EFFECT OF HIGH FAT DIET ON HINDBRAIN PROLACTIN-RELEASING PEPTIDE NEURONS AND SATIETY

by

Diana Roxana Olivos

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This thesis was presented

by

Diana Roxana Olivos

It was defended on

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and approved by

Charles C. Horn, PhD, Associate Professor, Medicine

Susan R. Sesack, PhD, Professor, Neuroscience

Committee Chair: Linda Rinaman, PhD, Professor, Neuroscience

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Rats fed high fat diet (HFD) eat larger meals compared to chow-fed rats before the onset of obesity, suggesting a reduced sensitivity to endogenous satiety signals (e.g., cholecystokinin (CCK)). CCK is released in response to nutrient absorption at the proximal small intestine and activates CCK receptors expressed on vagal afferents innervating gut mucosa, which then relay signals to the nucleus of the solitary tract (NTS), the site where vagal afferents terminate. Within the caudal NTS (cNTS), the A2 noradrenergic (NA) neuronal population is important for the regulation of food intake. Lesions of hindbrain A2 NA neurons eliminate the intake suppressive effects of CCK, suggesting that they play an important role in relaying satiety signals arising from the gut. A subset of A2 NA neurons is positive for prolactin-releasing peptide (PrRP), which suppresses food intake and body weight. This study investigates the effects of acute and chronic (2 and 7 weeks, respectively) HFD maintenance on the activation of hindbrain PrRP+ neurons and satiety. We tested the hypotheses that exposure to HFD blunts the ability of CCK to recruit PrRP neurons in the cNTS (experiment 1), and that chronic HFD exposure attenuates CCK's satiating effects by reducing recruitment of PrRP neurons in the cNTS (experiment 2). Both acute and chronic HFD-fed rats consumed significantly more calories and gained more weight compared to chow-fed rats. In experiment 2, behavioral testing revealed a trend towards an attenuation of CCK's intake suppressive effects in HFD-fed compared to chow-fed rats after 1 week and 6 weeks of diet exposure. Finally, after 2 or 7 weeks, animals were sacrificed after

injections of saline (2mL), 1μg/kg or 5μg/kg CCK, or no injection and brains were assessed for cFos and PrRP immunolabeling. Our data provide evidence that after chronic HFD maintenance, PrRP+ neuronal signaling at the level of the NTS is implicated, suggesting that the activity of the PrRP+ population is impaired and contributes to the hyperphagia and increased weight gain observed in the rats fed HFD.

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1.0 INTRODUCTION

Excessive caloric intake beyond energy expenditure promotes weight gain in both rats and humans. Overconsumption of calories has been documented in subjects provided high-fat (HF) diets compared to high-carbohydrate (HC) diets, suggesting that macronutrient composition affects satiety (Warwick, 1996). Satiety is the process by which a meal is terminated. When rats maintained on chow were offered non-fat or HF cake, where the palatability of the cake was rated equally during a two-choice test, rats in the HF group consumed more cake calories per day and consequently gained more weight than rats offered non-fat cake (Sclafani et al., 1993; Warwick, 1996). Furthermore, in two separate studies, lean and obese human participants consumed more calories when offered a HF (minimally 50% kcals from fat) compared to HC (minimally 50% kcals from carbohydrates) meal (Blundell et al., 1993; Warwick, 1996). Together these data suggest that satiety is influenced by meal macronutrient composition, such that HF meals are less able to promote satiety to terminate feeding.

HF diet (HFD) exposure, as well as intragastric delivery of HFD, where the diet is not tasted, promotes greater daily caloric consumption, suggesting that HFD increases the propensity of becoming overweight by changing the sensitivity to endogenous satiety signals (e.g. gastric distension and gastrointestinal (GI) hormones and peptides such as cholecystokinin (CCK); Warwick, 1996). While there are many gut peptides, CCK is a key player in providing intestinal feedback for the regulation of short-term feeding (Raybould, 2007). CCK is a GI derived anorectic peptide released by I cells of the upper small intestine in response to nutrient absorption (Moran, 2006; Moran, 2009; Moran and Dailey, 2011). In response to fat and protein in the intestine, endogenous CCK acts to inhibit gastric emptying, gastric acid secretion, and food intake (Raybould, 2007). CCK's ability to promote satiety relies on CCK_1 receptors on

vagal afferent fibers innervating gut mucosa that signal to the caudal brainstem (Moran, 2006; Moran and Dailey, 2009; Moran and Dailey, 2011; Grill and Hayes, 2012).

Volume and chemical composition of ingested contents is transmitted to vagal afferents, providing feedback about overall meal composition (e.g. nutrient content) to the brain (Moran, 2006). The interaction, integration and processing of these peripheral signals occurs within the caudal brainstem nucleus of the solitary tract (NTS), the site at which vagal afferents terminate (Grill and Hayes, 2012; de La Serre et al., 2016). The NTS receives and processes many signals involved in metabolic homeostasis, including GI satiation signals delivered by the vagus, energy-related hormonal signals (e.g. leptin and ghrelin) that circulate in the blood, and neural signals that originate in forebrain nuclei (e.g., lateral hypothalamus, arcuate nucleus, and paraventricular nucleus of the hypothalamus; Grill and Hayes, 2012). The NTS contains a phenotypically diverse population of neurons that process incoming signals and project to a variety of CNS nuclei, including nuclei involved in energy balance and reward (Grill and Hayes, 2012).

Of the various cell types in the NTS that ostensibly participate in satiety, the present study focuses on the A2 noradrenergic (NA) population located within intermediate and caudal levels of the NTS (Rinaman, 2011; Maniscalco et al., 2013). A2 NA neurons receive glutamatergic visceral sensory inputs from the cardiovascular, respiratory and alimentary systems (Rinaman, 2011). The A2 NA population is distinguished by positive immunolabeling for tyrosine hydroxylase and dopamine-β-hydroxylase (DbH; Rinaman et al., 1993; Rinaman, 2011). A2 NA neurons are activated to express cFos, a common marker of neuronal activation, in response to systemic administration of exogenous CCK at doses that suppress food intake (Rinaman et al., 1993; Rinaman et al., 1998). Interestingly, hindbrain NA lesions attenuate CCK's intake suppressive effects (Rinaman, 2003). A2 NA neurons project to many brain areas, including the

paraventricular and dorsomedial nuclei of the hypothalamus (PVN and DMH), arcuate nucleus, parabrachial nucleus, central nucleus of the amygdala, bed nucleus of the stria terminalis (BNST), ventral tegmental area, and nucleus accumbens (Rinaman, 2010; Rinaman, 2011). Importantly, A2 NA neurons appear to release more than just norepinephrine, as subsets of these neurons co-express markers for glutamate, neuropeptide Y, and prolactin-releasing peptide (PrRP; Rinaman, 2011).

PrRP is a brain- and peripherally-derived peptide that reduces food intake and body weight by suppressing meal size in rats and mice after central administration (Ellacott et al., 2003). PrRP is centrally expressed in the NTS A2 neurons, caudal DMH, and the ventrolateral medulla, and is expressed peripherally in adrenal chromaffin cells (Maruyama et al., 2001). PrRP's putative receptor, GPR10, is expressed centrally and in the periphery (Bechtold and Luckman, 2006; Takayanagi et al., 2008). Intracerebroventricular injections of PrRP reduce food intake and suppress body weight gain in both mice and rats, but the mechanism by which endogenous PrRP might interact with centrally and peripherally derived signals to terminate food intake is not well understood (Lawrence et al., 2000, 2002; Bechtold and Luckman, 2006). It has been reported that the lack of functional PrRP or GPR10 in genetically engineered mice results in hyperphagia, accompanied by greater weight gain and abdominal adiposity compared to wild-type controls (Takayanagi et al. 2008; Mochiduki et al., 2010). However, global knockouts of PrRP or GPR10 also likely induce changes in the periphery that may contribute to these effects. Interestingly, the hyperphagia observed in PrRP- or GPR10 knock-out mice is exacerbated when these animals are fed HFD, suggesting that in addition to HFD, the deficit of functional PrRP signaling attenuates the response to endogenous satiety signals, such as CCK.

To reveal changes in the behavioral sensitivity to CCK, many groups have administered intraperitoneal (i.p.) CCK to rodents (rats and mice) and studied their meal patterns, including meal size, meal frequency, and the time between meals. To examine the impact, many groups have used outbred Sprague Dawley rats (Covasa et al. 2000, Paulino et al. 2009). When maintained on HFD, some of these outbred rats gain significantly more weight than others (e.g., obese prone (OP) vs. obese resistant (OR)) after just 4 weeks of HFD maintenance. When OP rats are switched from chow to HFD, they tend to eat larger meals well before the onset of obesity, suggesting that reduced sensitivity to endogenous satiety signals is induced by HFD early on, before significant changes in body weight or adiposity (Paulino et al., 2009). One way to measure HFD-induced changes in sensitivity to CCK is through analysis of CCK-induced cFos immunoreactivity in the NTS. A second way to examine the satiating effects of exogenous CCK is to directly measure intake in animals after CCK administration. Covasa et al. (2000) assessed cFos immunoreactivity in the dorsal vagal complex (which includes the NTS, area postrema, and dorsal motor nucleus of the vagus) in response to a very low dose of CCK (0.25 μg/kg of body weight) and found that cFos expression was essentially absent in rats maintained for two weeks on a relatively HFD (34% kcals from fat) compared to moderate cFos activation in rats fed a very low fat diet (5% kcals from fat). Thus, reduced sensitivity to endogenous satiety signals may occur as an early consequence of diet exposure. The present study was designed to test the hypotheses that brief exposure to HFD blunts the ability of exogenous CCK to recruit PrRP-positive A2 neurons in the cNTS, with greater attenuation in OP rats (Experiment 1). We also hypothesized that chronic HFD exposure further attenuates CCK's satiating effects and ability to recruit PrRP-positive A2 neurons in the cNTS (Experiment 2).

1.1 MATERIALS AND METHODS

1.1.1 Subjects

Adult male outbred Sprague Dawley rats (Charles River; total n=112; 180-210g upon arrival) were individually housed in hanging wire cages in a temperature-controlled room with a 12h light: 12h dark cycle (lights on at 0500h). Rats were acclimated to laboratory conditions with ad libitum access to water and standard lab chow (Purina rat chow #5001, 3.35kcal/g; carbohydrates 56.7% of kcals, protein 29.8% of kcals, fat 13.4% of kcals) for one week prior to diet assignments (detailed below in *Feeding Protocol*). All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

1.1.2 Feeding protocol

Rats were handled daily to obtain body weight measurements and assigned to diet conditions 6 days after arrival [chow or high fat diet (D12451 Research Diets, NJ; 4.73kcal/g; carbohydrates 35% of kcals, protein 20% of kcals, fat 45% of kcals)]. Rats were initially weight-matched between diet groups. Body weight, food intake, and spillage were recorded daily. HFD-fed rats were retrospectively assigned as OP vs. OR via the median split of body weight gain expressed as a percentage compared to baseline when first assigned to the diet.

1.1.3 Experiment 1: 2-week study

On days 12-14 of their assigned diet condition, rats (n=76) were acclimated to 2mL intraperitoneal (i.p.) injections of 0.9% saline in the early afternoon after body weight and daily food intake measurements. On day 15, rats in each diet group were assigned to one of four

experimental injection conditions: i.p saline (chow n=9, HFD n=10), i.p. CCK [1µg/kg (chow n=9, HFD n=9) or 5µg/kg (chow n=9, HFD =9); Bachem)]or no injection (chow n=10, HFD n=11). Rats were anesthetized with Fatal Plus (39mg/mL/kg i.p.; Butler Schein) and perfused with fixative (detailed below) 90 minutes post-injection. Injections were administered between 0930-1100hr. See *Figure 1* for a detailed timeline.

1.1.4 Experiment 2: 7-week study

In addition to daily body weight and food intake recordings, the effect of CCK on 1h dark-onset food intake was assessed in a new group of rats (n=36, 18 per diet group) at three time points over a 7-week period of diet maintenance (i.e., during week 2, week 4, and week 7). Over four days at each of the three time periods, rats were injected i.p. (in randomized order, within-subjects design) with either saline, 1µg/kg or 5µg/kg CCK, or received no injection. Rats were acclimated to 2mL i.p. injections of 0.9% saline following afternoon body weight and food intake measurements for two days prior to each of the three time period assessments. At the beginning of week 8 (day 51), rats within each diet group were assigned to one of three final experimental injection conditions, either i.p. saline, CCK (1µg/kg), or no injection. Injections were administered between 0930-1100hr. Rats were anesthetized with Fatal Plus and perfused with fixative 90 minutes post-injection. As a measure of adiposity, inguinal fat pads were dissected and weighed following the perfusion. See *Figure 5* for a detailed timeline.

1.1.5 Perfusion

Rats in both experiments were sacrificed 90 minutes after i.p. injection, or at a similar time of day for non-injected controls. This time point was selected based on previous evidence that cFos

protein immunolabeling peaks 60–90 min after the onset of neural activation and persists at peak levels for at least 30 additional minutes (Chaudhuri et al., 2000). Anesthetized rats were transcardially perfused with 100 ml saline followed by 350-400 ml of 4% paraformaldehyde (PF; Sigma) containing 1.37% L-lysine (Sigma) and 0.21% sodium metaperiodate (Sigma) (McLean and Nakane, 1974). In Experiment 2, inguinal fat pads were dissected and weighed after fixation. Fixed brains were removed from the skull and stored overnight in 4% PF at 4 °C, then blocked, cryoprotected in 20% sucrose, frozen and sectioned at 35 μm using a sliding microtome. Sections were collected serially in six sets that each contained a complete rostrocaudal series of sections spaced by 210μm. Sections were stored at −20 °C in cryoproservant solution to await immunohistochemical processing (Watson et al., 1986).

1.1.6 Immunohistochemistry

Tissue sections were removed from cryopreservant, rinsed in 0.1M phosphate buffer (PB, pH 7.2), pre-treated in 0.5% sodium borohydride (Sigma) solution for 20 minutes, rinsed in PB, immersed in 0.5% H_2O_2 for 15 minutes, and rinsed again in PB. Primary and secondary antisera were diluted in PB containing 0.3% Triton X (Sigma), 1% donkey serum (Jackson ImmunoResearch), and 1% bovine serum albumin (BSA; Sigma).

Two sets of pre-treated sections from each rat were incubated overnight in primary cFos antiserum (1:20K; rabbit anti-cFos; Calbiochem/EMD) at room temperature. After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch) for 1 h at room temperature, rinsed, then incubated in avidin–biotin complex (Vectastain Elite reagents, Vector Labs) for 1.5h. After rinsing, tissue underwent an H_2O_2-catalyzed reaction in a solution of diaminobenzidine (DAB; Sigma) in 0.1M Na-Acetate buffer

intensified with nickel sulfate (Sigma) to produce a black/dark blue nuclear reaction product. One set of tissue sections from each rat was mounted and coverslipped as described below for quantification of cFos-positive cells in single-labeled tissue. For dual immunolabeling, the second set of cFos-labeled tissue was incubated overnight at room temperature in primary PrRP antiserum (1:10K; rabbit anti-PrRP; Phoenix). After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch) for 1 h at room temperature, rinsed, then incubated in avidin–biotin complex for 1.5h. After rinsing, tissue underwent an H_2O_2-catalyzed reaction in a solution of DAB in 0.1M Tris buffer to produce a brown cytoplasmic PrRP peroxidase reaction product.

After single or double immunolabeling, brain tissue sections were mounted onto adhesion Superfrost Plus Microscope Slides (Brain Research Laboratories), allowed to dry, then dehydrated and defatted in a series of graded ethanols followed by xylene. Slides were coverslipped with Cytoseal 60 mounting medium (Fisher Scientific) and stored at room temperature in covered boxes.

1.1.7 Quantification of feeding-induced neural activation

In double-labeled tissue sections from each rat, PrRP-positive neurons were counted within the caudal nucleus of the solitary tract (cNTS) through the mid level of the area postrema (AP) in 8-12 sections per rat that were spaced by 210μm, using a light microscope and a 20x objective. PrRP neurons were classified as cFos-positive if their nucleus contained visible blue-black immunolabeling, regardless of intensity. The number of PrRP-positive neurons and the proportion (percentage) of PrRP neurons activated to express cFos was determined in each experimental case. The other set of single-labeled tissue sections from each rat was used to

quantify the total number of cFos-positive cells within the cNTS at the same rostro-caudal levels expressed as an average per tissue section.

1.1.8 Data analysis

All of the data are expressed as mean ± SEM. One-way, two-way, and repeated measures analysis of variance (ANOVA) were conducted using SPSS (Version 22, IBM), followed by post-hoc Fisher's LSD tests as appropriate. The α level for significance was set to 0.05.

1.2 RESULTS

1.2.1 Experiment 1

1.2.1.1 Food intake and body weight

The timeline for Experiment 1 is depicted in Figure 1. After one week, HFD-fed rats consumed more calories overall and gained significantly more body weight compared to chow-fed rats ($p<0.05$; *Figure 2a*). The effect of HFD feeding on caloric intake and body weight gain persisted to the end of week 2 ($p<0.05$; *Figure 2b*). When the HFD-fed rats were retrospectively divided into OP vs. OR groups via median split, (i.e., "high body weight gainer" vs. "low body weight gainer"), OP rats consumed significantly more calories than OR and chow-fed rats ($p<0.05$; *Figure 2c*). Further, OP rats had already gained significantly more weight compared to OR rats by 4 days on HFD ($p<0.01$; *Figure 2d*), whereas OR rats gained body weight similar to rats maintained on chow. Thus, the ability of HFD to promote excessive caloric intake and increased body weight gain was observed in only a subset of rats, consistent with previous reports (Paulino et al., 2009; de Lartigue et al., 2012).

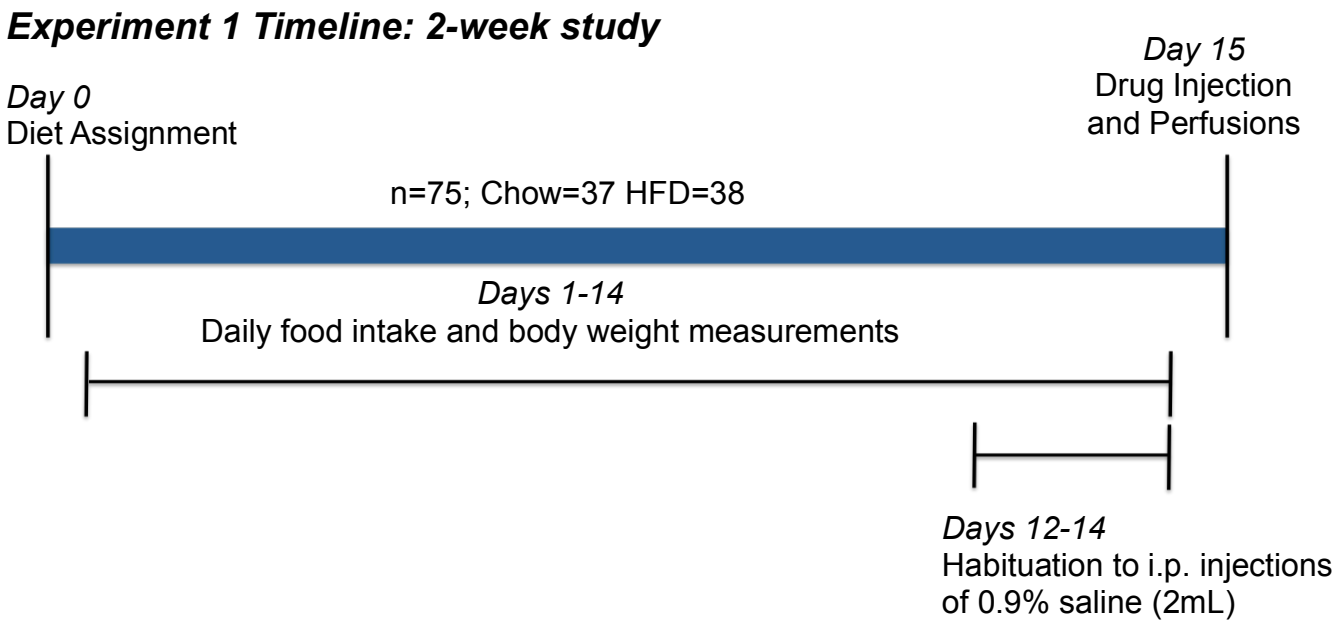
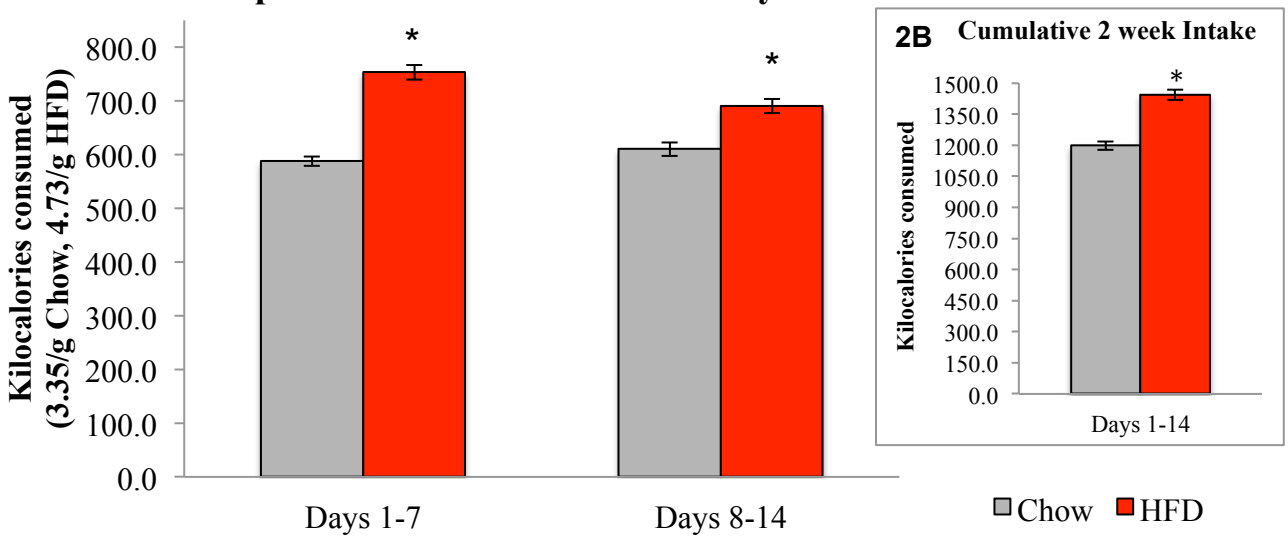


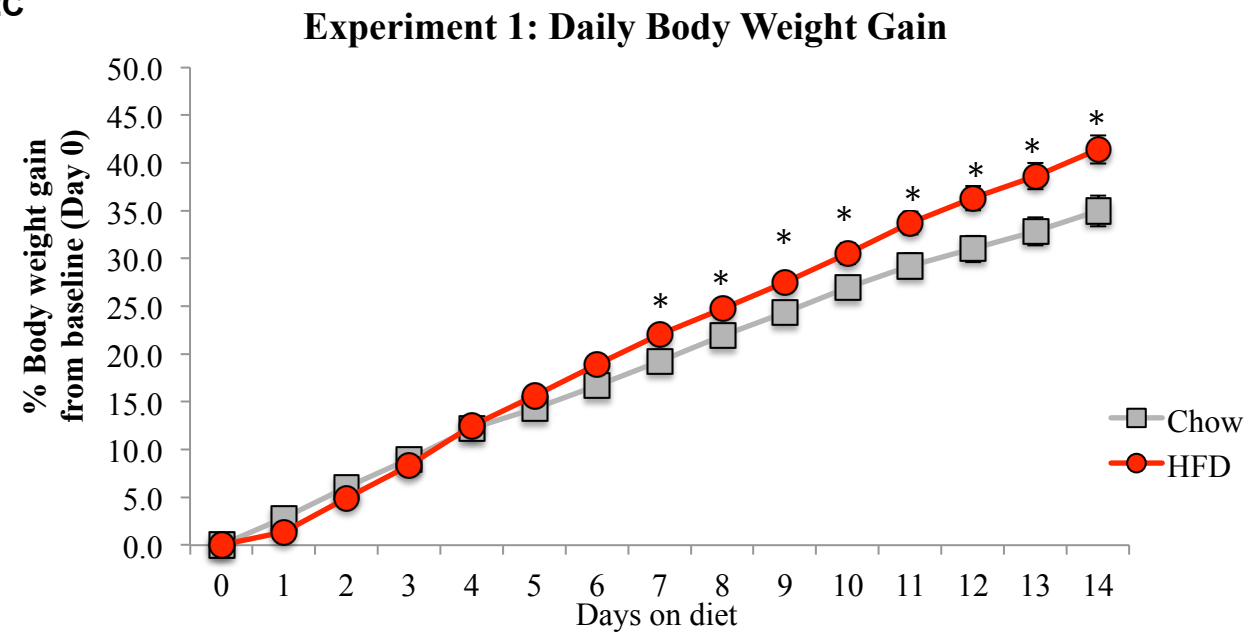
Figure 1. Timeline for Experiment 1

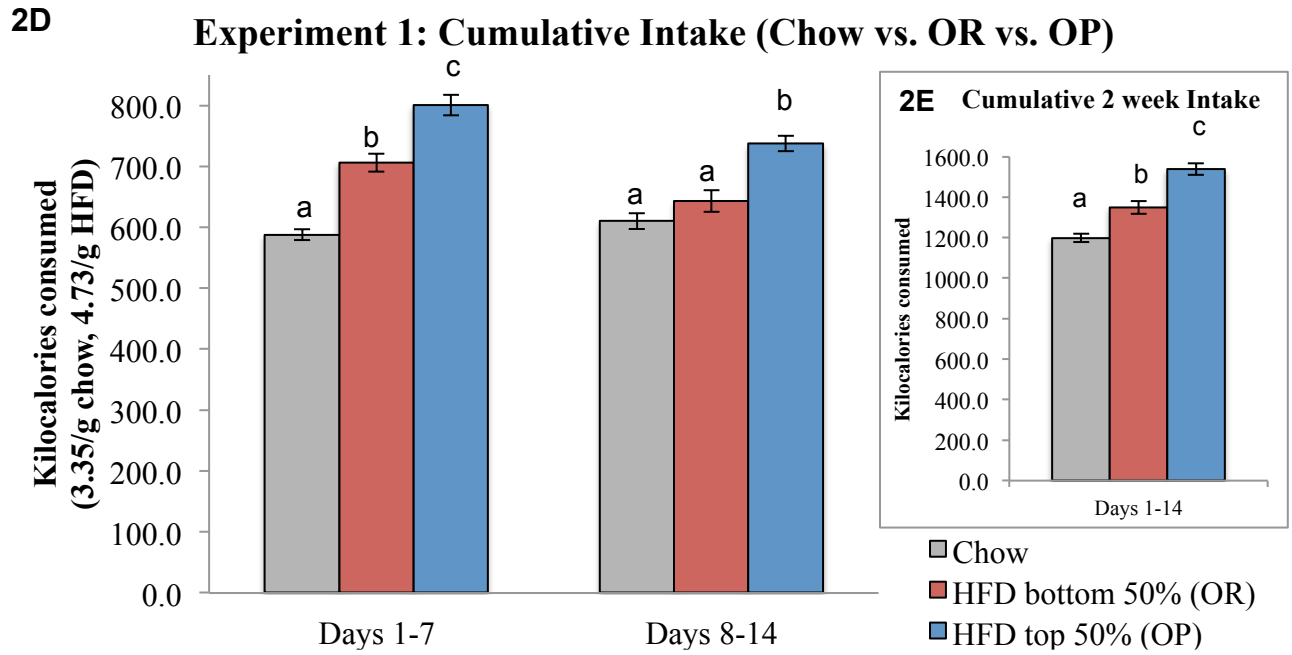
Rats were assigned to either chow or HFD after one week in the animal facility. Food intake and body weight were recorded daily, and on days 12-14 rats were habituated to i.p. injections. On day 15, rats were sacrificed after being assigned to one of four injection conditions: i.p. saline (chow n=5-8 HFD n=7-8), 1µg/kg CCK (chow n=5-6 HFD n=5-7), 5µg/kg CCK (chow n=7 HFD n=6-7), or no injection (chow n=7-9 HFD n=7).

2A


Experiment 1: Cumulative Weekly Intake
Kilocalories consumed (3.35/g Chow, 4.73/g HFD)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
*
*
Days 1-7
Days 8-14
Chow
HFD
2B
Cumulative 2 week Intake
Kilocalories consumed
1500.0
1350.0
1200.0
1050.0
900.0
750.0
600.0
450.0
300.0
150.0
0.0
*
Days 1-14

2C


Experiment 1: Daily Body Weight Gain
% Body weight gain from baseline (Day 0)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days on diet
*
Chow
HFD



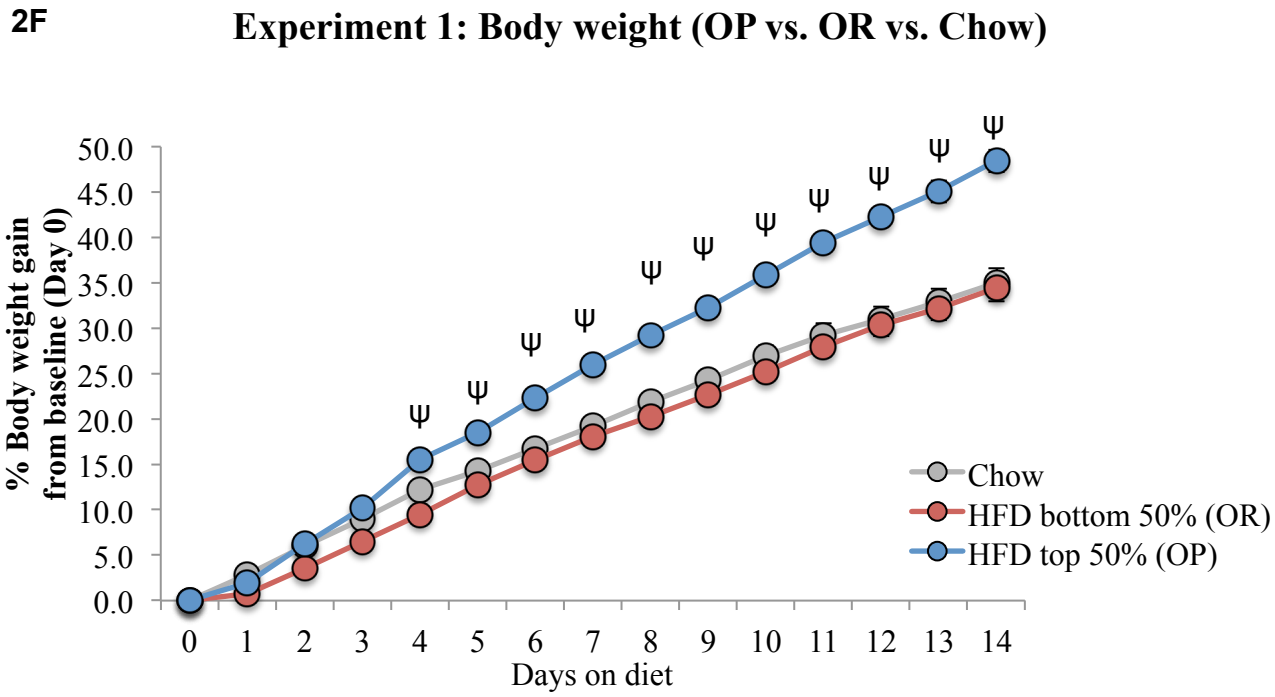


Figure 2. Experiment 1: Food intake and body weight

HFD-fed rats significantly consumed more calories and gained more weight compared to chow-fed rats. (A) HFD-fed rats consumed more calories during the first and second weeks compared

to chow-fed rats. (B) Cumulative 2-week intake ($p<0.001$). (C) HFD-fed rats had gained significantly more weight 7 days after diet assignment. (D) Caloric intake for chow-fed vs. OP (high BW gainer) vs. OR (low BW gainer) rats maintained on HFD. (E) Cumulative 2-week intake. (F) OP rats gained significantly more weight beginning day 4. * Indicates a significant difference ($p<0.05$). Different letters denote significant differences ($p<0.05$) between groups. Ψ Indicates a significant difference compared to chow and OR rats ($p<0.05$). Chow n=37, HFD=38: OR n=19, OP n=19.

1.2.1.2 CCK-induced cFos immunoreactivity

The total number of cFos-positive cNTS neurons was similar in non-injected and saline-injected controls, whereas CCK dose-dependently increased cFos immunolabeling within the cNTS in both chow- and HFD-fed rats (*Figure 3a*). ANOVA revealed a main effect of injection on the number of cFos-positive neurons ($p<0.001$; *Table* 1). However, ANOVA revealed no main effect of diet on cFos activation, even when HFD rats were divided into OP vs. OR groups (*Figure 3b*). Quantification of dual immunolabeled tissue with cFos and PrRP revealed no significant difference in the proportion of PrRP-positive neurons activated after CCK between rats fed chow compared to those fed HFD (*Figure* 4; *Table* 1). CCK did not activate more PrRP neurons than saline. Further, for all doses no difference was observed when HFD-fed rats were divided into OP vs. OR groups (data not shown).

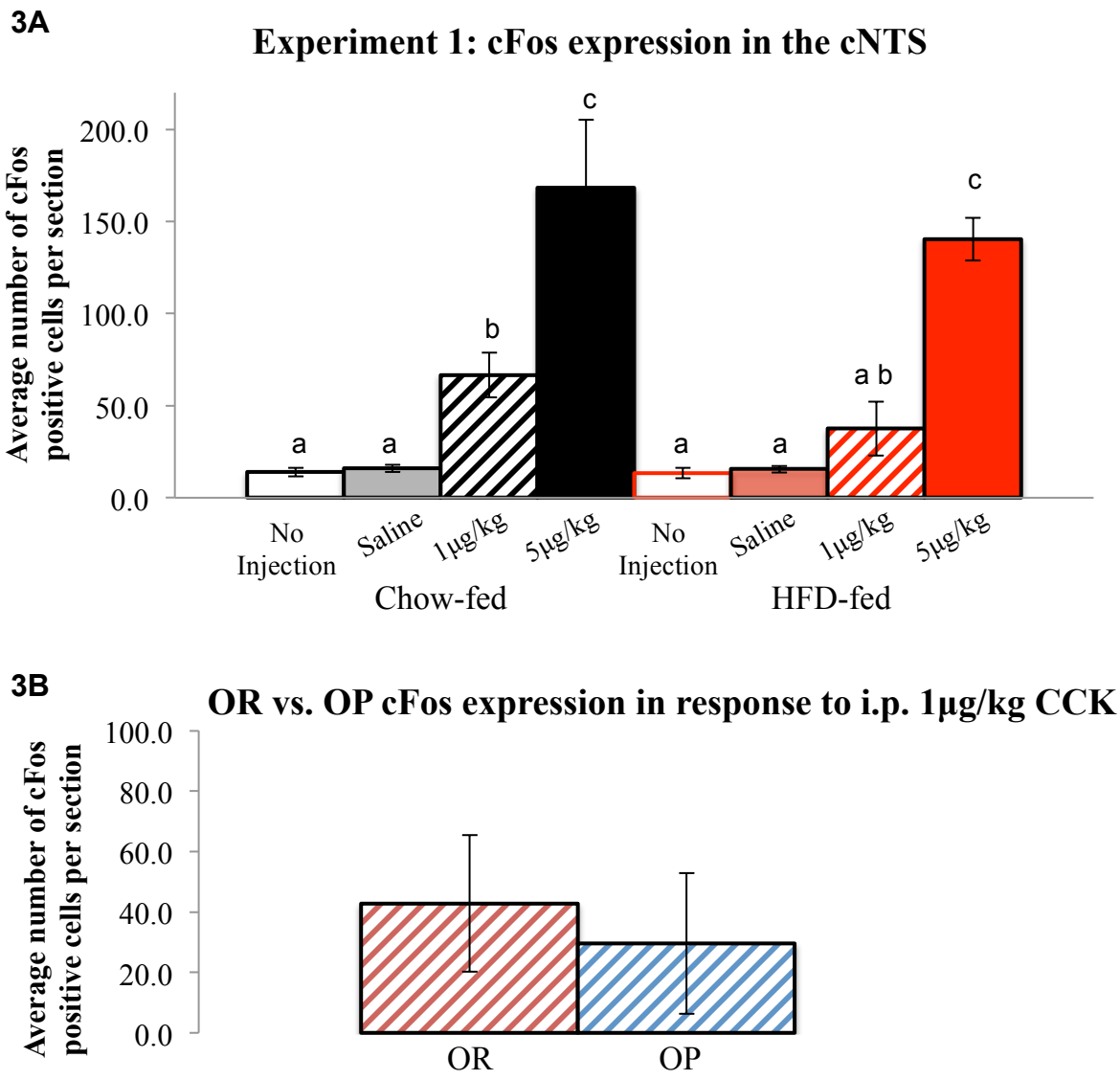


Figure 3. Experiment 1: Total cFos immunoreactivity in the cNTS

CCK-induced cFos in the cNTS (A) CCK dose-dependently increased cFos activation within the cNTS. Data presented as average cFos immunoreactivity per tissue section. A non-significant ($p=0.15$) attenuation in HFD- compared to chow-fed rats was observed for the 1µg/kg CCK dose. Chow *no injection* n=8, *saline* n=6, *1µg/kg* n=5, *5µg/kg* n=7. HFD *no injection* n=7, *saline* n=7, *1µg/kg* n=5, *5µg/kg* n=6. (B) ANOVA revealed no difference between OR (n=3) and OP (n=2) rats. Different letters denote significant differences ($p<0.05$) between groups.

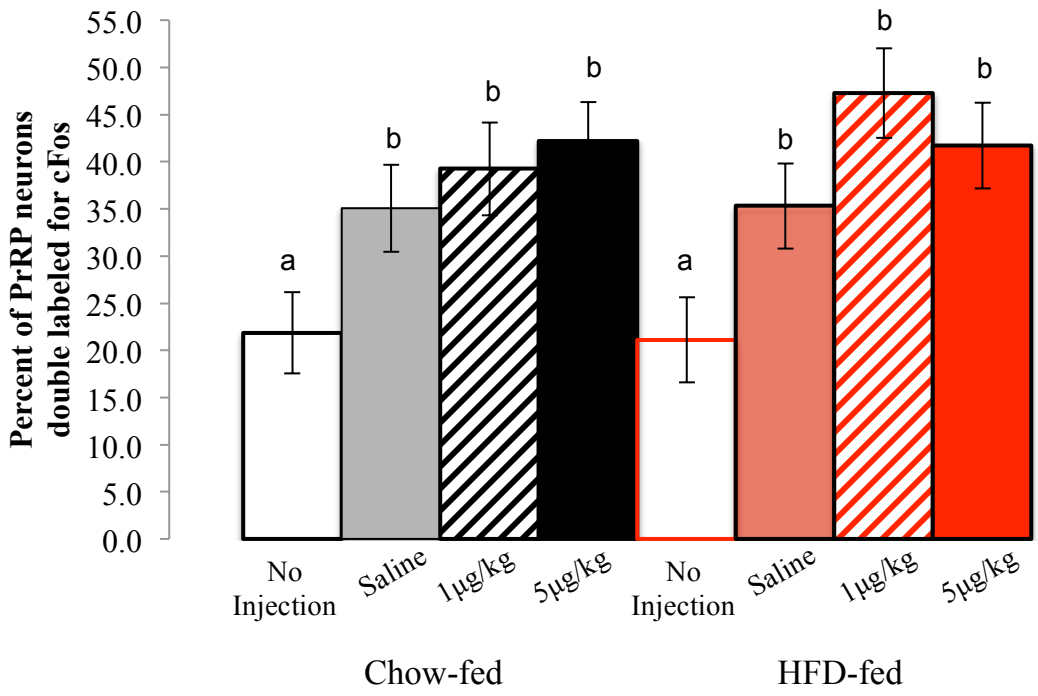


Figure 4. Experiment 1: Dual immunolabeling of cFos and PrRP

Quantification of dual immunolabeled tissue with cFos and PrRP revealed no significant difference between rats fed chow compared to those fed HFD. Different letters denote significant differences ($p<0.05$) between groups. Chow *no injection* n=9, *saline* n=8, *1µg/kg* n=6, *5µg/kg* n=7. HFD *no injection* n=7, *saline* n=8, *1µg/kg* n=7, *5µg/kg* n=7.

Table 1. Experiment 1: CCK-induced cFos immunoreactivity statistics

ANOVA F values and P values for CCK-induced cFos immunoreactivity. * Indicates statistical significance as determined by two-way ANOVA with Fisher's LSD post-hoc tests ($p<0.05$).

Effect source	F value	P value
Experiment 1: Total cFos within the cNTS		
Injection Condition		
Chow vs. HFD *(Sal vs. 1µg/kg CCK vs. 5µg/kg CCK vs. no injection)*	33.67	p <0.001*
Chow vs. OP vs. OR *(Sal vs. 1µg/kg CCK vs. 5µg/kg CCK vs. no inj)*	25.07	p <0.001*
Diet		
(Chow vs. HFD)	1.46	p = 0.23
(Chow vs. OP vs. OR)	0.60	p = 0.55
Interaction		
Chow vs. HFD *(Diet x Injection Condition)*	0.48	p = 0.70
Chow vs. OP vs. OR *(Diet x Injection Condition)*	0.27	p = 0.93
Experiment 1: Dual immunolabeling of cFos and PrRP		
Injection Condition		
Chow vs. HFD *(Sal vs. 1µg/kg CCK vs. 5µg/kg CCK vs. no injection)*	9.57	p <0.001*
Chow vs. OP vs. OR *(Sal vs. 1µg/kg CCK vs. 5µg/kg CCK vs. no inj)*	9.07	p <0.001*
Diet		
(*Chow vs. HFD)*	0.29	p = 0.59
(Chow vs. OP vs. OR)	0.09	p = 0.91
Interaction		
Chow vs. HFD *(Diet x Injection Condition)*	0.38	p = 0.77
Chow vs. OP vs. OR *(Diet x Injection Condition)*	0.34	p = 0.89

1.2.2 Experiment 2

1.2.2.1 Food intake, body weight, and adiposity

The timeline for Experiment 2 is depicted in Figure 5. Similar to results in experiment 1, HFD-fed rats consumed more calories ($p<0.01$; *Figure 6a*) and gained significantly more body weight than chow-fed rats ($p<0.05$; *Figure 6b*) after 7 days and 16 days, respectively. The magnitude of body weight difference continued to increase throughout the 7-week study. HFD- rats consumed significantly more calories throughout the entire experiment, reported as weekly intake over a 7-week period, compared to chow-fed rats (*Figure 6a*). Further, when HFD-fed rats were retrospectively assigned to OP or OR groups, OP rats consumed significantly more calories compared to OR rats (data not shown) and had already gained significantly more body weight than OR and chow-fed rats by day 14 ($p< 0.01$; *Figure 6c*). Inguinal fat pad dissections revealed a greater fat pad mass as a percentage of body weight in HFD- compared to chow-fed rats after seven weeks of diet maintenance (*Figure 6d*). There was a non-significant ($p=0.15$) difference in inguinal fat pad weight between HFD-fed OP vs. OR rats (data not shown).

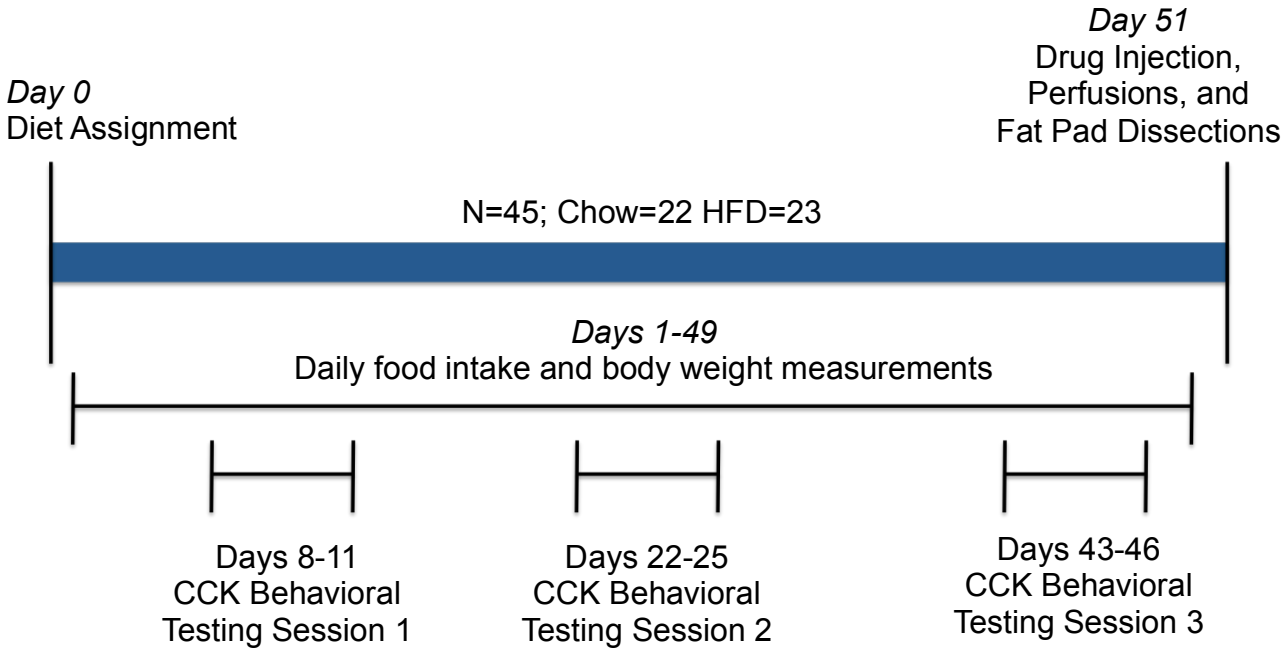
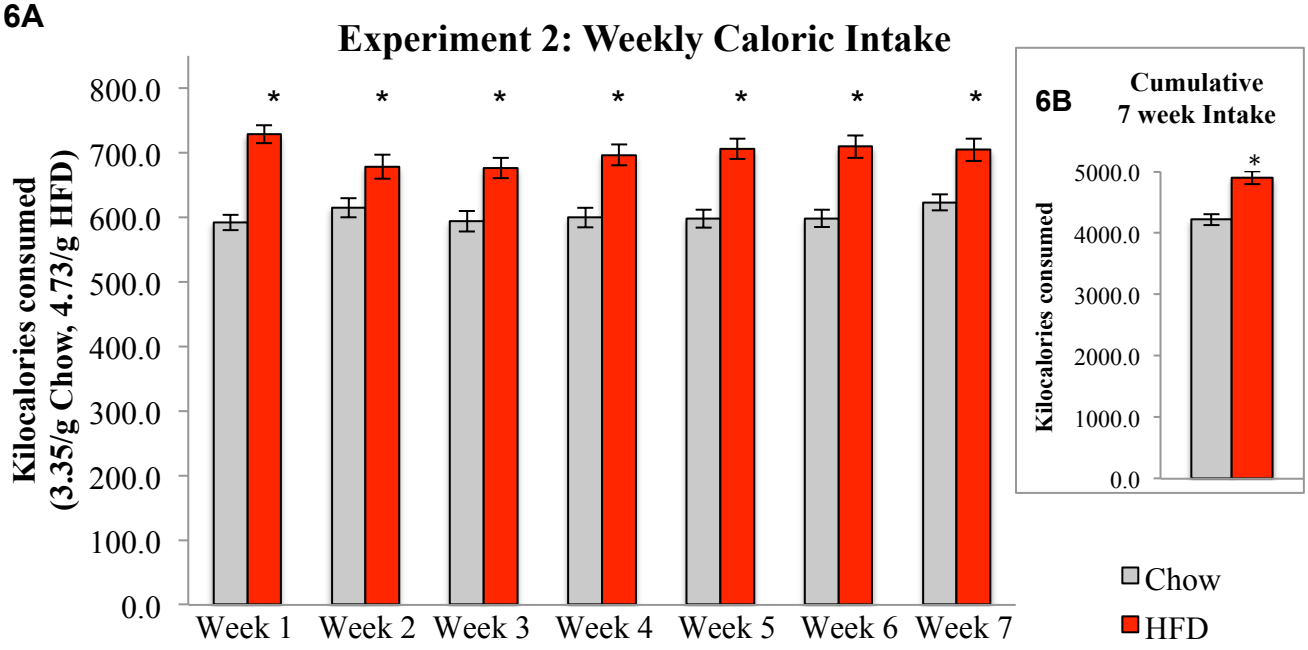


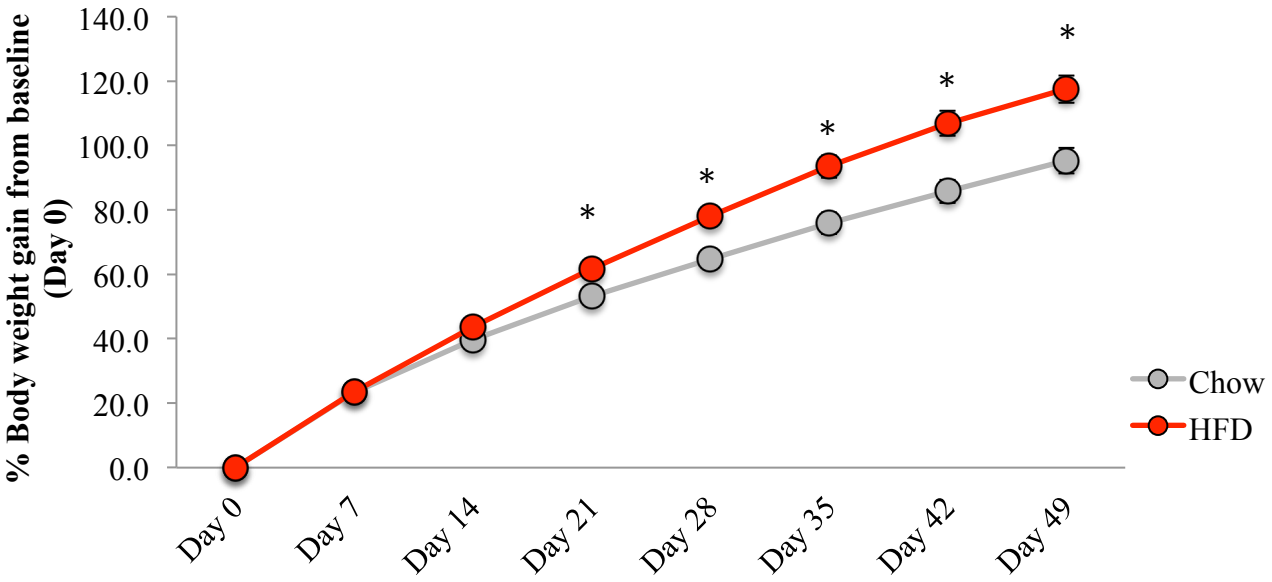
Figure 5. Timeline for Experiment 2

Rats were assigned to either chow or HFD after one week in the laboratory. Food intake and body weight was recorded daily. 2 days before beginning CCK sensitivity testing (on days 6-7, 20-21, and 41-42) rats were habituated to i.p. injections. During the testing week for all sessions, rats received all conditions (saline, 1μg/kg CCK, 5μg/kg CCK, and no injection). On day 51, rats were sacrificed after being assigned to one of three final injection conditions: i.p. saline (chow n=7-8, HFD n=7-8), 1μg/kg CCK (chow n=7, HFD n=5-6), or no injection (chow n=7, HFD n=6). Inguinal fat pads were dissected and weighed following perfusions.

6A


Experiment 2: Weekly Caloric Intake
Kilocalories consumed (3.35/g Chow, 4.73/g HFD)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
6B
Cumulative 7 week Intake
Kilocalories consumed
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
Chow
HFD

6C


Experiment 2: Weekly Body Weight Gain
% Body weight gain from baseline (Day 0)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Chow
HFD

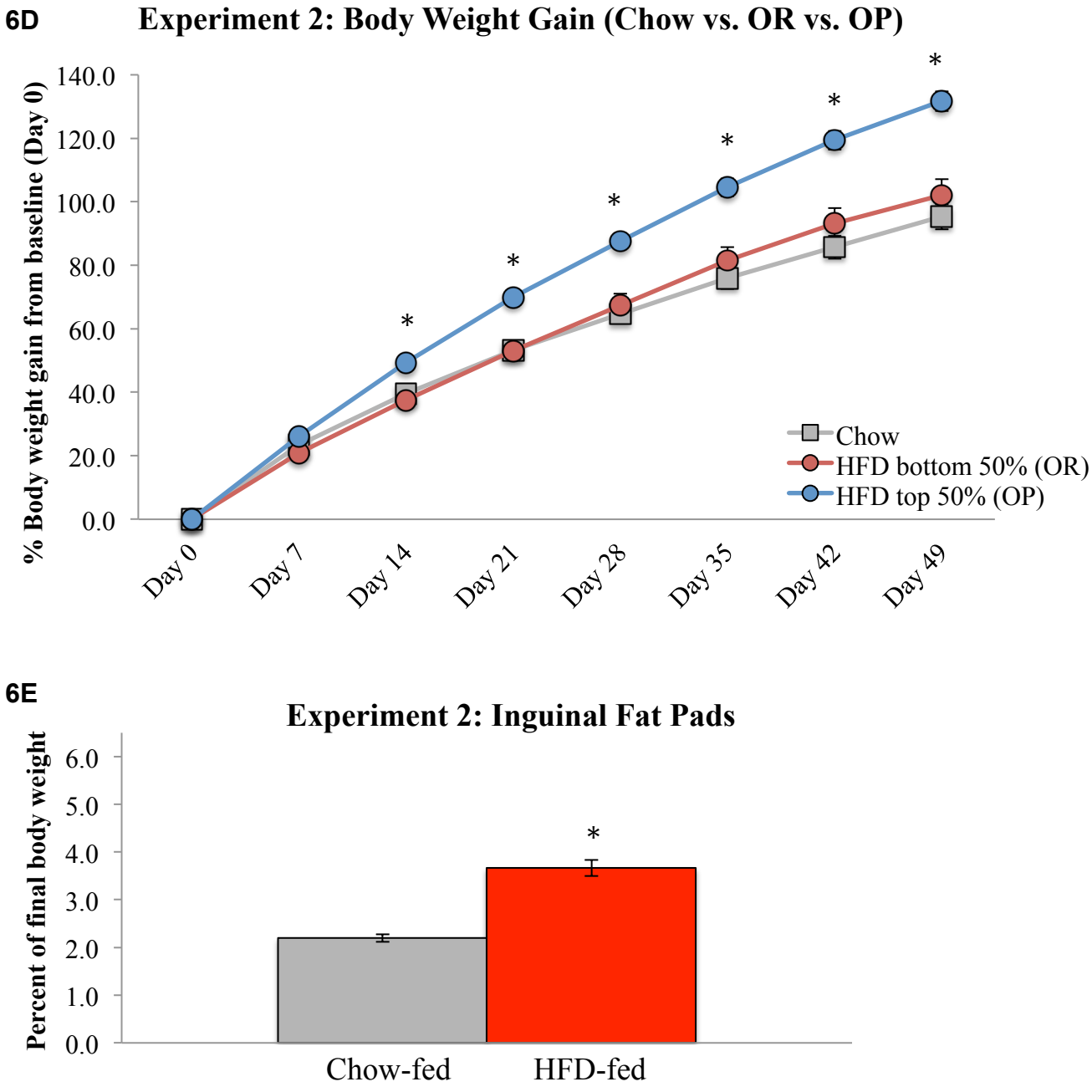


Figure 6. Experiment 2: Food intake and body weight

HFD-fed rats significantly consumed more calories and gained more weight compared to chow-fed rats. (A) HFD-fed rats consumed more calories after week 1. * Indicates a significant difference ($p<0.05$). (B) Cumulative 7-week intake. (C) HFD-fed rats gained significantly more

weight after 16 days. Mean percent body weight gain ± SEM chow 43.5±1.88 vs. HFD 49.3±2.04. (D) OP rats gained significantly more weight than OR as of day 14. (E) Inguinal fat pad weight, as a percentage of final body weight, was significantly greater in HFD- compared to chow-fed rats. * Indicates a significant difference ($p<0.05$). Chow n=22, HFD n=23: OR n=11, OP n=12.

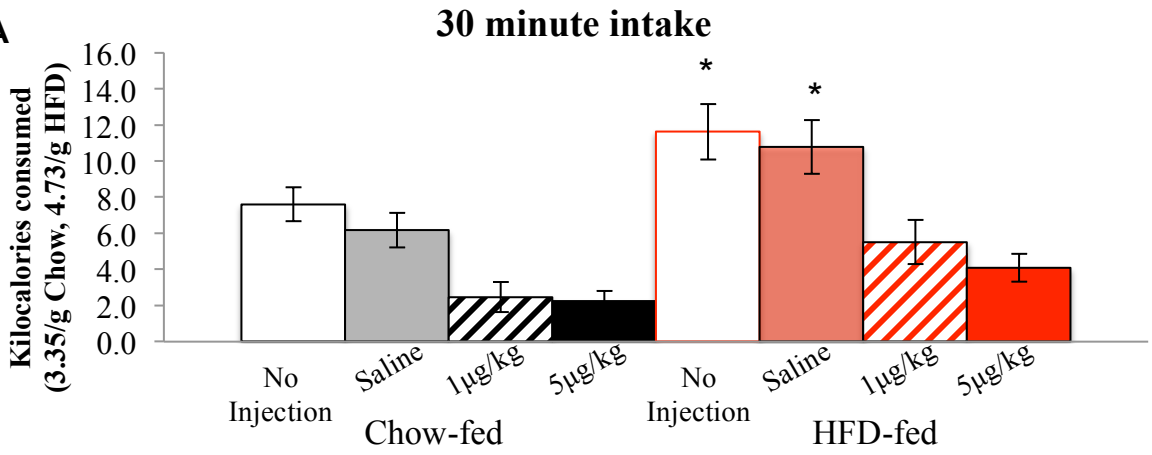
1.2.2.2 Behavioral sensitivity to CCK

30-minute intake is reported for the three behavioral testing sessions (*figures* 7a-c). During session 1 (assessed during the second week of diet exposure), 1μg/kg and 5μg/kg CCK significantly suppressed 30-minute food intake compared to non-injected and saline-injected controls, with no main effect of diet (*Figure* 7d; *Table* 2). A trending, but non-significant difference in intake between chow- and HFD-fed rats was observed after the 1μg/kg ($p=0.08$) and 5μg/kg doses ($p=0.15$). ANOVA revealed no significant difference in intake after any injection condition between OP and OR rats.

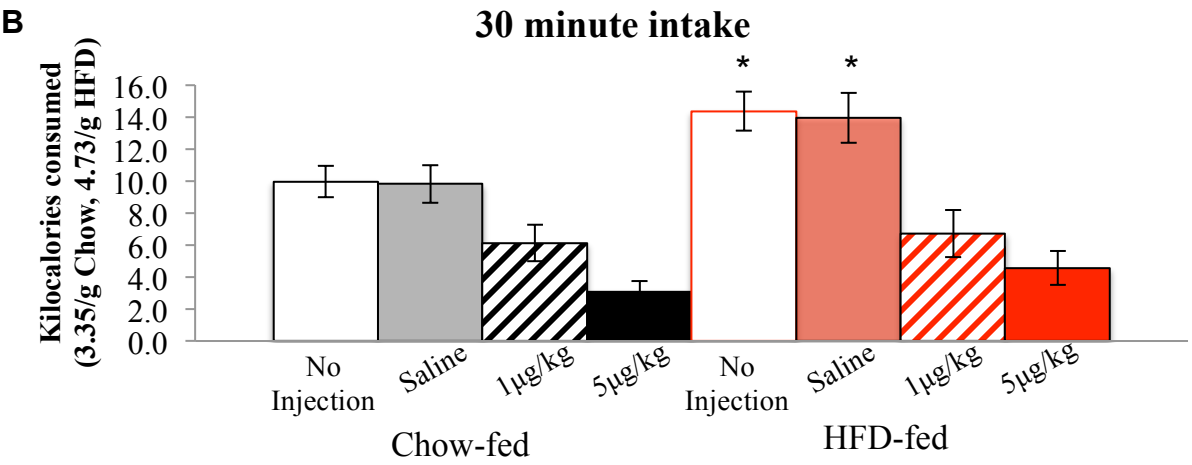
During session 2 (assessed during the fourth week of diet exposure), 1μg/kg and 5μg/kg CCK significantly suppressed 30-minute food intake with no main effect of diet (*figure* 7e; *Table* 2). A trending, but non-significant difference in intake suppression between chow- and HFD-fed rats was observed for the 1μg/kg dose. Surprisingly, a trend towards an attenuation was observed in chow- compared to HFD-fed rats for the effect of 1μg/kg CCK ($p=0.11$; *figure* 7e). When rats fed HFD were divided into higher and lower body weight gainers, the effect of 1μg/kg CCK was significantly attenuated in OP compared to OR rats ($p<0.05$; *figure* 7g*)*.

During testing session 3 (assessed during the seventh week of diet exposure), 1μg/kg and 5μg/kg CCK significantly suppressed 30-minute food intake with no main effect of diet (*figure* 7f; *Table* 2). However, a non-significant attenuation was observed for the 1μg/kg ($p=0.15$) and 5μg/kg ($p=0.18$) doses. Interestingly, when divided into higher and lower gainers, the hypophagic effect of the 1μg/kg CCK dose was significantly attenuated in OP compared to OR rats ($p<0.05$; *figure* 7h).

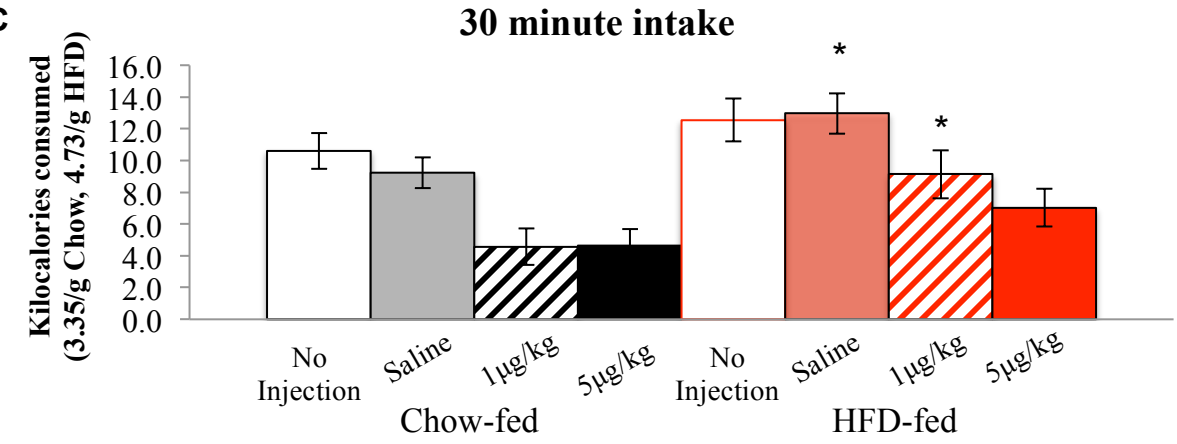
7A


30 minute intake
Kilocalories consumed (3.35/g Chow, 4.73/g HFD)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
*
No Injection
Saline
1µg/kg
5µg/kg
No Injection
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

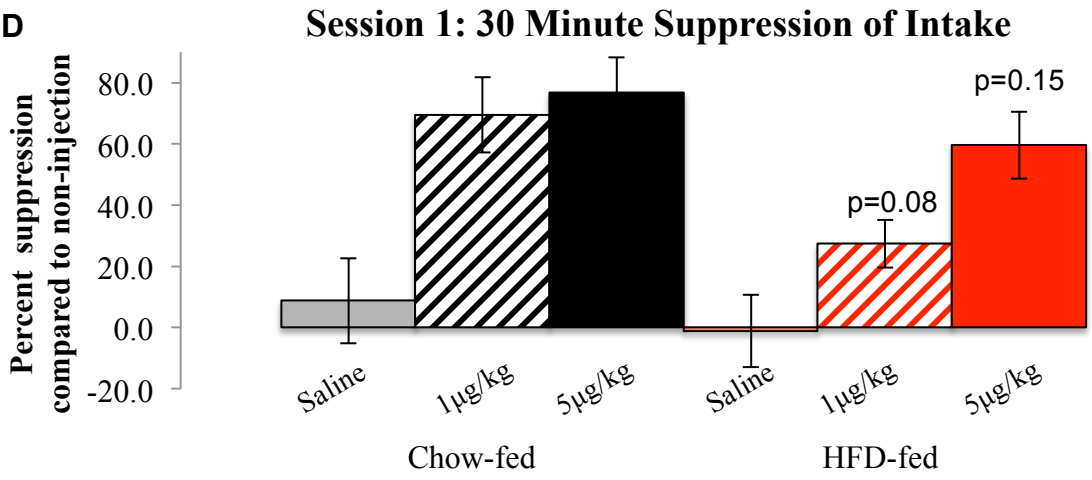
7B


30 minute intake
Kilocalories consumed (3.35/g Chow, 4.73/g HFD)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
*
No Injection
Saline
1µg/kg
5µg/kg
No Injection
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

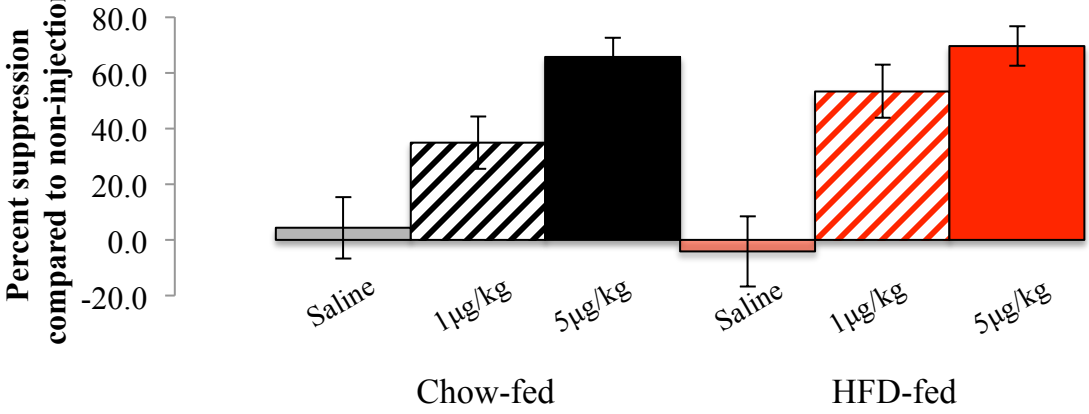
7C


30 minute intake
Kilocalories consumed (3.35/g Chow, 4.73/g HFD)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
*
No Injection
Saline
1µg/kg
5µg/kg
No Injection
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

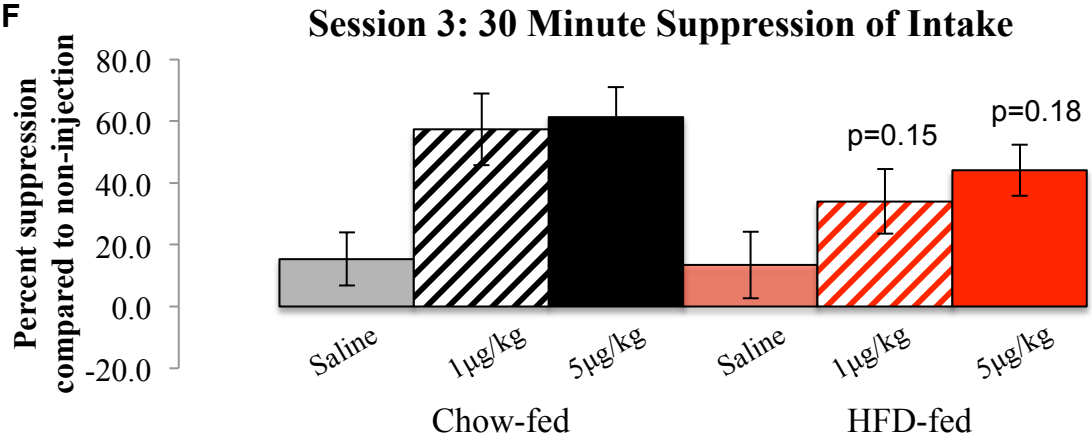
7D


Session 1: 30 Minute Suppression of Intake
Percent suppression compared to non-injection
80.0
60.0
40.0
20.0
0.0
-20.0
p=0.15
p=0.08
Saline
1µg/kg
5µg/kg
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

7E


Session 2: 30 Minute Suppression of Intake
Percent suppression compared to non-injection
80.0
60.0
40.0
20.0
0.0
-20.0
Saline
1µg/kg
5µg/kg
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

7F


Session 3: 30 Minute Suppression of Intake
Percent suppression compared to non-injection
80.0
60.0
40.0
20.0
0.0
-20.0
p=0.15
p=0.18
Saline
1µg/kg
5µg/kg
Saline
1µg/kg
5µg/kg
Chow-fed
HFD-fed

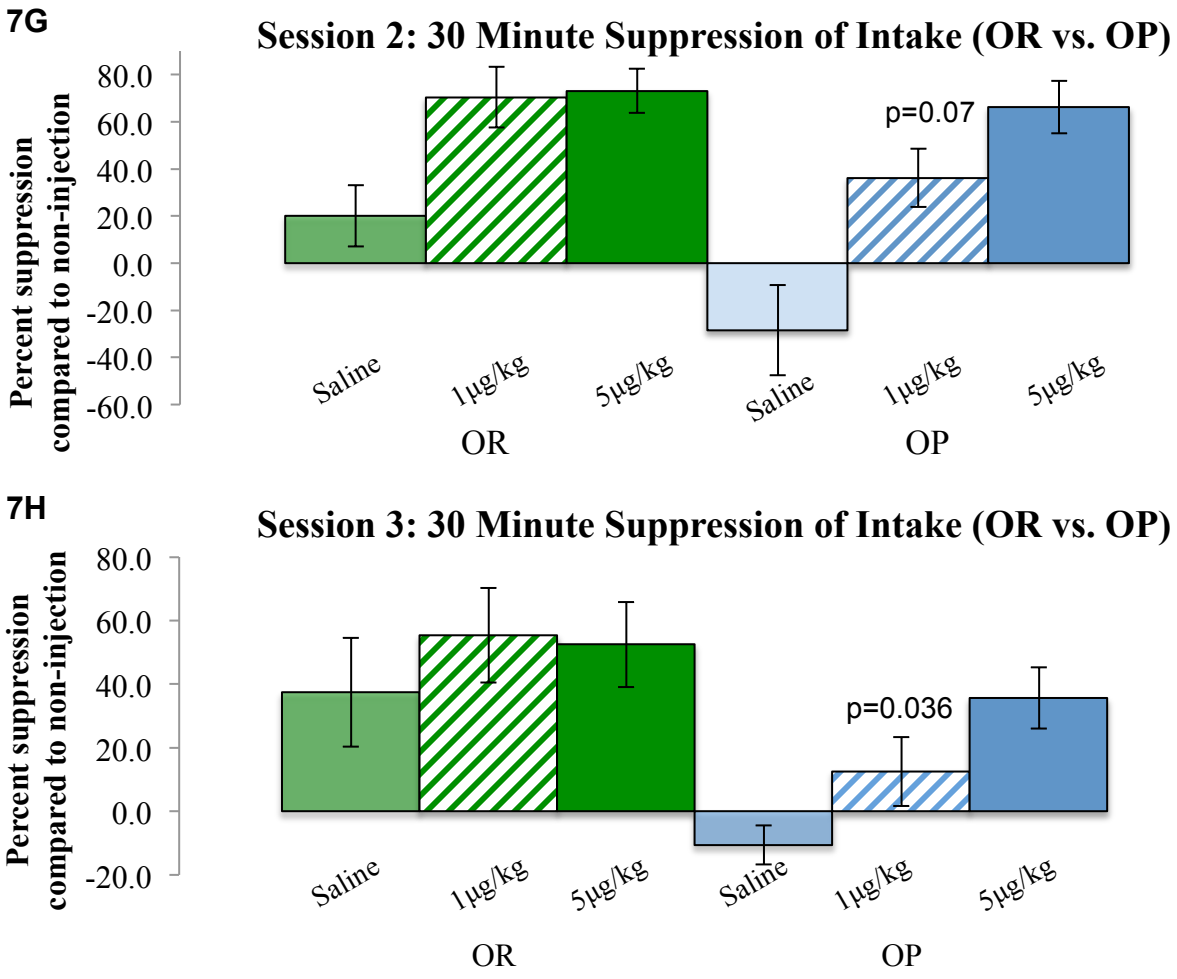


Figure 7. Experiment 2: Behavioral sensitivity to CCK

(A) Session 1 (after 1 week of diet exposure) intake during the first thirty minutes. (B) Session 2 (after 3 weeks of diet exposure) intake during the first thirty minutes. (C) Session 3 (after 6 weeks of diet exposure) intake during the first thirty minutes. (D) Session 1: Percent suppression compared to non-injection. Chow n=18, HFD n =21. (E) Session 2: Percent suppression compared to non-injection. Chow n=19, HFD n=20. (F) Session 3: Percent suppression compared to non-injection. Chow n=15, HFD n =16. (G) Session 2: OR (n=10) vs. OP (n=10) percent suppression compared to non-injection. (H) Session 3: OR (n=8) vs. OP (n=8) percent

suppression compared to non-injection. Rats were omitted if they were outliers (mean +/- 2SD).

* Indicates a significant difference ($p<0.05$) compared to chow for the same condition.

Table 2. Experiment 2: Behavioral sensitivity statistics

ANOVA F values and P values for behavioral sensitivity to CCK. * Indicates statistical significance as determined by one-way ANOVA with Fisher's LSD post-hoc tests ($p<0.05$).

Effect source	F value	P value
Experiment 2: Percent suppression compared to no-injection (Session 1)		
(Chow vs. HFD)		
Saline	0.47	p = 0.50
1μg/kg CCK	3.21	p = 0.08
5μg/kg CCK	2.12	p = 0.15
(Chow vs. OP vs. OR)		
Saline	0.47	p = 0.63
1μg/kg CCK	1.36	p = 0.27
5μg/kg CCK	1.03	p = 0.37
Experiment 2: Percent suppression compared to no-injection (Session 2)		
(Chow vs. HFD)		
Saline	0.44	p = 0.84
1μg/kg CCK	2.71	p = 0.11
5μg/kg CCK	0.34	p = 0.57
(Chow vs. OP vs. OR)		
Saline	2.54	p = 0.09
1μg/kg CCK	3.28	p < 0.05*
5μg/kg CCK	0.28	p = 0.76
Experiment 2: Percent suppression compared to no-injection (Session 3)		
(Chow vs. HFD)		
Saline	0.19	p = 0.89
1μg/kg CCK	2.23	p = 0.15
5μg/kg CCK	1.86	p = 0.18
(Chow vs. OP vs. OR)		
Saline	3.81	p = 0.03*
1μg/kg CCK	3.42	p < 0.05*
5μg/kg CCK	1.38	p = 0.27

1.2.2.3 CCK-induced cFos immunoreactivity

Only the low dose of CCK (1μg/kg) was used for the final experiment comparing cFos expression and PrRP neuronal activation in Experiment 2, given that the largest differences in behavioral sensitivity to CCK between diet groups was evident at this lower dose. Administration of CCK significantly ($p<0.001$) increased cFos expression within the cNTS compared to non-injected and saline-injected control conditions in both chow- and HFD-fed rats. ANOVA revealed no main effect of diet (*figure* 8; *Table* 3).

Quantification of dual immunolabeled tissue with cFos and PrRP revealed a significant difference between chow- and HFD-fed rats (*Figure* 9; *Table* 3). ANOVA revealed a significant diet effect ($p=0.015$), treatment effect ($p<0.001$), and interaction of diet*injection treatment ($p=0.034$). Surprisingly, there was no difference in cFos expression between OR and OP rats after any injection condition. Compared to Experiment 1, the percentage of double-labeled $PrRP^{+}$ neurons is similar in chow-fed rats, but most interesting is the reduced activation observed in HFD-fed rats, suggesting that exposure to the diet is changing the recruitment of this population to promote satiety (*Table* 3).

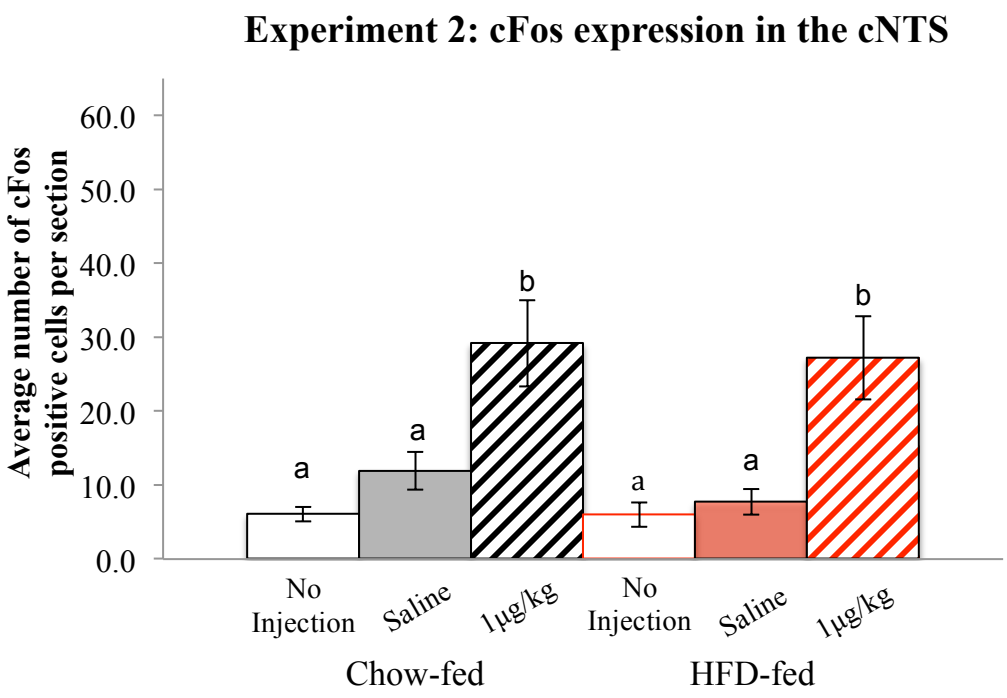


Figure 8. Experiment 2: Total cFos immunoreactivity in the cNTS

Diet does not affect cFos expression in the cNTS. Data presented as average cFos immunoreactivity per tissue section. i.p. *saline* (chow n=8, HFD n=8), *1µg/kg CCK* (chow n=7, HFD n=5), or *no injection* (chow n=7, HFD n=6). ANOVA revealed no main effect of diet. Different letters denote significant differences ($p<0.05$) between groups.

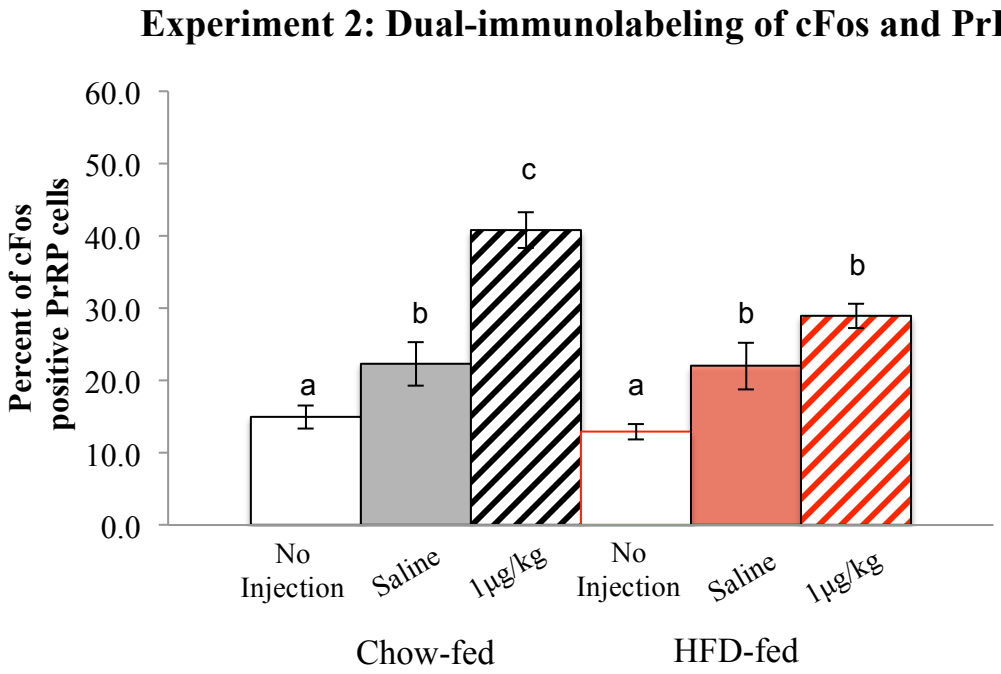


Figure 9. Experiment 2: Dual immunolabeling of cFos and PrRP

Dual-immunolabeling of cFos and PrRP suggests a difference due to diet. i.p. *saline* (chow n=7, HFD n=7), *1µg/kg CCK* (chow n=7, HFD n=6), or *no injection* (chow n=7, HFD n=6). ANOVA revealed a significant diet effect ($p=0.015$), treatment effect ($p<0.001$), and interaction of diet*injection treatment ($p=0.034$). Different letters denote significant differences ($p<0.05$) between groups.

Table 3. Experiment 2: CCK-induced cFos immunoreactivity statistics

ANOVA F values and P values for CCK-induced cFos immunoreactivity. * Indicates statistical significance as determined by two-way ANOVA with Fisher's LSD post-hoc tests ($p<0.05$).

Effect source	F value	P value
Experiment 2: Total cFos within the cNTS		
Injection Condition		
Chow vs. HFD *(Saline vs. 1μg/kg CCK vs. no injection)*	23.15	p <0.001*
Chow vs. OP vs. OR *(Saline vs. 1μg/kg CCK vs. no injection)*	19.62	p <0.001*
Diet		
(Chow vs. HFD)	0.92	p = 0.34
(Chow vs. OP vs. OR)	0.46	p = 0.64
Interaction		
Chow vs. HFD (Diet x Injection Condition)	0.227	p = 0.80
Chow vs. OP vs. OR (Diet x Injection Condition)	0.78	p = 0.55
Experiment 2: Dual immunolabeling of cFos and PrRP		
Injection Condition		
Chow vs. HFD *(Saline vs. 1μg/kg CCK vs. no injection)*	42.13	p <0.001*
Chow vs. OP vs. OR *(Saline vs. 1μg/kg CCK vs. no injection)*	28.67	p <0.001*
Diet		
(Chow vs. HFD)	6.54	p= 0.02*
(Chow vs. OP vs. OR)	3.73	p= 0.04*
Interaction		
Chow vs. HFD (Diet x Injection Condition)	3.73	p =0.03*
Chow vs. OP vs. OR (Diet x Injection Condition)	2.44	p =0.07

1.3 DISCUSSION

In the present study, the difference in body weight gain between chow- and HFD-fed rats was expected, but unlike other studies utilizing outbred Sprague Dawley rats, we observed a significant difference in body weight gain after 7 days. After retrospective assignment of HFD-fed rats to OP or OR groups, the difference in weight gain became evident after just 4 days of diet exposure, much earlier than previously reported (Paulino et al., 2009; de Lartigue et al. 2012). While a significant difference in body weight gain was evident before the end of the second week in Experiment 2, this difference did not reach significance until 16 days of diet maintenance. When HFD-fed rats were assessed retrospectively as OP vs. OR, a significant difference in weight gain was observed at day 14 of diet exposure.

Body weight results in the present study are consistent with previously published reports that maintenance on HFD promotes significant increase of body weight compared to body weight in chow-fed rats (Paulino et al., 2009; de Lartigue et al. 2012). Rats fed HFD consumed significantly more calories than chow-fed rats by the end of the first week in both experiments. Thus, HFD-induced effects on caloric intake precede the onset of obesity by several weeks, suggesting that HFD rapidly alters feedback signals to the brain that control food intake.

CCK is a very well studied gut peptide that contributes to feedback regulation of food intake (Ritter et al., 1994; Moran, 2006; Raybould, 2007; Moran and Dailey, 2011). In Experiment 2, exogenous CCK was used as a tool to assess changes in the ability of CCK to suppress feeding in chow- vs. HFD-fed rats at the beginning of the dark cycle, when rats naturally consume their largest meals. The significantly increased weight gain observed in HFD-fed rats in Experiment 1, after just seven days of diet exposure contributed to the selection of time points to assess behavioral sensitivity to CCK in Experiment 2. During each behavioral

testing session in Experiment 2 (i.e., after 1 week, after 3 weeks, and after 6 weeks of diet exposure), both doses of CCK significantly suppressed 30-minute food intake, with no main effect of diet. During session 1, a trending but non-significant difference between CCK-induced feeding suppression in chow- and HFD-fed rats was observed after both doses, but there was no significant difference between OP and OR rats. In session 2, a trending but non-significant difference between CCK-induced feeding suppression in chow- and HFD-fed rats was observed after the lower dose, but surprisingly unlike session 1, the trending attenuation was observed in rats fed chow and not the HFD-fed rats. When HFD-fed rats were divided into higher (OP) and lower (OR) body weight gainers, the feeding inhibitory effect of 1μg/kg CCK trended an attenuation in OP compared to OR rats. Interestingly, during session 3, the suppressive effect of 1μg/kg CCK on food intake was significantly attenuated in OP compared to OR rats, consistent with previously published data investigating changes in vagal sensory neuron sensitivity as a result of diet manipulation (Paulino et al., 2009; Nefti et al., 2009; de Lartigue et al., 2012; Kentish and Page, 2015; Ueno and Nakazato, 2016).

Another way to assess central neural sensitivity to systemic CCK is by quantifying CCK-induced cFos immunoreactivity. In Experiment 1, i.p. administration of CCK dose-dependently increased cFos activation in the cNTS, but there was no main effect of diet. There also was no significant difference in CCK-induced cFos activation when the HFD-fed rats were divided into OP vs. OR groups. When dual immunolabeled tissue was examined to assess activation of PrRP-positive neurons, there also was no difference between chow- and HFD-fed rats. Thus, despite the significantly increased caloric intake and body weight gain of HFD-fed compared to chow-fed rats after 1 week of diet exposure, there were no apparent differences in CCK-induced neural activation within the cNTS.

Given these results, along with the behavioral data obtained throughout Experiment 2, only one dose of CCK (1μg/kg) was used as one of the final injection treatments to assess diet-related effects on CCK-induced cFos activation in 7-week chow- and HFD-fed animals. CCK (1μg/kg body weight) significantly increased cFos activation in both chow- and HFD-fed rats, but there was no main effect of diet. It is interesting that in both diet groups and for each treatment the average of total cFos counts within the cNTS is approximately half of what was observed in Experiment 1, suggesting that both chow- and HFD-fed rats become less sensitive to i.p. injections after repeated treatments. While the behavioral sensitivity assessments suggest that the 1μg/kg dose continues to suppress intake compared to saline and non-injected conditions, it is possible that the attenuated cFos response is a result of repeated injections in both diet groups. Interestingly, however, the percentage of $PrRP^{+}$ neurons activated to express cFos after CCK treatment is similar in chow-fed rats in Experiments 1 and 2, whereas CCK activated smaller proportions of PrRP+ neurons in HFD-fed rats in Experiment 2 compared to Experiment 1. This supports the hypothesis that CCK loses some its ability to activate PrRP neurons, which may contribute to the blunted effect on food intake observed during the final testing session.

We expected to observe a reduced ability of CCK to activate cFos within the cNTS in HFD-fed rats based on Covasa et al.'s (2000) study. In that study, outbred Sprague Dawley rats were maintained on either a very low fat or a moderately HFD for two weeks before CCK-induced cFos was assessed in response to a very low dose (i.e., 0.25μg/kg). The authors reported that HFD essentially eliminated CCK-induced cFos expression in the NTS, However, it is critical to understand that their control rats were fed an unusually low fat diet (5% kcals from fat), approximately one-third of the fat content found in standard rat chow (13% kcals from fat). During our 2-week diet exposure a non-significant, but trending, difference in CCK-induced

cFos activation between chow- and HFD-fed rats was observed in the response to the 1μg/kg CCK dose. While this dose is larger than the one administered by Covasa et al., it increases plasma levels of CCK to reported peak endogenous levels in rats after meal consumption (Zittel et al., 1999). Incorporating our results, and data obtained from other groups using similar diet manipulation paradigms in rats, it is possible that Covasa et al. (2000) did not find reduced satiety in HFD-fed rats, but rather an increased sensitivity in rats maintained on an isocaloric very low fat diet (Torregrossa and Smith, 2003).

Rats in our study were designated OP or OR retrospectively, with the top 50% body weight gainers designated as OP and the bottom 50% body weight gainers as OR. While a standard method has not been declared by the ingestive behavior field, it is not uncommon to retrospectively assign animals to OP or OR with 50% as the dividing factor (Levin, 1993; de Lartigue et al., 2012). Further, this median split kept the low body weight gainers on HFD (OR) equivalent to the percentage in body weight gain recorded for rats fed chow.

Our experimental model is based on a proposed role for impaired NTS signaling to be the main contributor to HFD-induced hyperphagia and body weight gain (*Figure 10*), but it is important to recognize that the vagus also exhibits changes in response to HFD. Several published studies using rats and mice have focused on diet-induced changes in nodose ganglion vagal sensory neurons, focusing on receptor expression profiles, which include: CCK_1, cannabinoid type 1, glucagon-like peptide 1, ghrelin, peptide YY type 2, melanin-concentrating hormone, leptin, and orexin receptors (Nefti et al., 2009; Paulino et al., 2009; de Lartigue et al. 2012; Ronveaux et al., 2015). It has previously been reported that mice deficient in CCK_1 receptors exhibit a shorter latency to initiate a meal, suggesting an increased drive to eat that is especially pronounced when animals are fed HFD (Donovan et al., 2007). If HFD exposure

results in immediate changes to the receptor profile of physiologically relevant feeding-related peptides at the nodose, such as CCK, leptin, and ghrelin, then chronic HFD maintenance is likely to maintain or further drive changes in receptor expression. Therefore, chronic HFD maintenance not only changes the sensitivity to endogenous satiety signals which alters signaling to the NTS via the vagus, but it also induces changes within the vagus, altogether contributing to impaired NTS signaling.

In conclusion, our data are consistent with previously published data reporting differences in intake and body weight between chow- and HFD-fed rats, but the apparent difference in body weight gain as early as 4 days is novel. Further, behavioral sensitivity to CCK assessments revealed a difference between OP and OR rats after 7 weeks of diet maintenance, and dual-immunolabeling of cFos and PrRP suggest that the A2 NA $PrRP^{+}$ neurons in the cNTS may be involved in the diminished sensitivity. Future studies should further explore the role of A2 NA $PrRP^{+}$ neurons by selectively activating and inactivating the population via Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) as well as by selectively lesioning the population and examining food intake, particularly meal size and manipulations with CCK (Urban and Roth, 2014).

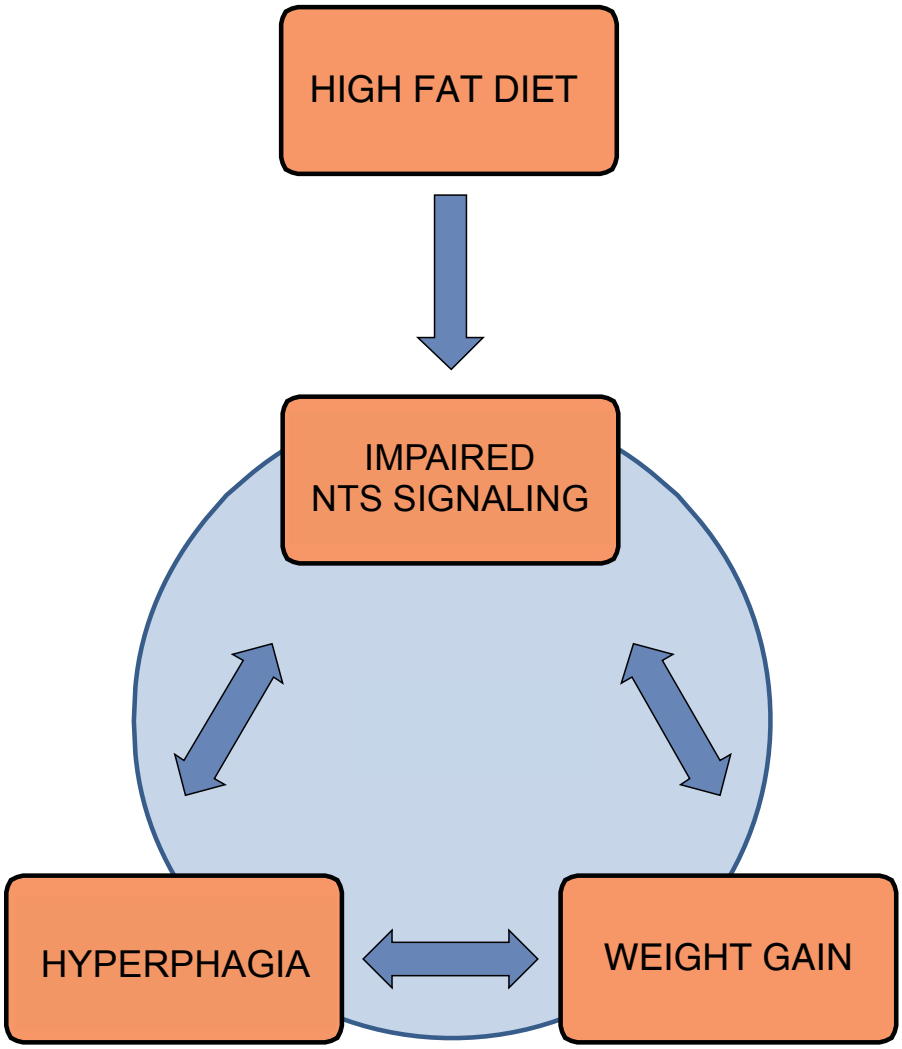


Figure 10. Proposed model of how HFD contributes to hyperphagia and weight gain

Maintenance on HFD changes the signal being relayed via the vagus nerve to the NTS. A reduced signal through the vagus will impair the satiety signaling at the level of the NTS, contributing to increased food consumption and weight gain. Figure is adapted from Kentish and Page (2015).

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