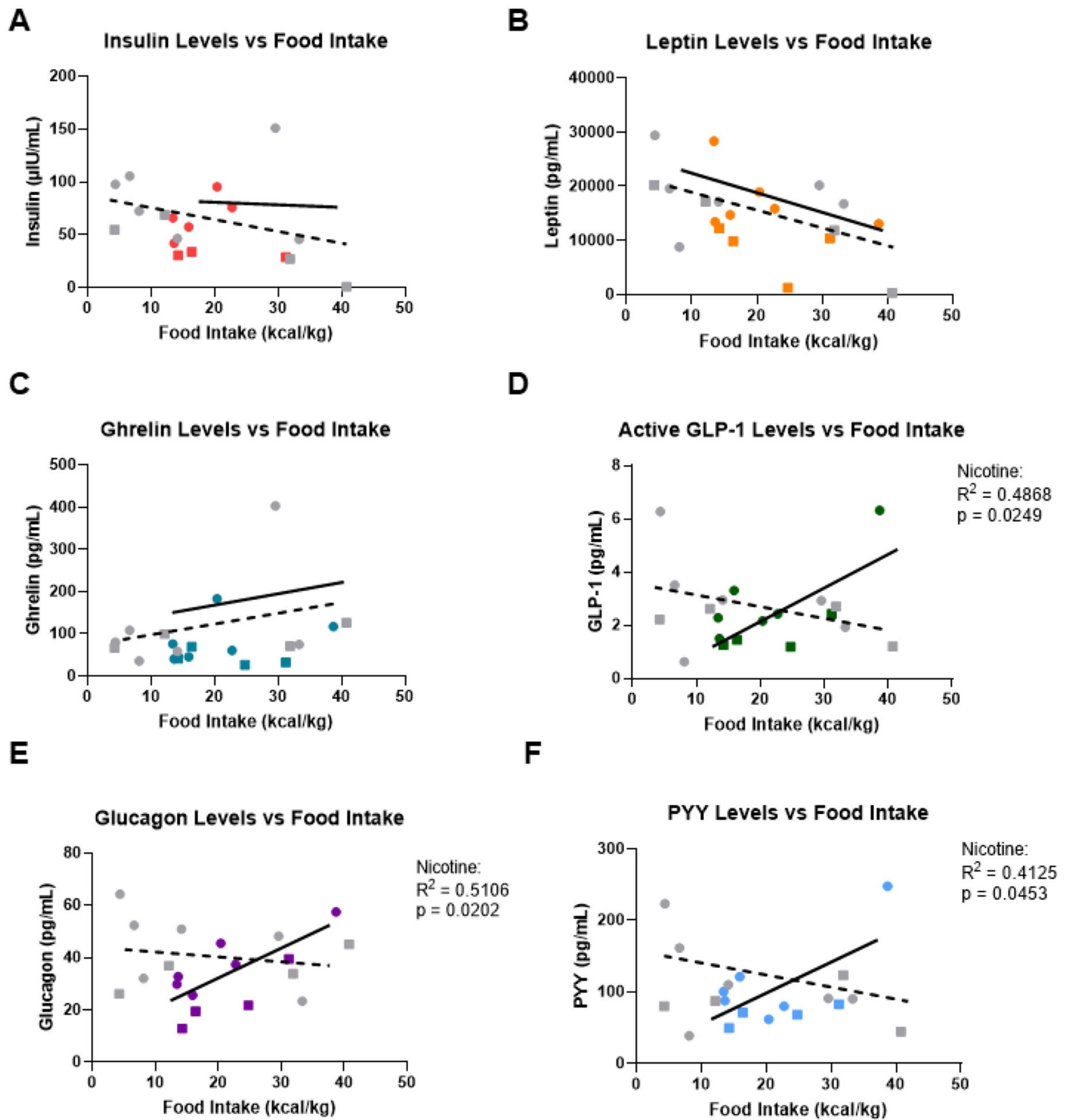

Figure 6.

Correlation of plasma levels of hormones 10 min following acute nicotine injection with short-term food intake 30 min following injection in dependent rats. Squares represent females, circles represent males. A) Insulin levels vs. food intake following saline (gray dots) or nicotine (pink dots) injection. B) Leptin levels vs. food intake following saline (gray dots) or nicotine (orange dots) injection. C) Ghrelin levels vs. food intake following saline (gray dots) or nicotine (blue dots) injection. D) GLP-1 levels vs. food intake following saline (gray dots) or nicotine (green dots) injection. Nicotine injection p = 0.0249, R^2 = 0.4868, Pearson's correlational analysis. E) Glucagon levels vs. food intake following saline (gray dots) or nicotine (purple dots) injection. Nicotine injection p = 0.0202, R^2 = 0.5106, Pearson's correlational analysis. F) PYY levels vs. food intake following saline (gray dots)

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or nicotine (blue dots) injection. Nicotine injection p = 0.0453, R^2 = 0.4125, Pearson's correlational analysis. Dashed line represents best-fit line of saline data points. Solid line represents best-fit line of nicotine data points. N = 10-12 per group.

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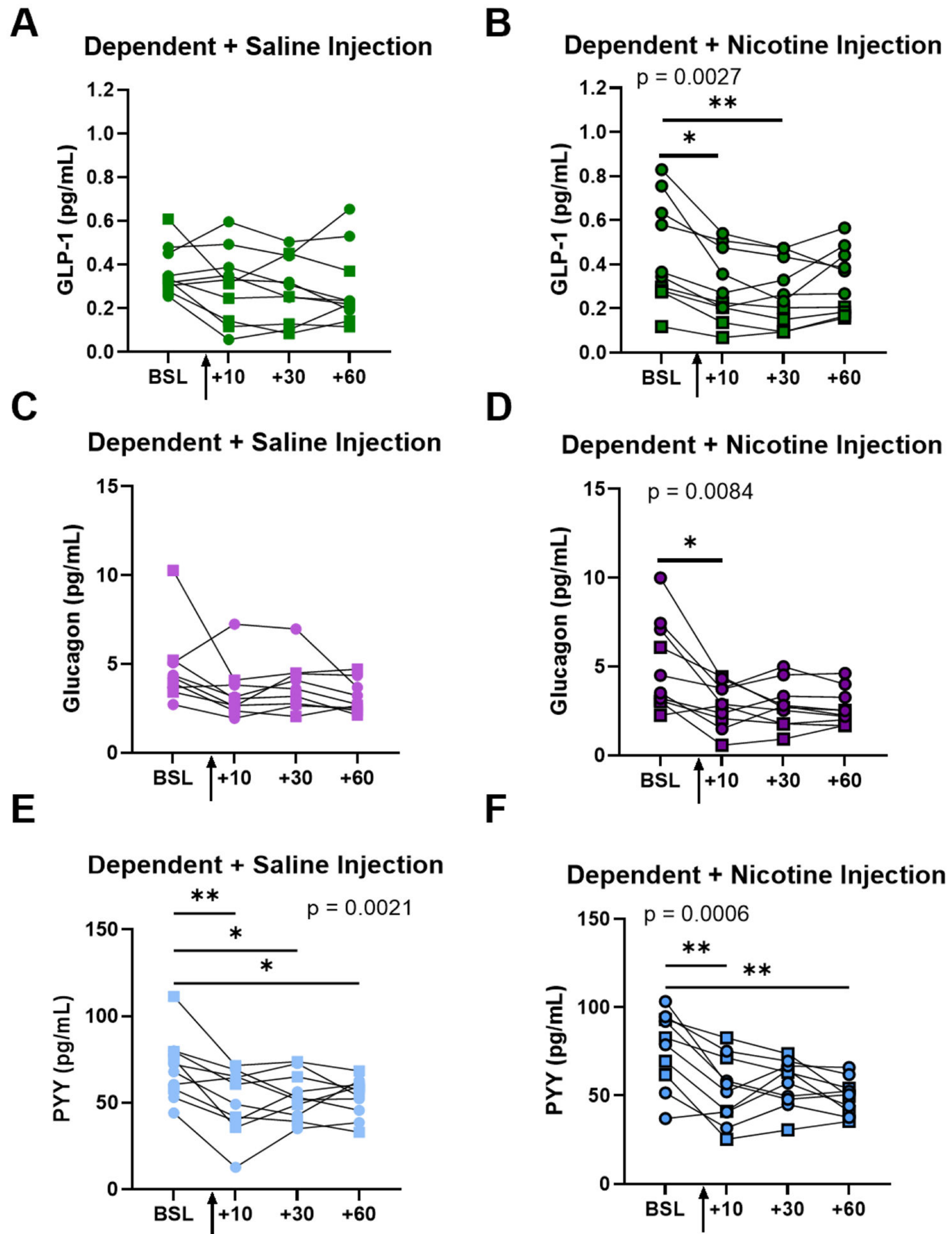

Figure 7.

Time course of plasma levels of hormones following acute nicotine injection in dependent rats. Graphs show paired samples for each rat. Acute injection of nicotine or saline is represented at time t = 0 by black arrow on x-axis. A) GLP-1 levels following saline injection. B) GLP-1 levels following nicotine injection. p = 0.0027, 1-way RM ANOVA. C) Glucagon levels following saline injection. D) Glucagon levels following nicotine injection. p = 0.0084, 1-way RM ANOVA. E) PYY levels following saline injection. p = 0.0021, 1-way RM ANOVA. F) PYY levels following nicotine injection. p = 0.0006, 1-way RM ANOVA. *p < 0.05, **p < 0.01, Tukey's multiple comparisons post hoc. N = 12 per group.

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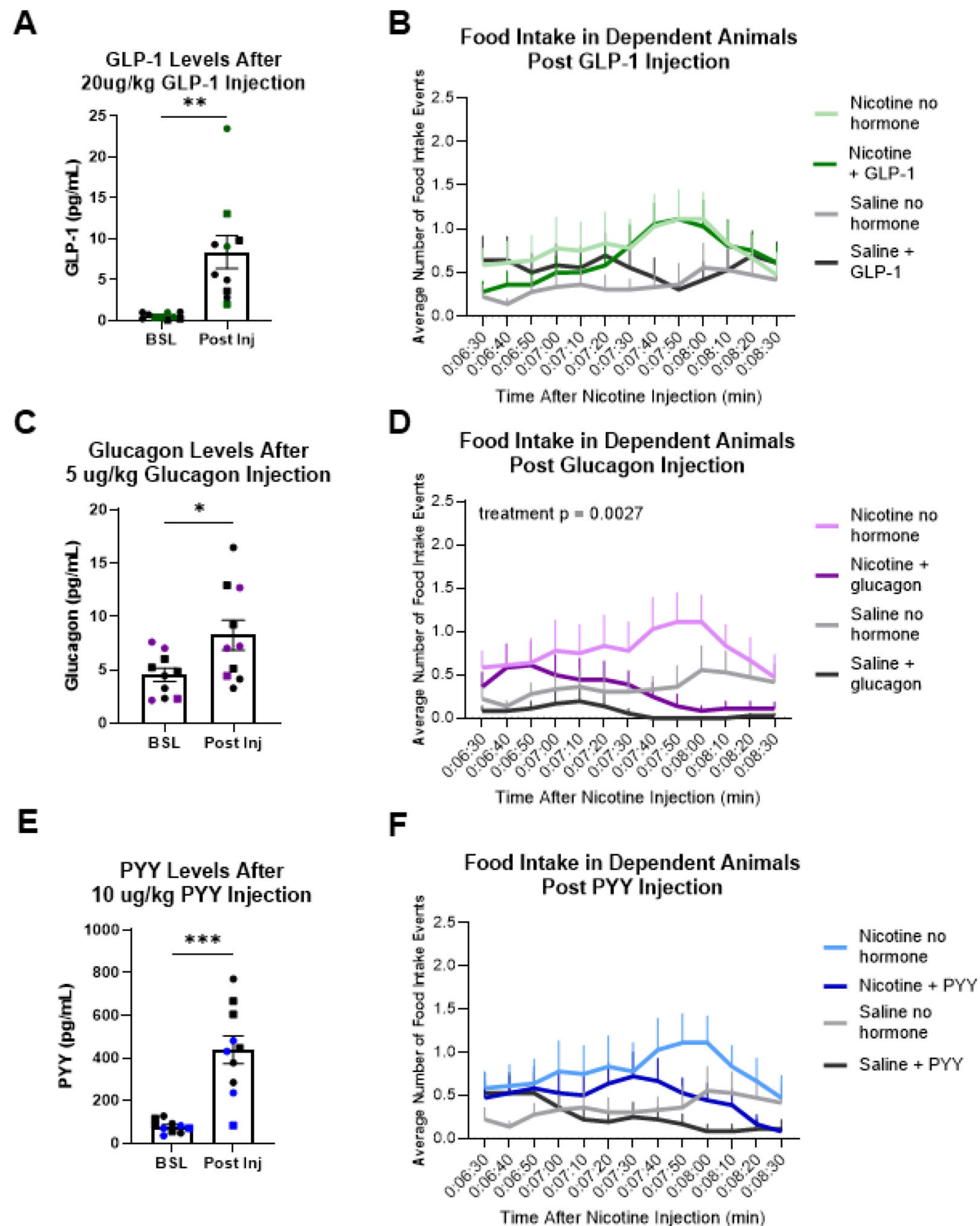

Figure 8.

Food intake following hormone treatment and acute nicotine injection in dependent rats. Squares represent females, circles represent males. A) GLP-1 levels following GLP-1 injection. $p = 0.0035$, paired t-test. Black squares represent rats subsequently receiving saline injection, colored squares represent rats subsequently receiving nicotine injection. B) Food intake events following injection of GLP-1 and either saline or nicotine. C) Glucagon levels following glucagon injection. $p = 0.0142$, paired t-test. Black squares represent rats subsequently receiving saline injection ($N = 6$), colored squares represent rats subsequently receiving nicotine injection ($N = 6$). D) Food intake events following injection of glucagon and either saline or nicotine. Main effect of treatment $p = 0.0027$, 2-way RM ANOVA.

E) PYY levels following PYY injection. $p = 0.0003$, paired t-test. Black squares represent rats subsequently receiving saline injection, colored squares represent rats subsequently receiving nicotine injection. F) Food intake events following injection of PYY and either saline or nicotine. $N = 6$ per group.

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Table 1.

Feeding-related hormones and their role in increasing or decreasing food intake.

Hormone	Role in Feeding	Source	Function	References
Ghrelin	Hunger	Stomach and hypothalamic neurons[20-22]	Activates AgRP neurons, stimulates feeding behavior	Serrenho et al., 2019[23]
Glucagon	Satiety	Pancreas	Stimulates hepatic glucose production, decreases food intake by reducing meal size	Jong et al.[24] Al-Massadi et al., 2019
Glucagon-like peptide 1 (GLP-1)	Satiety	Intestine and NTS neurons	Stimulates insulin production, inhibits glucagon secretion, decreases food intake	Kreymann et al., 1987 Wang et al., 1995 Gutniak et al., 1992 Skibicka, 2013
Insulin	Satiety	Pancreas	Reduces blood glucose levels, decrease food intake through stimulation of anorexigenic neurons	Woods et al., 2006[25] Air et al., 2002[26] Foster et al., 1991[27] Dodd and Tiganis, 2017[28]
Leptin	Satiety	Subcutaneous white adipose tissue[29]	Decreases feeding behavior through activation of anorexigenic neurons and inhibition of orexigenic neurons	Penicaud et al., 2012[30] Coll et al., 2007[31]
Peptide YY (PYY)	Satiety	Small and large intestine	Inhibits food intake through binding to Y2 receptors on vagal afferents and in the arcuate nucleus and NTS	Koda et al., 2005[32] Nonaka et al., 2003[33] Blevins et al., 2008[34]