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Access to nicotine in drinking water reduces weight gain without changing caloric intake on high fat diet in male C57BL/6J mice

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Abstract

Nicotine and tobacco use is associated with lower body weight, and many smokers report concerns about weight. In animal studies, nicotine reduces weight gain, reduces food consumption, and alters energy expenditure, but these effects vary with duration and route of nicotine administration. Previous studies have used standardized nicotine doses, however, in this study, male and female mice had free access to nicotine drinking water for 30 days while fed either a high fat diet (HFD) or chow, allowing animals to titrate their nicotine intake. In male mice, HFD increased body weight and caloric intake. Nicotine attenuated this effect and decreased weight gain per calorie consumed without affecting overall caloric intake or acute locomotion, suggesting metabolic changes. Nicotine did not decrease weight in chow-fed animals. In contrast, the same paradigm did not result in significant differences in weight gain in female animals, but did alter corticosterone levels and locomotion, indicating sex differences in the response to HFD and nicotine. We measured levels of mRNAs encoding nicotinic acetylcholine receptor subunits, uncoupling proteins (UCP) 1–3, and neuropeptides involved in energy balance in adipose tissues and the arcuate nucleus of the hypothalamus (ARC). HFD and nicotine regulated UCP levels in adipose tissues and ARC from female, but not male, mice. Regulation of agouti-related peptide, neuropeptide-Y, melanin-concentrating hormone, and cocaine- and amphetamine-regulated transcript in ARC varied with diet and nicotine in a sex-dependent manner. These data demonstrate that chronic consumption of nicotine moderates the effect of HFD in male mice by changing metabolism rather than food intake, and identify a differential effect on female mice.

Keywords

food intake; nicotine; high fat diet; uncoupling proteins; neuropeptides; sex differences

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1. Introduction

Smoking is the leading cause of preventable death worldwide, followed closely by obesity, yet up to 20% of the global population currently smokes cigarettes (Centers for Disease Control and Prevention [CDC], 2016). A subset of these smokers cite concerns about weight as a reason for beginning to smoke or declining to quit (Cavallo, et al., 2010; Copeland & Carney, 2003; Klesges et al., 1988; Klesges, Elliott, & Robinson, 1997; Robinson, Klesges, Zbikowski, & Glaser, 1997). The perception that smoking can control body weight is widespread, even among adolescents, who are vulnerable to initiating smoking (Klesges, et al., 1997). These beliefs are substantiated by research indicating that smokers, on average, weigh less than non-smokers (Albanes et al., 1987; Chiolero et al., 2008).

In preclinical models, nicotine, the most well-studied of the more than 4,000 chemicals in cigarette smoke, affects both food intake and body weight. Sustained, chronic nicotine exposure achieved with implanted osmotic mini-pumps reduces food intake, body weight, and weight of stored fat in male and female rats, although effects were more pronounced in female animals (Bowen, Eury, & Grunberg, 1986; Grunberg, Bowen, & Winders, 1986; Miyata, Meguid, Varma, Fetissoff, & Kim, 2001; Winders & Grunberg, 1990). Comparable changes in food consumption and body weight have also been seen with implanted nicotine pellets (Levin et al., 1987) and chronic, daily subcutaneous injections in rats (Grunberg, Winders, & Popp, 1987), as well as daily intraperitoneal injections in male C57BL/6J mice (Mineur, et al., 2011).

Smokers, like the American population in general, are exposed to high fat and high sugar foods, which interact with nicotine to affect food intake and weight gain. Although light to moderate smokers weigh less than non-smokers, heavy smokers weigh more than other groups, possibly due to a clustering of behaviors such as increased consumption of fatty foods in addition to smoking (Chiolero, et al., 2008; Filozof, Fernández Pinilla, & Fernández-Cruz, 2004). Obese individuals may also smoke more to achieve the same level of reward as leaner individuals, highlighting the importance of individual differences in nicotine consumption (Blendy, et al., 2005). In male mice and rats, nicotine prevents weight gain more dramatically when animals have access to high fat diet (HFD) than when only chow is available, indicating possible protective effects of nicotine under conditions that lead to obesity (Mangubat, et al., 2012; Wellman, et al., 2005). However, these animals were not able to self-regulate their nicotine dose.

The current study was designed to determine the combined effects of freely consumed nicotine and HFD on food intake, weight gain, and physiological markers of energy metabolism in male and female mice. We measured food intake and body weight for 30 days, using a chronic nicotine drinking paradigm and free access to HFD or standard lab chow. We also evaluated behaviors (locomotion, light-dark test, and marble burying) and blood corticosterone (CORT) levels to determine whether stress or anxiety contribute to any observed sex differences in HFD and nicotine response. To examine a link between nicotine, HFD and metabolism, we measured levels of mRNAs encoding uncoupling protein 1–3 (UCP) in both brown and white adipose tissues (BAT and WAT). Finally, we measured levels of mRNAs encoding UCP 1–3, nicotinic acetylcholine receptor subunits and feeding related

neuropeptides in the arcuate nucleus of the hypothalamus (ARC) to identify molecular changes in a brain region involved in regulation of food intake. These studies indicate that self-regulated nicotine consumption interacts with diet to alter weight gain without changing caloric intake in male mice and has a sex-dependent interaction with HFD leading to changes in UCP and neuropeptide expression.

2. Materials & Methods

2.1 Animals

8–9 week old male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to habituate to the colony room for 1 week prior to the start of experiments. Animals were single-housed and maintained on a 12-hour light/dark cycle (lights-on at 7am) with *ad libitum* access to food and water for the duration of experiments. Body weight and food consumption were measured daily. Water consumption was measured daily from the onset of nicotine administration. All experimental procedures were approved by the Yale University Institutional Animal Care and Use Committee.

2.2 Nicotine and Diet regimens

Eight days prior to the start of nicotine treatment, animals were randomly assigned to receive either regular chow (CHOW; Harlen Laboratories, Diet 2018, 18% kCal from fat) or HFD (Research Diets, Diet D12451, 45% kcal from fat). After the habituation phase, all animals received new drinking water. Half of the animals in each diet group received a nicotine solution (NIC; 200 µg/mL free base concentration in 2% saccharin vehicle) as has been reported previously (Jung, et al., 2016; King, Caldarone, & Picciotto, 2004). Control animals received a matched molar solution of L-(+)-tartaric acid (2.5 mM; SigmaAldrich, St. Louis, MO) in 2% saccharin (SAC). NIC and SAC solutions were adjusted to pH 7. (–)-Nicotine hydrogen tartrate salt was provided through the NIDA drug supply program. HFD pellets were changed every 3 days to prevent dehydration, and drinking water solutions were refreshed every 7 days.

2.3 Behavioral Assays

The start of nicotine treatment was considered to be Day 0. Aside from daily measurements of body weight, food weight, and water consumption, animals were left undisturbed for 23 days after the start of nicotine drinking. Open field, marble burying, and light-dark tests were conducted on days 24, 25, and 26 respectively. For all behavioral assays, mice were allowed to habituate to the testing room for at least 30 min prior to testing. Only one behavioral test was performed each day, and the least stressful tests were performed first.

2.4 Open Field Test

The open-field test was used to measure locomotor activity. Mice were allowed to explore a novel open-field measuring 46 cm × 46 cm for 15 min. Video recordings of open field behavior were analyzed with EthoVision XT10 tracking software (Noldus). A smaller “center-zone” was designated 7.6 cm in from all borders of the apparatus. Locomotor activity was measured as total distance traveled by the animal during the first 5 min of the test. Time spent in the center-zone and the edges of the open field was also calculated.

2.5 Light-Dark Test

The light-dark apparatus consisted of a two-chamber box with one dark and one light chamber. Mice were released into the light chamber and allowed to explore the apparatus for 5 min. Latency to the first entry into the dark chamber as well as overall time spent in the light chamber were recorded by an observer blind to treatment status. A mouse was determined to be in the light chamber if all four paws were in that chamber.

2.6 Marble Burying Test

Mice were placed in a 29 × 41 cm open field containing approximately 5 cm of bedding. Twenty glass marbles were placed on top of the bedding equally spaced in a 4 × 5 matrix. Animals were allowed to explore the field and the marbles for 30 min. The total number of marbles buried was counted at the end of each 30 min trial. A marble was considered to be buried if more than 2/3 of the marble was under the bedding.

2.7 Tissue collection

30 days after the start of nicotine treatment, mice were euthanized by rapid decapitation and tissues were collected and stored for biochemical analysis. The whole hypothalamus (HYP) was dissected from the ventral surface of the brain. WAT was collected from the gonadal visceral fat deposit, epididymal adipose in males or gonadal adipose in females, and weighed before freezing and storage. Scapular BAT was dissected by removing the fat pad from between the shoulder blades and separating any surrounding WAT. All tissues were frozen over dry ice and kept at −80°C until processing for RNA extraction. For serum extraction, trunk blood was collected at the time of sacrifice, allowed to sit at room temp for at least 30 min then centrifuged at 1500 rcf for 30 min. The resulting supernatant was collected and frozen at −80°C.

2.8 Biochemical Analyses

To ensure consistency, a piece of WAT not exceeding 100 mg, one half of the recovered BAT (one “wing”), and a 1.2 mm punch of central hypothalamus containing the arcuate nucleus (ARC) were homogenized for mRNA extraction from each animal. Tissue was hand homogenized using either a Dounce homogenizer (WAT and BAT) or a disposable pestle (ARC) in Qiazol. Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit following the manufacturer’s protocol (Qiagen, Valencia, CA). The concentration of extracted mRNA was measured with a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Waltham, MA) and converted to cDNA with a QuantiTect Reverse Transcriptase Kit following the manufacturer’s protocol (Qiagen, Valencia, CA). Varying volumes of extracted mRNA were used in the RT reaction so that resulting cDNA concentrations were consistent for each tissue type across animals within sex.

Relative expression of target genes was measured with real-time quantitative polymerase chain reaction (PCR) on a 96-well StepOne Plus qPCR machine with Power SYBR Green Mix (Applied Biosystems, Foster City, CA). All primers were designed to yield only one PCR product, had ~100% efficiency in a standard curve, and resulted in single melt peaks (see Table 1 for primer pair sequences). Samples were run in triplicate and were averaged to yield one Ct value per animal. Calculations of relative gene expression, expressed as fold

change, were conducted with the 2^{-Ct} method (Goni et al., 2009). β -glucuronidase (Gusb) was used as a reference gene, and samples were normalized to the CHOW-SAC group. The resulting fold change measure was graphed and used for statistical analysis between groups.

Expression of agouti-related peptide (AgRP) and neuropeptide-Y (NPY) were used in tandem with notes about tissue dissection to control for the localization and quality of ARC punches. Samples with noted ambiguity in dissection and whose Ct values varied by 4 or more compared to average Ct value of AgRP and NPY were excluded from analyses of gene expression. This resulted in exclusion of 5 samples of ARC mRNA: one animal each from the male CHOW-SAC, HFD-SAC, and HFD-NIC and 2 from the female CHOW-NIC group.

Serum was tested using commercially available kits for glucose (EnzyChrom Glucose Assay Kit, BioAssay Systems, Hayward, CA) and corticosterone (Corticosterone ELISA Kit, Enzo, Farmingdale, NY) following manufacturer protocols. Colorimetric results were read using an ELx800 plate reader and Gen5 microplate reader software (BioTek, Winooski, VT). Both glucose and corticosterone concentrations were extrapolated using the appropriate standard curve analysis described in the assay instructions. After glucose and corticosterone were measured, 25–50 μ L of serum remained which was used to measure nicotine and cotinine levels by reversed-phase HPLC with ion pairing as described previously (Caldarone et al., 2008). The small amount of serum used was close to the limit of detection for nicotine, therefore nicotine was not consistently detected and levels are not reported here. Positive readings above threshold for cotinine are used here as a rough proxy for validation of nicotine consumption.

2.9 Statistical Analyses

All data are presented as the mean $\pm$ SEM. Three-way ANOVA was used to test differences in body weight over time, two-way ANOVAs were used to compare the main effects and interactions of nicotine and HFD exposure, and unpaired t-tests were used to compare nicotine dose between groups. $P < 0.05$ was regarded as significant. Tukey's HSD post hoc tests were used when main effects or interactions were indicated. Statistical analyses were carried out using R for the three-way ANOVA of body weight over time, and Graph Pad Prism 7 for all other tests.

3. Results

3.1 Response to NIC and HFD in male mice

In male animals striking differences in body weight gain between treatment groups were observed over the course of this study (Fig. 1A). At the start of the study no differences were observed between treatment groups, and after 7 days of habituation to the HFD there were still no significant weight differences between groups. All animals gained some weight over the treatment period (main effect of time $F(37,814) = 52.03$, $p < 0.001$ in males), however in the group on SAC, 30 additional days of access to HFD resulted in a significant increase in body weight. Access to NIC drinking water blocked this weight gain (significant interaction of time $\times$ food $\times$ water $F(37,814) = 1.926$, $p = 0.001$). *Post hoc* analysis indicated that the SAC-HFD group had begun to differ from the NIC-CHOW group by day 17 of nicotine

exposure, the SAC-CHOW group by day 20, and these differences remained significantly elevated for the duration of the experiment. The SAC-HFD group showed a trend toward higher body weight compared to the HFD-NIC group, but this difference did not reach significance when corrected for multiple comparisons. However, there were no significant differences in body weight between groups fed regular lab chow or the HFD-NIC group across the duration of the experiment. Net weight change from the start of nicotine access reflected a similar relationship, with animals in the HFD-SAC group gaining significantly more weight than either chow group, although this group did not gain significantly more weight than the HFD-NIC group. Group averages in grams $\pm$ SEM were: CHOW-SAC 1.33 ± 0.20 , CHOW-NIC 1.42 ± 0.36 , HFD-SAC 4.60 ± 1.07 , HFD-NIC 1.9 ± 1.82 (ANOVA Main effect of Diet $F(1,22)=14.3$, $p=0.001$).

To determine if this increase in body mass was a result of an increase in stored fat, we weighed gonadal visceral fat at the conclusion of the experiment. As expected, male mice on HFD had larger WAT pads compared to CHOW-fed controls (Fig. 1B, main effect of diet $F(1,22) = 10.45$, $p = 0.004$). Although male mice with access to NIC and HFD weighed less than animals on SAC and HFD, there was no significant interaction with WAT weight.

To determine if changes in weight gain were due to changes in calorie intake, total caloric intake was calculated for all groups. Because the HFD contained more calories per gram, raw grams of food consumed were used to calculate calorie intake, to facilitate comparison between groups. There was a main effect of diet, such that the animals on HFD consumed more calories than those given CHOW ($F(1,22) = 5.211$, $p = 0.033$); however, there was no difference in caloric intake between male animals with access to SAC versus NIC water (Fig. 1C). These results indicate that the changes in weight over the course of nicotine administration cannot be accounted for by caloric intake alone. This is more apparent when body weight gained over the course of the experiment was normalized to the total calories consumed (Fig. 1D). Male mice on HFD showed an increase in grams of body weight gained per calorie consumed (Main effect of diet: $F(1,22) = 20.43$, $p < 0.001$), and this was attenuated by NIC treatment (Interaction: $F(1,22) = 4.445$, $p=0.0466$). *Post hoc* analysis indicated that the HFD-SAC group gained significantly more weight/calorie than either CHOW group (adjusted $p < 0.05$) and more weight/calorie than the HFD-NIC group (adjusted $p = 0.0589$). Thus, while nicotine access did not alter caloric intake in HFD-fed male mice, it did alter metabolism such that less weight was gained per calorie consumed.

Because the animals in this behavioral paradigm were able to self-regulate their exposure to nicotine via their water intake, fluid consumption was monitored over the duration of NIC exposure to assess nicotine intake. These data were collapsed into a single measure to compare the total water intake across all 30 days of exposure to compare between groups (Fig. 1E). There were main effects of both the drug and diet on fluid intake, but no interaction between these factors (Diet $F(1,22) = 11.43$, $p, 0.0027$; Drug $F(1,22) = 8.974$, $p=0.0067$). Male mice consumed less fluid when their water contained nicotine, which may indicate the animals regulated their intake of the drug. Further, mice on HFD consumed less water than animals on CHOW regardless of which type of water they had, which is likely due to the fact that the HFD pellets have a higher water content than CHOW pellets. Although there were differences in water consumption, there was no difference between the

CHOW and HFD groups in the total amount of nicotine ingested in mg over the course of the experiment (Fig. 1F: $t=1.567$, $p>0.05$). When examined over the time course of the experiment, there is a main effect of diet on nicotine intake, with the HFD group overall consuming less nicotine, due to their reduced water consumption (Fig 1F: $F(1,12)=6.491$, $p=0.0256$), however no individual day showed significantly different intake upon *post-hoc* analysis. There was also variation in intake over days, but this was comparable in both groups, suggesting natural variation across days more generally. When calculated as a daily dose, male animals in both groups received an average of ~40 mg/kg/day of nicotine. Because there were no differences in total nicotine intake between groups, the differences in fluid intake were unlikely to have a significant impact on the other measures of the study. To ensure that animals were exposed to biologically-active levels of nicotine that were not modified by diet, we measured serum cotinine levels. We did not observe significant differences in serum cotinine between Chow and HFD groups (mean ng/mL $\pm$ SE Chow = 164.2 $\pm$ 81.1, HFD= 154.7 $\pm$ 49.1; $t=0.093$, $p>0.05$), and no cotinine was detected in animals receiving SAC control water.

To understand further the metabolic and biochemical changes that are associated with increased body weight, and thus might be affected by nicotine, we examined both serum glucose and CORT levels at the time of sacrifice. Changes in serum glucose are associated with increased body fat and exposure to HFD (Lin et al., 2000). Animals in the current study were not fasted before the collection of serum, so glucose concentrations reflect a fed state. Consistent with increased weight gain, male mice exposed to HFD had significantly elevated serum glucose concentrations compared to CHOW fed males (Fig. 1G), indicating a main effect of diet ($F_{(1,22)} = 14.3$, $p = 0.001$). However, there was no effect of NIC on serum glucose levels. Serum CORT levels were also measured (Fig. 1H) to determine if increased stress might be a cause of changes in body weight and fat accumulation due to nicotine and HFD. Despite the differences in weight gain, there were no differences in serum CORT across groups, indicating that these weight changes are likely driven by metabolic changes rather than by an increase in HPA-axis signaling. Further, this indicates that the male animals with the greatest weight gain did not reach the threshold for diet-induced obesity, where increased weight begins to result in metabolic stress. Further the changes in body weight between groups did not alter open field locomotion in a 5 min test (Fig. 1I). There were also no differences in time spent in the center portion of the open field (data not shown), again indicating no broad changes in stress reactivity.

Additional behavioral tests were conducted to evaluate the effects of HFD and NIC exposure on anxiety and stress reactivity, which could affect nicotine intake or weight. The light-dark test is commonly used as a test of anxiety-related behavior, and behavior in this paradigm is sensitive to nicotine administration (Kutlu et al., 2015). Neither NIC nor HFD had an effect in male mice on time to enter the dark chamber or time spent in the dark chamber (data not shown). In contrast, there was a difference in the number of marbles buried in the marble-burying test (Fig. 2), which has been validated as a measure sensitive to anxiolytic administration, with more marbles buried indicating higher levels of anxiety-like behavior (Deacon, 2006). NIC but not HFD had a significant main effect of decreasing number of marbles buried by male mice ($F_{(1,22)} = 4.58$, $p = 0.044$), indicating reduced anxiety-like behavior in male mice with access to NIC.

3.2 Response to NIC and HFD in female mice

When the same nicotine and HFD/NIC exposure paradigm was repeated in female animals, results varied considerably from the male animals. As with male mice, all female groups started with equivalent baseline body weights, and over the duration of the experiment the animals did gain some weight over time (main effect of time $F(37,814) = 48.68$, $p < 0.001$). However, female animals with access to HFD did not gain significantly more weight than their CHOW-fed counterparts (Fig. 3A), and there were no significant interactions of diet or NIC over time. NIC did not reduce body weight gain in animals on either diet. The lack of effect of HFD compared to CHOW in this group may be due to a delayed response to HFD diet in female mice, since in previous studies substantial weight gain in female C57BL/6 mice did not occur until more than 6 weeks on HFD (Krishna et al., 2016; Lee et al., 2010; Sparks and Pauly, 1999; Yang et al., 2014). Net weight change from the start of nicotine access reflected a similar relationship, with no differences across groups. Group averages in grams $\pm$ SEM were: CHOW-SAC 2.18 ± 0.30 , CHOW-NIC 1.90 ± 0.32 , HFD-SAC 2.63 ± 0.69 , HFD-NIC 1.82 ± 0.63 . Consistent with the lack of body weight differences between female groups, there were no differences observed in female WAT weight (Fig. 3B). Further, there were no differences in caloric intake across groups (Fig. 3C), or in weight gained per calorie consumed (Fig. 3D), suggesting that female mice titrated their caloric intake on the HFD in this study. As with the male animals, there were no decreases in caloric intake on CHOW, indicating that nicotine given with free-access does not decrease weight on a lower calorie diet in either sex.

Similar to the male animals, the female animals showed differences in water consumption during the course of the experiment (Fig. 3E). The effect of the type of water (SAC v NIC) on water intake did not reach significance in these animals, however, there was an effect of diet ($F_{(1,21)} = 30.94$, $p < 0.001$), with the animals on HFD drinking less water than the animals with CHOW. Unlike in the male animals, this significantly affected the total nicotine consumed between groups in the female animals, with the animals on HFD taking in less nicotine than their CHOW-fed controls (Fig. 3F: $t=3.09$, $p = 0.0114$). As in male mice, there were daily fluctuations in nicotine intake that were roughly the same across diet groups. There was also a main effect of diet on intake over time ($F(1,10)=9.55$, $p=0.0114$), with significantly reduced intake on days 14, 17, and 20 (adjusted $p<0.05$). When calculated as a daily dose, female mice on the HFD consumed approximately 15 mg/kg less nicotine per day than female mice on CHOW (CHOW average = 55.28 mg/kg/day, HFD average = 40.5 mg/kg/day). Because comparable differences were seen in the consumption of the SAC water between diet groups, the decrease in NIC intake seems to be driven by hydration rather than a change in nicotine seeking. Further, even though the female mice on HFD consumed less nicotine, their daily dose was comparable to that seen in the male animals. Likely because the days on which nicotine intake differed significantly were 10 days or more before the conclusion of the experiment, at the time of sacrifice serum cotinine levels were not different between diet groups ($t=1.487$, $p>0.05$), as observed in male animals (mean ng/mL $\pm$ SE Chow = 69.5 ± 17.0 , HFD = 125.6 ± 33.1). No serum cotinine was detected in animals receiving SAC control water. When compared in a two-way ANOVA with male animals, there were no differences between male and female mice or diet groups.

When female serum was analyzed, there were no main effects or interaction observed in glucose levels, consistent with their resistance to the effects of HFD compared to male mice (Fig. 3G). Unlike in the male mice, there was a significant main effect of diet on serum CORT levels, with HFD animals showing reduced serum CORT levels compared to CHOW fed controls (Fig. 3H: $F_{(1,21)} = 9.10$, $p = 0.007$).

There were also differences in behavior after this regimen of diet and nicotine in female mice compared to male animals. There was an interaction between NIC and HFD in open field locomotion (Fig. 3I; $F_{(1,21)} = 5.63$, $p = 0.027$). NIC did not affect overall locomotion in CHOW fed female mice, but there was a trend toward an increase in locomotion in HFD fed female mice, with the group exposed to NIC and HFD showing the highest locomotor activity. As in male mice, neither NIC nor HFD had an effect on time spent in the center zone of the open field, time to enter the dark chamber in the light-dark test, or the total time spent in the dark chamber (data not shown). Unlike in the male animals, there were no effects of NIC or HFD on the number of marbles buried in the marble burying test (data not shown). These data indicate that even though HFD fed female animals had reduced CORT levels in serum, this did not alter this stress-related behavior.

3.3 Changes in mRNA levels after NIC and HFD in male and female mice

To understand how nicotine might affect weight without changing food intake, we examined the levels of mRNAs encoding numerous metabolic proteins and feeding peptides in multiple tissues (for a full summary of results see Table 2). There were no significant differences between groups of male mice in any of the UCP proteins in BAT, WAT, or ARC, however there was a trend for nicotine exposure to decrease UCP1 mRNA levels in WAT ($F_{(1,22)} = 2.952$, $p = 0.10$). Surprisingly there were multiple differences observed in UCP expression in female mice (Fig. 4). HFD reduced expression of UCP3 independent of NIC exposure in BAT (Fig. 4A: $F_{(1,21)} = 5.79$, $p = 0.025$). In WAT, the variability observed in UCP1 mRNA expression in the CHOW-SAC group resulted in highly variable fold changes for all other groups, yielding no significant group differences. This high variability may represent individual differences in the response to HFD and NIC that might precede changes in weight gain. While UCP3 expression was reduced in BAT of female animals on HFD, it was significantly increased in WAT of HFD-fed females, again independent of NIC exposure (Fig. 4B: $F_{(1,21)} = 4.506$, $p = 0.047$). In ARC, HFD and NIC interacted to regulate UCP1 expression (Fig. 4C: $F_{(1,18)} = 6.672$, $p = 0.019$). UCP2 expression did not change between groups in any of the tissues studied.

Levels of mRNAs encoding the neuropeptides AgRP, NPY, and melanin concentrating hormone (MCH), which signal hunger, and cocaine- and amphetamine-related transcript (CART), which signals satiety, were measured in ARC punches. AgRP mRNA levels were decreased by HFD in male mice independent of NIC exposure (Fig. 5: $F_{(1,20)} = 8.117$, $p = 0.001$). NPY expression was also decreased by HFD ($F_{(1,19)} = 9.848$, $p = 0.005$), again with no effect of NIC. CART expression was increased by HFD ($F_{(1,19)} = 4.653$, $p = 0.044$), with expression in the HFD-SAC group significantly elevated in a *post-hoc* comparison to the CHOW-SAC group (adjusted $p = 0.0133$). There was also a significant interaction with NIC ($F_{(1,19)} = 6.717$, $p = 0.018$), such that mice on NIC did not show robust increases in CART

expression compared to animals receiving SAC. These CART expression differences mirrored the weight gained in the male animals.

In female mice (Fig. 6), AgRP mRNA levels were also decreased by HFD ($F_{(1,19)} = 5.2$, $p = 0.034$) mice, independent of NIC exposure, however HFD did not reduce NPY expression significantly ($F_{(1,19)} = 2.504$, $p = 0.130$). There was no effect of HFD or NIC on CART expression in female mice. While there were no effects of NIC or HFD on MCH mRNA in male animals, there was a significant interaction in MCH expression between HFD and NIC in females ($F_{(1,19)} = 7.341$, $p = 0.0139$). Female mice in the CHOW-NIC group showed an increase in MCH expression that was significantly greater by *post-hoc* analysis than all of the other groups, including the HFD-NIC group, which did not show this increase.

We also examined levels of the mRNAs encoding key nAChR subunits in the ARC. There were no significant changes in expression of the $\alpha 7$ or $\beta 2$ subunit in either male or female mice. In female mice, there was a trend toward an interaction of HFD and NIC in expression of the $\beta 4$ subunit ($F_{(1,19)} = 3.95$, $p = 0.062$), but this was not paralleled in male animals.

4. Discussion

In the current study, male mice on a HFD gained more weight compared to CHOW-fed mice, and this weight gain was blocked by concurrent nicotine access. Weight was not altered by nicotine exposure in CHOW-fed animals. Caloric intake was not affected by either diet or NIC, however, male mice on HFD-NIC did not gain as much weight per calorie consumed as HFD-SAC controls, suggesting NIC exposure altered metabolism in these animals. Consistent with increased weight gain, male mice on HFD showed increased serum glucose, but this was not affected by NIC. Serum CORT levels and behavioral testing indicated that changes in weight were not due to increased stress or anxiety. Surprisingly, there were no interactions with UCP expression, implying that the HFD-NIC mice did not remain lean due to increased UCP expression. HFD caused predicted decreases in mRNAs encoding feeding-related neuropeptides NPY and AgRP in ARC, yet neither peptide was changed by NIC exposure. NIC and HFD interacted to change expression of CART mRNA, with HFD increasing expression and NIC returning levels back towards baseline levels.

Female mice in this paradigm were resistant to the effects of HFD on body weight and serum glucose levels. Animals fed HFD had lower serum CORT than CHOW-fed controls. Diet and NIC interacted to affect locomotion, with HFD fed female animals on SAC moving less than their NIC exposed counterparts, although female mice showed no changes in anxiety-related behaviors. Despite resisting weight gain, female mice fed HFD showed changes in UCP mRNA expression in WAT, BAT, and ARC along with an expected decrease in the hypothalamic feeding peptide AgRP. Unlike male mice, there was no change in NPY or CART expression, but NIC did increase MCH only in CHOW-fed animals. Thus, a number of physiological measures differed greatly between female and male mice fed HFD, which may underlie the significant sex differences in weight gain and response to nicotine in the current study.

Although numerous studies have evaluated the effects of nicotine on body weight and food intake, few have examined these measures during chronic, self-regulated nicotine delivery. It is important to note that using this route of administration does force consumption of at least some nicotine as the nicotine water is the animals' only source of hydration. The nicotine consumed in this experiment, ~40 mg/kg/day, is within the range of nicotine consumed in previous studies using this route of administration and nicotine dose (Caldarone et al., 2008; Huang et al., 2015; King et al., 2004; Sparks and Pauly, 1999). This single-bottle dosing regimen does increase nicotine intake compared to experiments where animals have a nicotine-free drinking water option, as all daily liquid intake contains nicotine (Robinson et al., 1996). The dose of nicotine used in these studies (200 µg/mL) was chosen because it has been shown to increase plasma concentrations of nicotine and cotinine reliably, and to increase nicotine binding to high affinity nAChRs in the brains of both male and female C57BL/6J mice (Caldarone et al., 2008; King et al., 2004; Sparks and Pauly, 1999). Using serum cotinine as a rough read out of nicotine exposure in the current study, we observe that both male and female mice are exposed to significant levels of nicotine and that HFD does not change circulating levels of cotinine. Although differences in nicotine exposure could account for the weight gain differences observed in this study, our data do not indicate significant exposure differences between groups by sex or diet.

One previous study used a drinking-water paradigm of nicotine administration to examine nicotine's interaction with HFD, however, metabolic measures in that study focused on changes in male rats following nicotine withdrawal (Fornari, et al., 2007). Nicotine delivery in drinking water allows animals to titrate both the timing of nicotine delivery and the dose, in contrast to daily injections or implanted mini-pumps. Allowing animals to titrate their nicotine exposure may provide a paradigm more similar to human nicotine intake, since HFD can alter the rewarding properties of nicotine (Blendy, et al., 2005; Morganstern, Ye, Liang, Fagan, & Leibowitz, 2012). In the same way that self-titrated nicotine did not change caloric intake in the current study, when rats are allowed to self-administer IV nicotine in 1 hr daily sessions, their body weight decreased without a change in food intake (Rupprecht et al., 2016). This difference in dosing method may explain why nicotine did not decrease weight gain in animals fed CHOW or decrease caloric intake, as previously observed with experimenter-administered nicotine (Chen, et al., 2005, 2008; Frankish, et al., 1995; Mangubat, et al., 2012; Sinha-Hikim, et al., 2014). The ability of nicotine to blunt weight gain in male mice on HFD is consistent with past studies despite the difference in route of nicotine administration (Mangubat, et al., 2012; Sinha-Hikim, et al., 2014; Wellman, et al., 2005).

The current study examined weight gain in both male and female animals to determine whether there were sex differences in responses to NIC and HFD. With this paradigm we did not see robust weight gain in female mice. This is consistent with studies in which female C57B/6 mice did not out-gain their low fat fed counterparts until 6 to 9 weeks on HFD (Krishna, et al., 2016; Lee, et al., 2010; Yang, et al., 2014). In previous experiments with fixed doses of nicotine, female rats tended to be more sensitive to nicotine's effects of on body weight and food intake than males and were especially vulnerable to increased food intake and weight gain during nicotine withdrawal (Bowen, et al., 1986; Grunberg, Popp, & Winders, 1988). In the current study, in which animals could control their exposure to

nicotine, we did not see reduced intake or weight gain in females on nicotine on either diet. Even in male mice, the 38 days of HFD in the current experiment results in diet-induced weight gain, but is not sufficient for full insulin and leptin resistance to develop (Krishna, et al., 2016; Lin, et al., 2000). As expected, changes in serum glucose associated with this HFD were observed in male animals, indicating the beginning stages of diet-induced obesity, but female mice did not show changes in glucose at this time point, consistent with a previous study (Lin et al., 2000). Thus, female mice may need longer exposure to HFD to show comparable weight gain to males and further experiments with a paradigm more tailored to female animals would be necessary to fully understand this sex difference. It would also be beneficial for future studies to determine the effect of this type of nicotine dosing regimen on the timeline of development of other changes associated with diet-induced obesity, specifically in insulin, leptin, and ghrelin levels, in both female and male mice.

Long-term exposure to HFD has been associated with increased markers of stress and anxiety, including increased CORT levels (Dutheil et al., 2015; Sivanathan et al., 2015), and prolonged increases in CORT can cause weight gain (Karatsoreos, et al., 2010). In the current study, the only behavioral change across treatment groups was a decrease in marble burying in male mice on NIC, which is thought to reflect reduced anxiety-like behavior. There were no differences in serum CORT in male mice, indicating that the blunted weight gain in HFD-NIC male animals was likely not due to effects on stress pathways. Female mice showed a difference in serum CORT, but HFD lowered, rather than increased, CORT levels, which is not consistent with previous studies. Despite this difference, there were no changes in tests of anxiety-like behavior. Because this study required individual monitoring of food and water intake, animals were single-housed. While all animals were maintained under the same conditions and comparisons between groups were well controlled, it is important to note that group-housed animals may respond differently to nicotine or HFD exposure. Single-housed C57BL/6 mice have been shown to have reduced corticosterone levels as compared to group-housed animals (Kamakura et al., 2016), possibly due to within cage fighting in group-housed animals, however there are effects on some tests of anxiety after single-housing. Interestingly, single housing has been shown to reduce food intake mildly, yet increase adiposity, while not altering overall body weight significantly in C57BL/6 mice (Sun et al., 2014).

Multiple neurobiological mechanisms and pathways have been implicated in nicotine's regulation of body weight and food intake and its interaction with diet since nAChRs are expressed on multiple cell types throughout the brain and body, including sensory inputs, neurons and non-neuronal cells in throughout the brain, the peripheral nervous system, and non-neuronal cells in many peripheral tissues (for review see Zoli & Picciotto, 2012). One way to maintain homeostasis is to modify energy expenditure. UCPs are mitochondrial transporter proteins originally named for UCP1's role in uncoupling ATP synthase from the electron transport chain, causing energy to be dissipated as heat and regulating resting metabolism. While the primary function of UCP1 is to regulate non-shivering and cold-induced thermogenesis, UCP2 and 3 have roles regulating diet-induced thermogenesis, free radical scavenging, insulin secretion, fatty acid metabolism, and controlling metabolic rate (Rousset, 2004). Diet-induced obesity has been associated with increased thermogenesis and leptin-induced BAT activation, including increased UCP1 expression (Cannon and

Nedergaard, 2004). In the current study HFD did not increase BAT or WAT UCP1 mRNA levels in male mice, perhaps because animals were not on HFD long enough to induce all of the changes of diet-induced obesity. Although previous studies have shown changes in UCP1 and 3 in both BAT and WAT after nicotine exposure (Chen, et al., 2005, 2006, 2008; Martínez De Morentin, et al., 2012), no such changes were observed with nicotine treatment in male mice in the current study, consistent with the findings of Fornari, et al. (2007). Nicotine did not alter UCP expression in female mice, however, HFD decreased UCP3 mRNA in BAT and increased it in WAT, which could indicate changes in fat metabolism, possibly explaining the initial resistance of female animals to weight gain in this paradigm.

Compared to UCP function in adipose tissue, the role of UCPs in other tissues is less firmly established. For example, it was previously assumed that UCP1 was not expressed in neuronal tissues, yet studies have now shown expression in mouse cortex (Lengacher et al., 2004; Mehler-Wex et al., 2006). Further, CNS UCPs have been implicated in neurotransmission, synaptic plasticity, neurodegenerative processes, and recovery after injury (Andrews et al., 2005). Previous studies examining expression of UCPs in response to HFD or nicotine have not measured UCP expression in neural tissues. The regulation of UCP1 in the ARC of female animals may play a role in neuronal signaling in the hypothalamus that contributes to the resistance to weight gain on HFD seen in this paradigm in female mice.

Another way that energy homeostasis and weight are regulated is through central signaling in the hypothalamus. Nicotinic signaling in the ARC can reduce food intake (Mineur, et al., 2011), and both nicotine and HFD can regulate feeding peptides in the ARC and other hypothalamic nuclei. The current finding that HFD decreases expression of mRNAs encoding AgRP and NPY in male mice is consistent with previous studies (Giraudou, Kotz, Grace, Levine, & Billington, 1994; Lee, et al., 2010; Wang, Storlien, & Huang, 2002), and the current study demonstrates that this is also true for AgRP in female animals, with NPY trending in the same direction. However, changes in expression of these feeding peptides did not correlate with observed weight gain.

The literature is more mixed with respect to nicotine's effects on NPY mRNA levels. The observation that nicotine had no effect on NPY mRNA levels in ARC is consistent with some experiments (Chen, et al., 2005, 2006; Fornari, et al., 2007; Hur, Hong, Choi, Shin, & Chun, 2010), but other groups have found that nicotine can increase NPY mRNA and peptide levels (Chen, et al., 2008; Li et al., 2000), while still others show that nicotine decreases NPY (Frankish, et al., 1995; Martínez De Morentin, et al., 2012), indicating that dose, route of administration, and diet affect nicotine's effects on expression of feeding peptides. In the current study, nicotine had no effect on AgRP levels in either male or female animals, which is in contrast to the decrease seen by Martínez, et al. (2012) and the increase seen by Hur, et al. (2010). Both of these studies, however, used subcutaneous nicotine injections for shorter time periods than the current study. MCH is a feeding peptide that, like AgRP, acts in opposition to α -melanocyte stimulating hormone and has also been implicated in stress reactivity (Hervieu, 2003). MCH was not affected by nicotine exposure in male mice in the current study, consistent with previous findings (Fornari, et al., 2007), however, nicotine increased MCH in female animals fed CHOW, which may be related to its role in

stress reactivity and the altered CORT and locomotion changes observed in the female animals. Finally, male mice exhibited increased expression of mRNA encoding CART with HFD that was normalized by nicotine, which mirrored the pattern in weight gain in these animals. Normally both orexogenic and anorexigenic peptides are reduced in animals on HFD, maintaining their balance (Lin, et al., 2000; Wang, et al., 2002). The current study is consistent with previous experiments showing that nicotine disrupts this balance leading to increased CART and α -MSH mRNA along with reduced NPY and AgRP in some cases (Chen, Saad, Sandow, & Bertrand, 2012; Martínez De Morentin, et al., 2012) or increases in all three peptides in others (Hur, et al., 2010).

In conclusion, this study demonstrates that self-titrated nicotine can reduce weight gain without altering caloric intake in male mice on a HFD, and this may result from changes in signaling in the feeding regions of the hypothalamus. Further, female animals do not respond in the same way as males to 30 days of freely-titrated nicotine and HFD, and sex differences are also observed at the gene transcription level. Human studies have established that nicotine affects males and females differently, and these differences create unique challenges in smoking cessation for both sexes (McKee et al., 2015). Understanding the mechanisms that underlie sex-differences in nicotine's effects on energy metabolism and weight gain could help explain differences in smoking behaviors seen in men and women, which in the long term may help in designing more targeted cessation aids, especially for weight concerned smokers.

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Highlights

- Nicotine in drinking water decreases weight gain on HFD, but not chow, in male mice.
- Self-titrated nicotine does not change calorie intake in male or female mice.
- Nicotine and HFD change the expression of feeding peptides in the arcuate nucleus.

Figure 1. Physiological responses of male animals after exposure to HFD and NIC

A. Body weight over time in animals consuming CHOW and SAC (CS), CHOW and NIC (CN), HFD and SAC (HS) or HFD and NIC (HN) (mean $\pm$ SEM for each day). Day-6 indicates baseline weight and the start of HFD for HFD groups. Day 0 (dashed line) indicates the start of NIC treatment. Behavioral tests were conducted on Days 24–26, and animals were sacrificed after weighing on Day 30. For clarity, only data for every 3rd day is presented on the graph, although all daily time points were used for statistical analyses. B. Weight of gonadal visceral fat after dissection at the conclusion of NIC exposure. C. Cumulative caloric intake for the duration of NIC exposure. D. Normalized energy intake represented as grams of weight gain per calorie consumed over the duration of NIC exposure. E. Cumulative water intake for the duration of NIC exposure. F. Cumulative NIC intake for the duration of NIC exposure (left) and daily nicotine intake over the duration of nicotine access (right). NIC groups only: solid line, CHOW; dashed line, NIC. G. Resting serum glucose levels after 30 days of NIC or SAC drinking water. H. Resting serum corticosterone levels after 30 days exposure to NIC or SAC drinking water. I. Locomotor activity represented as total distance traveled over a 5 min testing period in a novel environment. All bars represent mean $\pm$ SEM, $n = 6-7$ animals per group, individual animals represented as individual data points, open bars = SAC water and grey bars = NIC water.

Figure 2. Behavioral differences in male mice exposed to NIC and HFD

Total number of marbles buried in a 30 min marble burying test. All bars represent mean $\pm$ SEM, $n = 6-7$ animals per group, individual animals represented as individual data points, open bars = SAC water and grey bars = NIC water.

Figure 3. Physiological responses of female animals after exposure to HFD and NIC

A. Body weight over time in animals consuming CHOW and SAC (CS), CHOW and NIC (CN), HFD and SAC (HS) or HFD and NIC (HN) (mean $\pm$ SEM for each day). Day-6 indicates baseline weight and the start of HFD for HFD groups. Day 0 (dashed line) indicates the start of NIC treatment. Behavioral tests were conducted on Days 24–26, and animals were sacrificed after weighing on Day 30. For clarity, only data for every 3rd day is presented on the graph, although all daily time points were used for statistical analysis. B. Weight of gonadal visceral fat after dissection at the conclusion of NIC exposure. C. Cumulative caloric intake for the duration of NIC exposure. D. Normalized energy intake represented as grams of weight gain per calorie consumed over the duration of NIC exposure. E. Cumulative water intake for the duration of NIC exposure. F. Cumulative NIC intake for the duration of NIC exposure (left) and daily nicotine intake over the duration of nicotine access (right). NIC groups only: solid line, CHOW; dashed line, NIC. G. Resting serum glucose levels after 30 days of NIC or SAC drinking water. H. Resting serum corticosterone levels after 30 days exposure to NIC or SAC drinking water. I. Locomotor activity represented as total distance traveled over a 5 min testing period in a novel environment. All bars represent mean $\pm$ SEM, $n = 6-7$ animals per group, individual animals represented as individual data points, open bars = SAC water and grey bars = NIC water. “I” indicates a significant interaction in a 2-way ANOVA.

Figure 4. Relative levels of significantly regulated mRNAs encoding UCP proteins in WAT, BAT and ARC in female mice

A. UCP3 mRNA levels in BAT. B. UCP3 mRNA levels in WAT. C. UCP1 mRNA levels in ARC. Box plots represent the min, max and quartiles for fold change using the CHOW-SAC group as the control group to which all other groups were normalized; CHOW and SAC (CS), CHOW and NIC (CN), HFD and SAC (HS) or HFD and NIC (HN). n = 4–7 animals per group as noted. “I” indicates a significant interaction in a 2-way ANOVA.

Figure 5. Relative levels of mRNAs encoding feeding-related neuropeptides in ARC in male mice
Levels of AgRP, NPY, CART, and MCH mRNAs. Box plots represent the min, max and quartiles for fold change using the CHOW-SAC group as the control group to which all other groups were normalized; CHOW and SAC (CS), CHOW and NIC (CN), HFD and SAC (HS) or HFD and NIC (HN). n = 5–6 animals per group as noted.

Figure 6. Relative levels of mRNAs encoding feeding related neuropeptides in ARC in female mice

Levels of AgRP, NPY, CART, and MCH mRNAs. Box plots represent the min, max and quartiles for fold change using the CHOW-SAC group as the control group to which all other groups were normalized; CHOW and SAC (CS), CHOW and NIC (CN), HFD and SAC (HS) or HFD and NIC (HN). n = 4–7 animals per group as noted.

Table 1

Nucleotide sequences of primer sets used for qPCR reactions

Gene	Forward Sequence	Reverse Sequence
Gusb	5' GAGCCTTCCTCTGCTCTGAA 3'	5' ATATTGGCGTTGCTCACAA 3'
Ucp1	5' AGGTGTGGCAGTGTTCATTGG 3'	5' TCTGTGGTGGCTATAACTCTGTAAG 3'
Ucp2	5' TCTGGATACCGCCAAGGT 3'	5' TTGTAGAGGCTGCGTGGA 3'
Ucp3	5' CCTACGACATCATCAAGGAGAAGTT 3'	5' TCCAAAGGCAGAGACAAAGTGA 3'
Chrna7	5' GCCTA GTGGACCAGGATCA 3'	5' CTCGGAAGCCAATGTAGAGC 3'
Chrb2	5' ATCCCCTGCGTACTCATCAC 3'	5' GTACTTTCCCACCAGCGGTA 3'
Chrb4	5' CCTGCTCCTCGTCTCTCTGT 3'	5' CAGGTTGTTGTAGCGGGTTT 3'
AgRP	5' AGGTGCTAGATCCACAGAAC 3'	5' CATTGAAGAAGCGGCAGTAG 3'
NPY	5' TCGTGTGTTTGGGCATTCTG 3'	5' TCTGGTGATGAGATTGATGTAGTG 3'
CART	5' AGCCCTGGACATCTACTCTG 3'	5' ATCGGAATGCGTTTACTCTTG 3'
MCH	5' AGGTGTATGCTGGAAGAGTC 3'	5' GCACAAGTTATAGCAACATCAAGG 3'

Table 2

Fold change $\pm$ SEM in mRNA expression and significant differences for all measured genes in male and female WAT, BAT and ARC

			CHOW-SAC	CHOW-NIC	HFD-SAC	HFD-NIC	Main Effects		Interaction
							Diet	Nicotine	
Male	WAT	UCP1	1.312 $\pm$ 0.360	0.544 $\pm$ 0.096	0.742 $\pm$ 0.251	0.673 $\pm$ 0.140			
		UCP2	1.141 $\pm$ 0.208	0.643 $\pm$ 0.139	1.057 $\pm$ 0.244	1.028 $\pm$ 0.197			
		UCP3	1.226 $\pm$ 0.208	1.024 $\pm$ 0.182	1.22 $\pm$ 0.197	1.308 $\pm$ 0.115			
	BAT	UCP1	1.006 $\pm$ 0.049	1.050 $\pm$ 0.107	1.079 $\pm$ 0.076	1.117 $\pm$ 0.045			
		UCP2	1.027 $\pm$ 0.108	1.125 $\pm$ 0.194	0.815 $\pm$ 0.149	1.009 $\pm$ 0.079			
		UCP3	1.027 $\pm$ 0.094	1.088 $\pm$ 0.070	0.828 $\pm$ 0.096	1.032 $\pm$ 0.089			
	ARC	UCP1	1.004 $\pm$ 0.040	1.291 $\pm$ 0.203	1.179 $\pm$ 0.244	1.113 $\pm$ 0.134			
		UCP2	1.083 $\pm$ 0.193	0.828 $\pm$ 0.170	1.097 $\pm$ 0.174	1.192 $\pm$ 0.317			
		UCP3	1.013 $\pm$ 0.075	1.080 $\pm$ 0.177	1.039 $\pm$ 0.090	1.234 $\pm$ 0.153			
		$\alpha 7$	1.007 $\pm$ 0.052	0.834 $\pm$ 0.118	1.017 $\pm$ 0.098	1.140 $\pm$ 0.126			
		$\beta 2$	1.059 $\pm$ 0.163	0.959 $\pm$ 0.100	1.160 $\pm$ 0.208	1.069 $\pm$ 0.153			
		$\beta 4$	1.143 $\pm$ 0.268	1.185 $\pm$ 0.268	1.007 $\pm$ 0.124	0.887 $\pm$ 0.269			F(1, 19) = 3.95, p=0.062
		AgRP	1.118 $\pm$ 0.255	1.337 $\pm$ 0.229	0.760 $\pm$ 0.044	0.624 $\pm$ 0.163	F(1, 20) = 8.117 P=0.0099		
Female	WAT	NPY	1.038 $\pm$ 0.129	1.094 $\pm$ 0.179	0.644 $\pm$ 0.047	0.644 $\pm$ 0.159	F(1, 19) = 9.848 P=0.0054		
		CART	1.017 $\pm$ 0.082	1.266 $\pm$ 0.117	1.542 $\pm$ 0.160	1.218 $\pm$ 0.052	F(1, 19) = 4.653 P=0.0440		F(1, 19) = 6.717 P=0.0179
		MCH	1.007 $\pm$ 0.051	1.041 $\pm$ 0.147	1.087 $\pm$ 0.155	1.181 $\pm$ 0.166			
	BAT	UCP1	2.742 $\pm$ 1.072	0.346 $\pm$ 0.189	3.205 $\pm$ 2.191	2.443 $\pm$ 2.135			
		UCP2	1.125 $\pm$ 0.192	0.932 $\pm$ 0.133	0.836 $\pm$ 0.137	1.071 $\pm$ 0.110			
		UCP3	1.053 $\pm$ 1.145	1.480 $\pm$ 0.223	1.838 $\pm$ 0.364	1.698 $\pm$ 0.176	F(1, 21) = 4.506 P=0.0458		
	ARC	UCP1	1.009 $\pm$ 0.052	1.089 $\pm$ 0.115	1.053 $\pm$ 0.063	1.084 $\pm$ 0.080			
		UCP2	1.006 $\pm$ 0.044	1.167 $\pm$ 0.062	1.060 $\pm$ 0.135	1.063 $\pm$ 0.140			
		UCP3	1.026 $\pm$ 0.088	0.999 $\pm$ 0.075	0.841 $\pm$ 0.085	0.821 $\pm$ 0.035	F(1, 21) = 5.79 P=0.0254		
	ARC	UCP1	1.097 $\pm$ 0.181	1.533 $\pm$ 0.152	1.626 $\pm$ 0.230	1.166 $\pm$ 0.068			F(1, 18) = 6.672 P=0.0188
		UCP2	1.039 $\pm$ 0.117	1.018 $\pm$ 0.139	1.073 $\pm$ 0.165	0.929 $\pm$ 0.102			
		UCP3	1.050 $\pm$ 0.142	1.042 $\pm$ 0.158	0.996 $\pm$ 0.105	1.061 $\pm$ 0.169			
		$\alpha 7$	1.049 $\pm$ 0.131	1.055 $\pm$ 0.126	1.083 $\pm$ 0.076	1.033 $\pm$ 0.167			

		CHOW-SAC	CHOW-NIC	HFD-SAC	HFD-NIC	Main Effects		Interaction Diet × Nicotine
						Diet	Nicotine	
	β2	1.050 ± 0.152	1.092 ± 0.110	1.070 ± 0.080	1.078 ± 0.161			
	β4	1.124 ± 0.190	1.588 ± 0.112	2.110 ± 0.435	1.466 ± 0.164			
	AgRP	1.095 ± 0.186	0.927 ± 0.319	0.631 ± 0.128	0.557 ± 0.106	F(1, 19) = 5.2 P=0.0343		
	NPY	1.116 ± 0.224	0.918 ± 0.295	0.765 ± 0.198	0.603 ± 0.100			
	CART	1.073 ± 0.151	1.392 ± 0.161	1.247 ± 0.234	1.050 ± 0.098			
	MCH	1.140 ± 0.219	2.392 ± 0.420	1.188 ± 0.263	1.058 ± 0.140	F(1, 19) = 6.357 P=0.0208	F(1, 19) = 4.849 P=0.0402	F(1, 19) = 7.341 P=0.0139

Note: Fold change was calculated using the 2^{-Ct} method normalizing to the CHOW-SAC group within gene and sex. Main effects and interactions were calculated using two-way ANOVA.