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High Fat Diet Attenuates Cholecystokinin-Induced cFos Activation of Prolactin-Releasing Peptide-Expressing A2 Noradrenergic Neurons in the Caudal Nucleus of the Solitary Tract

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Abstract

Cholecystokinin (CCK) released from the small intestine increases the activity of vagal afferents that relay satiety signals to the caudal nucleus of the solitary tract (cNTS). A caudal subset of A2 noradrenergic neurons within the cNTS that express prolactin-releasing peptide (PrRP) have been proposed to mediate CCK-induced satiety. However, the ability of exogenous CCK to activate cFos expression by PrRP neurons has only been reported in rats and mice after a very high dose (i.e., 50 µg/kg BW) that also activates the hypothalamic-pituitary-adrenal stress axis. The present study examined the ability of a much lower CCK dose (1.0 µg/kg BW, i.p) to activate PrRP-positive neurons in the rat cNTS. We further examined whether maintenance of rats on high fat diet (HFD; 45% kcal from fat) alters CCK-induced activation of PrRP neurons, since HFD blunts the ability of CCK to suppress food intake. Rats maintained on HFD for 7 weeks consumed more kcal and gained more BW compared to rats maintained on Purina chow (13.5% kcal from fat). CCK-treated rats displayed increased numbers of cFos-positive cNTS neurons compared to non-injected and saline-injected controls, with no effect of diet. In chow-fed rats, a significantly larger proportion of PrRP neurons were activated after CCK treatment compared to controls; conversely, CCK did not increase PrRP neuronal activation in HFD-fed rats. Collectively, these results indicate that a relatively low dose of exogenous CCK is sufficient to activate PrRP neurons in chow-fed rats, and that this effect is blunted in rats maintained for several weeks on HFD.

Keywords

rat; satiety; PrRP; CCK

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INTRODUCTION

Caloric intake that exceeds energetic expenditure promotes body weight (BW) gain in both rats and humans. Subjects maintained on a high-fat diet (HFD) for approximately three weeks consumed more calories per meal and per day compared to subjects maintained on a high-carbohydrate diet, evidence that dietary fat promotes increased caloric intake (Warwick, 1996). Satiety is the process by which a meal is terminated, and depends on negative feedback signals delivered to the central nervous system. When laboratory rats maintained on standard chow were able to consume one of two equally palatable and calorically-matched treats *ad libitum* along with chow, rats with access to HF treats for roughly one month consumed more total calories per day and consequently gained more BW than rats consuming fat-free treats; rats administered HFD intragastrically or through normal intake also gained more weight and consumed more kCals during meals of longer duration (Sclafani et al., 1993; Warwick, 1996). Similarly, both lean and obese human subjects consumed more calories when offered a single high-fat vs. high-carbohydrate meal (Warwick, 1996). These findings support the view that dietary fat promotes greater caloric intake by reducing within-meal satiety signaling generated by gastrointestinal (GI) distension and gut hormones that are released during and after food intake. Indeed, intragastric delivery of HFD (bypassing the oral cavity) also promotes greater cumulative daily caloric intake (Warwick, 1996). Thus, HFD may reduce both neural and behavioral sensitivity to endogenous satiety signals in a manner that promotes obesity in susceptible individuals (Paulino et al., 2009).

Among several GI hormones that are implicated in gut-brain signaling and feeding control, cholecystokinin (CCK) is recognized as a key satiety signal that regulates the size of individual meals (Raybould, 2007). CCK is released by enteroendocrine cells of the upper small intestine in response to absorption of dietary nutrients (Raybould, 2007; Moran, 2009). CCK inhibits vagally-mediated gastric emptying and food intake by binding to CCK-1 receptors expressed in the peripheral axons of gastric distension-sensitive vagal afferents whose cell bodies occupy the nodose ganglia, and whose central axons synapse within the caudal nucleus of the solitary tract (cNTS) (Raybould, 2007; Moran, 2009; Grill and Hayes, 2012). The cNTS contains a phenotypically diverse population of neurons that receive and process sensory signals from the GI tract, and relay these signals to a variety of brain regions involved in food intake, energy balance, stress responses, and reward (Rinaman, 2010; Grill and Hayes, 2012). The A2 noradrenergic (NA) cell group within the cNTS has been especially implicated in these diverse functions, including responses to CCK (Rinaman et al., 1993, 1995; Onaka et al., 1995; Rinaman, 2010; Maniscalco and Rinaman, 2013). A2 NA neurons receive excitatory synaptic input from CCK-sensitive vagal afferents (Appleyard et al., 2007), and are activated to express cFos in direct proportion to feeding-induced gastric distension (Rinaman et al., 1998). A2 NA neurons also express cFos in rats after systemic administration of high doses of CCK (i.e., 10–100 µg/kg BW) that robustly suppress food intake while also activating the hypothalamic-pituitary-adrenal (HPA) stress axis (Luckman, 1992; Rinaman et al., 1993, 1995; Myers and Rinaman, 2002; Maniscalco and Rinaman, 2013). Chemically-specific lesions of A2 NA neurons reduce the hypophagic effect of a 10 µg/kg dose of CCK, and also reduce its ability to activate cFos expression

within the paraventricular nucleus of the hypothalamus, the apex of the HPA axis (Rinaman, 2003).

A2 NA neurons are glutamatergic (Stornetta et al., 2002), and the most caudal subset within the cNTS is immunoreactive for prolactin-releasing peptide (PrRP) (Hinuma et al., 1998; Chen et al., 1999; Maruyama et al., 1999; Lawrence et al., 2002). Central administration of synthetic PrRP reduces food intake and meal size (Lawrence et al., 2002; Ellacott et al., 2003; Davis and Grill, 2018), and PrRP-positive A2 neurons are activated to express cFos in mice and rats after food intake (Takayanagi et al., 2008; Kreisler et al., 2014). Further, whole-body knockout mice that lack PrRP expression display hyperphagia, increased BW gain, and a blunted hypophagic response to exogenous CCK compared to wild-type controls (Takayanagi et al., 2008), and these effects are normalized after targeted rescue of PrRP expression in brainstem NA neurons (Dodd et al., 2014). In addition, mice which lack the PrRP receptor gene GPR10 become obese and display diminished responsiveness to exogenous CCK application (Bechtold and Luckman, 2006). Thus, endogenous central PrRP signaling pathways arising from A2 NA neurons within the cNTS may contribute to normal meal-induced satiety, such that reduced or absent PrRP signaling reduces the impact of CCK and other negative feedback signals that normally constrain food intake.

Interestingly, the behavioral and physiological effects of whole-body PrRP knockout are especially pronounced when mice are maintained on HFD (Takayanagi et al., 2008). Covasa and colleagues (2000) reported that a very low dose of CCK (i.e., 0.25 µg/kg BW) failed to increase cFos expression within the cNTS in Sprague-Dawley rats that had been maintained for two weeks on a relatively HFD (34% kcals from fat), whereas this low dose of CCK did increase cNTS cFos counts in rats fed a very low-fat diet (i.e., 4% kcals from fat). It is unclear whether those results reflect HFD-induced suppression or low-fat diet-induced increase in neural sensitivity to CCK; however, they support the view that altered behavioral sensitivity to CCK may occur as a consequence of altered dietary fat (Savastano and Covasa, 2005), perhaps by altering the ability of CCK to activate PrRP neurons within the cNTS. An earlier study reported the effects of only a very high dose of CCK (i.e., 50 µg/kg) to activate PrRP neurons in mice and rats (Lawrence et al., 2002). Given that similarly high CCK doses activate the HPA stress axis in rats (Verbalis et al., 1991; Kamilaris et al., 1992), and given that cNTS PrRP neurons are activated by stressful stimuli (Morales et al., 2000; Maruyama et al., 2001; Zhu and Onaka, 2002; Morales and Sawchenko, 2003; Maniscalco et al., 2015), the present study sought to determine whether a much lower dose of CCK (i.e., 1 µg/kg) that reduces meal size in rats without eliciting a significant stress response (Verbalis et al., 1991; Kamilaris et al., 1992) is sufficient to activate cFos in PrRP neurons. We further examined whether chronic exposure of rats to HFD (45% kcals from fat) blunts the ability of exogenous CCK to recruit PrRP-positive and other cNTS neurons, compared to CCK effects in rats maintained on standard Purina chow.

EXPERIMENTAL PROCEDURES

Subjects

Adult male outbred Sprague-Dawley rats (Charles River, total $n = 38$, 180–210 g upon arrival) were individually housed in a temperature-controlled room with a 12 h light:12 h

dark cycle (lights on at 0500 h). Rats were acclimated to laboratory conditions with *ad libitum* access to water and standard chow (Purina rat chow #5001, 3.35 kcal/g; carbohydrates 56.7% of kcals, protein 29.8% of kcals, fat 13.5% of kcals) for at least one week prior to experimental diet assignment (detailed below in *Feeding Protocol*). All procedures involving rats were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (where the study was initiated) and at Florida State University (where the study was completed), in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Feeding protocol

Rats were assigned to one of two diet conditions [i.e., either standard Purina chow or a more energy-dense HFD that is comparatively higher in fat and lower in carbohydrate and protein (D12451; Research Diets, NJ; 4.73 kcal/g; carbohydrates 35% of kcals, protein 20% of kcals, fat 45% of kcals)]. Rats were initially weight-matched between diet groups, such that average BW and BW range were similar between groups. Over the course of 7 weeks, BW and 24 h food intake (corrected for spillage) were recorded daily for 5 days each week, approximately 2 h before lights out. Rats had continuous *ad libitum* access to water and their assigned diet, except for ~1 h on days that intake and BW data were collected.

CCK-induced cFos activation after seven-week diet maintenance

The experimental timeline is illustrated in Fig. 1. Beginning on day 14 after diet assignment, rats maintained on Purina chow or HFD were habituated to i. p. injection (0.9% saline, 2.0 ml; 9 injections per rat) over the course of three weeks, with injections administered immediately after daily BW and food intake data were collected. On day 50, rats within each diet group were randomly assigned to one of three final treatment conditions: i.p. saline (chow $n = 6$, HFD $n = 8$), 1.0 $\mu\text{g/kg}$ CCK (chow $n = 7$, HFD $n = 6$), or no injection (chow $n = 7$, HFD $n = 6$). Injections were administered between 0930–1100 h; food was removed at the time of injection (and at a similar time in non-injected controls) to prevent potential feeding-related effects on cFos activation during the post-injection interval. Rats were anesthetized with pentobarbital sodium (Fatal Plus; 39 mg/ml/kg, i.p.; Butler Schein) and perfused with fixative (detailed below, "Perfusion") 90 min post-injection, or at a similar time for non-injected controls. This time point was selected based on evidence that nuclear cFos protein immunolabeling peaks 60–90 min after the onset of treatment-induced neural activation (Sagar et al., 1988; Morgan and Curran, 1991; Hoffman et al., 1993). Inguinal fat pads from rats in both diet groups were dissected and weighed post-mortem to provide an index of adiposity after 50 days of diet maintenance.

Perfusion

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) [PLP fixative; (McLean and Nakane, 1974)]. 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 coronally at 35 μm using a sliding microtome. Sections were collected serially in six sets, each containing a complete rostrocaudal series of

sections spaced by 210 μm . Sections were stored at -20°C in cryopreservant solution (Watson et al., 1986) before immunohistochemical processing.

Immunohistochemistry

Tissue sections were removed from cryopreservant, rinsed in 0.1 M phosphate buffer (PB, pH 7.2), pre-treated in 0.5% sodium borohydride (Sigma) solution for 20 min, rinsed in PB, immersed in 0.5% H_2O_2 for 15 min, 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 (Sigma). Two sets of pre-treated sections from each rat were incubated overnight in primary cFos antiserum [either 1:20 K; rabbit anti-cFos; Calbiochem/EMD (catalog #PC05; AB_565444) diluted 1:20 K, or rabbit anti-cFos; Cell Signaling Technology (catalog #2250; AB_2247211) diluted 1:10 K] at room temperature. Pilot studies confirmed that both antibodies generated equivalent cFos labeling in alternate tissue sections from the same rat, and both were used to generate cFos labeling in equivalent numbers of cases from each diet and i.p. injection group. After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch) for 1 h at room temperature, rinsed in PB, then incubated in avidin—biotin complex (Vectastain Elite reagents; Vector Labs) for 1.5 h. After rinsing, tissue underwent an H_2O_2 -catalyzed reaction intensified with nickel sulfate (Sigma) in a solution of diaminobenzidine (DAB; Sigma) in 0.1 M sodium acetate buffer to produce a dark blue-black nuclear reaction product. One set of cFos-labeled tissue sections from each rat was mounted and coverslipped as described below, and used to quantify the total number of cFos-positive cells within the cNTS.

For dual immunolabeling of PrRP neurons, the second set of cFos-labeled tissue sections from each rat was incubated overnight at room temperature in primary rabbit anti-PrRP antiserum [1:10 K; Phoenix (catalog #H-008-52; AB_2728727)]. 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 1.5 h. After rinsing, tissue underwent an H_2O_2 -catalyzed reaction in a solution of plain DAB in 0.1 M Tris buffer to produce a brown cytoplasmic PrRP peroxidase reaction product. After single or double immunolabeling, 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.

In single-labeled tissue sections from each rat, cFos-positive neurons were photographed and counted within the cNTS from the pyramidal decussation through the mid-level of the area postrema (AP) in sections spaced by 210 μm (8–12 sections/rat), using a light microscope (Keyence) equipped with a 20x objective. Labeling was quantified manually in digitized images using ImageJ [Fiji] (Schindelin et al., 2012; Schneider et al., 2012). Total cNTS cFos counts in each rat were then divided by the number of sections from which counts were obtained. The other set of double-labeled tissue sections from each rat was used to quantify the total number of PrRP-positive cNTS neurons, and the proportion that was positive for

cFos. Using ImageJ [Fiji] and a software plug-in that effectively separates blue-black labeling from brown labeling in digitized images (Schindelin et al., 2012; Schneider et al., 2012), PrRP neurons were identified based on brown cytoplasmic labeling and were classified as cFos-positive if their nucleus contained detectable blue-black immunolabeling, regardless of intensity.

Data analysis

Quantitative data are reported as group mean $\pm$ SEM. Two-way ANOVA and linear regressions were conducted using GraphPad Prism 7, with Tukey and Sidak corrections for multiple comparisons. An α level of 0.05 was used to describe effects, interactions, and differences as statistically significant.

RESULTS

Food intake, BW gain, and adiposity

Two-way ANOVA revealed main effects of both diet [$F(1,36) = 27.59$, $P < 0.0001$] and time [$F(6,36) = 3.454$, $P = 0.0028$] on cumulative kcal intake across all 7 weeks, with a significant interaction between the two factors [$F(6,36) = 5.087$, $P < 0.0001$] (Fig. 2A). Post-hoc Sidak's multiple comparisons-corrected tests confirmed that HFD-fed rats consumed more kcals each week compared to their chow-fed counterparts ($P < 0.05$ for each comparison). Regarding the percent of BW gain from diet assignment day 0, two-way ANOVA revealed a significant main effect of weeks maintained on diet [$F(7, 36) = 146.6$, $P < 0.0001$]; corrected post-hoc tests revealed significant differences between diet groups at week 4 after diet assignment and throughout the remainder of the experiment ($P < 0.05$ each week; Fig. 2B). By week 7, HFD-fed rats had increased their BW by $\sim 120\%$, compared to a $\sim 97\%$ increase in chow-fed rats ($P < 0.0001$; Fig. 2B).

Within each diet group, significant between-subjects variability was evident in both the distribution of weekly kcal intake (Fig. 2A) and cumulative BW gain (Fig. 2B), with no obvious stratification of data within either group. For both measures, the magnitude of between-subjects variability evident at the end of the study was similar within each diet group [total BW gain at day 49, chow vs. HFD within-group variability: $F(19,17) = 0.9799$, $P > 0.05$; kcals consumed during week 7, chow vs. HFD within-group variability: $F(19,17) = 0.48$, $P > 0.05$].

After perfusion fixation on day 51 of diet maintenance, HFD-fed rats had significantly heavier inguinal fat pads expressed as a proportion of total BW (3.65% vs. 2.20% for HFD- vs. chow-fed rats, respectively; $P < 0.0001$), evidence for increased adiposity in the HFD-fed group.

CCK-induced cFos expression within the cNTS (total counts)

After 7 wk diet maintenance, two-way ANOVA revealed a main effect of i.p. treatment on the total number of cFos-positive neurons counted within the cNTS [$F(3,33) = 17.98$, $P < 0.0001$], but no main effect of diet, and no interaction. Within both diet groups, rats injected with CCK displayed more cFos-positive cNTS cells compared to counts in non-injected and

saline-injected controls (Fig. 3A). To examine potential within-subjects relationships between kcal intake and CCK-induced neural activation, total cFos counts within the cNTS (Fig. 3A) were correlated with each rat's cumulative kcal intake during the final week before perfusion (i.e., week 7; Fig. 2A). None of these relationships were statistically significant within any of the three final i.p. treatment groups when data were analyzed separately within each diet group, or when data from rats in both diet groups were combined (not shown).

CCK-induced activation of PrRP-positive neurons

Table 1 summarizes cell count data within each maintenance diet and i.p. injection group. These data include the total number of PrRP-positive neurons, double-labeled (i.e., PrRP/cFos-positive) neurons, and cFos-positive neurons, as well as the proportion of cFos-positive neurons that are PrRP-positive (Table 1). As expected, there were no significant interactions [$F(2,33) = 0.17$, $P = 0.85$] and no main effects of either maintenance diet [$F(1,33) = 2.38$, $P = 0.13$] or i.p. treatment [$F(2,33) = 0.30$, $P = 0.49$] on the total number of PrRP-positive neurons counted within the cNTS (Table 1). However, two-way ANOVA revealed significant main effects of both i.p. treatment [$F(2,33) = 9.13$, $P < 0.0001$] and maintenance diet [$F(1,33) = 7.46$, $P = 0.01$] on the proportion of PrRP neurons activated to express cFos, as well as a significant interaction between these factors [$F(2,33) = 4.34$, $P = 0.02$]. Post-hoc tests revealed that a smaller proportion of PrRP neurons expressed cFos in HFD-fed vs. chow-fed rats treated with CCK (Fig. 3B). The ability of CCK to activate PrRP-positive neurons is illustrated in Fig. 4. In both diet groups, a non-significant trend towards larger proportions of PrRP neurons expressing cFos in i.p. saline-injected vs. non-injected control rats was evident (likely due to the acute stress of i.p. injection), but there was no difference between diet groups in PrRP neural activation in either control condition (Fig. 3B). However, compared to activation after no injection or after i.p. saline, CCK treatment significantly increased PrRP neural activation in chow-fed rats, but not in HFD-fed rats (Fig. 3B).

When data from both diet groups were combined, linear regression analysis to assess within-subjects relationships between kcal intake and PrRP neural activation revealed a significant inverse relationship between kcals consumed during week 7 (Fig. 2A) and the ability of CCK treatment to activate PrRP neurons ($R = -0.628$, $P = 0.039$) (Fig. 3C), such that higher caloric intake was associated with less CCK-induced activation of cFos in PrRP neurons. However, this within-subjects relationship did not reach significance when data were analyzed separately for each diet group. There also were no significant relationships between week 7 kcal intake and PrRP neural activation in saline-injected or non-injected control rats (data not shown).

DISCUSSION

In rodents, long term maintenance on HFD reduces the ability of CCK and other satiety signals to suppress food intake, contributing to hyperphagia and increased BW gain (Warwick, 1996; Covasa and Ritter, 1998; Covasa et al., 2000; Savastano and Covasa, 2005). However, the mechanisms through which dietary fat reduces satiety signaling are not fully understood. Evidence suggests that PrRP signaling from hindbrain NA neurons may

contribute to meal-induced satiety, since whole-body deletion of PrRP or its putative receptor (GPR10) increases food intake and BW gain in mice and reduces behavioral sensitivity to high doses of exogenous CCK (Lawrence et al., 2002; Gu et al., 2004; Watanabe et al., 2005; Bechtold and Luckman, 2006; Takayanagi et al., 2008; Onaka et al., 2010). The present study used rats to investigate whether PrRP-positive neurons within the cNTS are sensitive to a relatively low dose of exogenous CCK, and whether maintenance of rats on HFD maintenance reduces that sensitivity. Compared to Purina chow-fed controls, rats maintained on HFD consumed more kcals/ week and gained more BW, consistent with previous reports (Levin et al., 1997; de Lartigue et al., 2012). Our new findings reveal that a relatively low systemic dose of CCK (i.e., 1 μ g/kg BW) is sufficient to activate cFos expression in PrRP-positive cNTS neurons in chow-fed rats, but not in rats maintained on HFD. These novel data support the view that high dietary fat promotes hyperphagia, at least in part, by suppressing the ability of CCK and other endogenous satiety signals to activate PrRP neurons.

Our data are consistent with published evidence that HFD maintenance promotes gradual and persistent increases in BW over several weeks, despite stable weekly kcal intake within each diet group (de Lartigue et al., 2012; Haleem and Mahmood, 2019). In our study, stable HFD-induced hyperphagia and increased BW gain was evident within 2 weeks after diet assignment (Fig. 2), somewhat earlier than in a previous report using the same rat model and maintenance diets (i.e., Purina chow vs. 45% HFD) (de Lartigue et al., 2012). In that study, male Sprague-Dawley rats that gained the most BW after several weeks on HFD displayed reduced behavioral sensitivity to low doses of exogenous CCK, concurrent with HFD-induced leptin resistance in vagal afferent neurons (VANs) within the nodose ganglia. Since leptin signaling increases both behavioral and VAN sensitivity to exogenous CCK (Wang et al., 1997; Emond et al., 1999; Peters et al., 2006a,b; Williams et al., 2009), HFD-induced leptin resistance in VANs (de Lartigue et al., 2012) likely contributes to reduced transmission of satiety signals to postsynaptic neurons within the cNTS. Indeed, the ability of exogenous CCK to activate cFos expression within the cNTS is affected by dietary fat concentration in Sprague-Dawley rats (Covasa et al., 2000). In that study, rats that were maintained on an unusually low-fat control diet (i.e., 5% kcals from fat) displayed a larger hypophagic response and more overall cFos activation within the cNTS after a very low dose of CCK (i.e., 250 ng/kg) compared to rats maintained on a relatively HFD (i.e., 34% kcal from fat). Conversely, in the present study, the effect of a higher CCK dose (1 μ g/kg) on the overall (total) number of cFos-positive cells within the cNTS was similar in rats maintained for 7 weeks on either Purina chow (14% kcal from fat) or HFD (45% kcal from fat). While this result appears to contradict the previously published findings (Covasa et al., 2000), the very low-fat "control" diet used in the Covasa study may have served to *increase* VAN and cNTS neural sensitivity to the very low dose of CCK (250 ng/kg) that was administered. While an overall diet-related difference in the total number of cNTS neurons expressing cFos after CCK treatment was not found in the present study, it is important to note that cNTS neurons activated by exogenous CCK and by food intake are phenotypically heterogeneous (Rinaman et al., 1993; Grill and Hayes, 2009, 2012; Rinaman, 2010; Maniscalco et al., 2013; Kreisler et al., 2014), and PrRP-positive neurons comprise only a small caudal subset of NA neurons within the A2 cell group, which extends through most of

the rostral-caudal extent of the NTS. Thus, while diet-related differences in PrRP neural activation may be functionally relevant to feeding control, differential recruitment of neurons within this small cell group is masked when overall/total cFos responses are quantified within the cNTS.

Ellacott et al. (2002) reported that a high dose of CCK (i.e., 50 µg/kg) that activates the HPA stress axis in rats (Verbalis et al., 1991; Kamilaris et al., 1992) robustly activates the majority (i.e., 84%) of PrRP-positive cNTS neurons in chow-fed rats. Conversely, the lower 1 µg/kg dose of CCK used in the present study activated only ~40% of PrRP neurons in chow-fed rats. PrRP-positive neurons are activated to express cFos in rats not only after consumption of average- to large-sized meals (Kreiser et al., 2014), but also in response to physical and cognitive threats that recruit the HPA stress axis and activate significantly larger proportions of PrRP neurons (Morales et al., 2000; Maruyama et al., 2001; Zhu and Onaka, 2002; Morales and Sawchenko, 2003; Maniscalco et al., 2013, 2015). In this regard, the CCK dose used in the present study is sub-threshold for activating the HPA axis in rats (Kamilaris et al., 1992), and is perhaps a better model for vagally-mediated satiety signaling compared to the stressful effects of higher CCK doses. In our study, a significantly larger proportion of PrRP-positive cNTS neurons was activated to express cFos in chow-fed rats after CCK treatment compared to non-injected or saline-injected controls. Conversely, compared to neural activation in controls, CCK did not activate PrRP neurons in rats maintained on HFD. Thus, the documented effect of HFD to reduce behavioral sensitivity to CCK and other satiety signals (Warwick, 1996; Covasa and Ritter, 1998; Covasa et al., 2000; Savastano and Covasa, 2005) may be explained, at least in part, by a reduced ability of vagally-mediated satiety signals to recruit PrRP-expressing A2 neurons and their central signaling pathways.

In conclusion, results from the present study are consistent with previously published data reporting significant differences in caloric intake and in BW gain between chow- and HFD-fed rats. Our new demonstration that HFD maintenance blunts the ability of CCK to activate PrRP neurons within the cNTS supports the view that reduced sensitivity of PrRP-positive A2 neural activation by CCK and other vagally-mediated satiety signals contributes to increased caloric intake and BW gain in HFD-fed rats. Additional work will be necessary to reveal the central PrRP neural projections and receptor-mediated signaling pathways that underlie these dietary effects on physiology and behavior.

Abbreviations:

AP	area postrema
BW	body weight
CCK	cholecystokinin
cNTS	caudal nucleus of the solitary tract
DAB	diaminobenzidine
GI	gastrointestinal

HFD	high-fat diet
HPA	hypothalamic-pituitaryadrenal
NA	noradrenergic
PB	phosphate buffer
PrRP	prolactin-releasing peptide
VANs	vagal afferent neurons

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Fig. 1.

Experimental timeline. Rats were assigned to either chow or HFD on Day 0, after one week acclimation to the animal housing facility. Each rat's 24 h food intake and body weight (BW) was recorded daily. Rats were habituated to i.p. saline injections between days 14–35. On day 50, rats were anesthetized and perfused with fixative 60–90 min after i.p. injection of saline (chow $n = 6$, HFD $n = 8$), CCK ($1.0 \mu\text{g/kg}$; chow $n = 7$, HFD $n = 4$), or no injection (chow $n = 7$, HFD $n = 6$). Inguinal fat pads were dissected and weighed postmortem.

Fig. 2.

A, HFD-fed rats ($n = 18$) consumed more calories each week than chow-fed rats ($n = 20$). B, HFD-fed rats gained significantly more BW than chow-fed rats; this effect became significant during week 3, and persisted through week 7. Each symbol represents data from one rat, while bars represent the group mean $\pm$ SE. *indicates significant differences ($P < 0.05$) between diet groups.

Fig. 3.

A, Quantification of treatment-induced cFos activation (total counts) within the cNTS in rats injected i.p. with saline (chow $n = 6$, HFD $n = 8$), CCK (1.0 µg/kg; chow $n = 7$, HFD $n = 5$), or no injection (chow $n = 7$, HFD $n = 6$). In both diet groups, CCK activated significantly more cNTS neurons compared to activation in saline- or non-injected controls, with no main effect of diet group on total cFos activation. B, In both chow- and HFD-fed rats, i.p. saline activated more PrRP neurons than were activated in non-injected controls. In chow-fed rats, CCK activated a significantly larger proportion of PrRP-positive A2 neurons than were

activated in either saline- or non-injected controls. However, the ability of CCK to increase PrRP activation (vs. i.p. saline) was absent in HFD-fed rats. In both panels, different letters (a, b, c) denote significant within- and between-diet group differences ($P < 0.05$) between groups. C, When data from CCK-treated rats in both diet groups were combined, an inverse correlation was evident between kcals consumed during week 7 and the ability of CCK to activate PrRP neurons (Pearson's $R = 0.628$; $*P = 0.039$).

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Fig. 4.

Double immunolabeled sections (blue-black nuclear cFos, brown cytoplasmic PrRP) in representative CCK-treated rats. A, Section through the cNTS (just caudal to the area postrema) in a CCK-treated, chow-fed rat. B, Section through the cNTS at a slightly more caudal level in a CCK-treated, HFD-fed rat. In each panel, regions indicated by asterisks are shown at higher magnification in the insets; arrows point out double-labeled (i.e., cFos-positive) PrRP neurons. Quantified data are presented in Fig. 3; the number of PrRP neurons counted in each experimental subgroup are provided in Table 1.

Table 1.

Summary of cNTS cell count data

Maintenance diet	i.p. drug treatment (<i>N</i>)	Total number of PrRP+ neurons (mean $\pm$ SE)	Total number of PrRP+/cFos+ neurons (mean $\pm$ SE)	Total number of cFos+ neurons (mean $\pm$ SE)	Proportion of cFos+ neurons that are PrRP+ (mean $\pm$ SE)
Purina chow	none (7)	167.0 $\pm$ 15.3	25.6 $\pm$ 4.5	86.1 $\pm$ 13.1	26.5% $\pm$ 6.3
	saline (6)	158.8 $\pm$ 14.9	35.0 $\pm$ 9.3	183.0 $\pm$ 30.3	22.3% $\pm$ 4.7
	CCK (7)	176.3 $\pm$ 19.4	74.6 $\pm$ 12.3	404.0 $\pm$ 58.8	19.6% $\pm$ 3.0
HFD	none (6)	177.0 $\pm$ 17.9	22.2 $\pm$ 2.0	60.5 $\pm$ 13.1	32.5% $\pm$ 6.4
	saline (8)	185.6 $\pm$ 11.1	41.5 $\pm$ 7.1	118.7 $\pm$ 28.7	46.1% $\pm$ 8.1
	CCK (5)	203.7 $\pm$ 12.9	59.0 $\pm$ 5.3	397.2 $\pm$ 85.2	20.7% $\pm$ 8.8