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Hyperphagia induced by sucrose: Relation to circulating and CSF glucose and corticosterone and orexigenic peptides in the arcuate nucleus

V.A. Gaysinskaya, O. Karatayev, J. Shuluk, S.F. Leibowitz *

The Rockefeller University, 1230 York Avenue, New York, NY 10065, United States

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ABSTRACT

Sucrose-rich diets compared to starch-rich diets are known to stimulate overeating under chronic conditions. The present study in normal-weight rats established an acute "preload-to-test meal" paradigm for demonstrating sucrose-induced hyperphagia and investigating possible mechanisms that mediate this behavioral phenomenon. In this acute paradigm, the rats were first given a small (15 kcal) sucrose preload (30% sucrose) for 30 min compared to an equicaloric, starch preload (25% starch with 5% sucrose) and then allowed to freely consume a subsequent test meal of lab chow. The sucrose preload, when compared to a starch preload equal in energy density and palatability, consistently increased food intake in the subsequent test meal occurring between 60 and 120 min after the end of the preload. Measurements of hormones, metabolites and hypothalamic peptides immediately preceding this hyperphagia revealed marked differences between the sucrose vs starch groups that could contribute to the increase in food intake. Whereas the sucrose group compared to the starch group immediately after the preload (at 10 min) had elevated levels of glucose in serum and cerebrospinal fluid (CSF) along with reduced expressions of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus (ARC), the subsequent effects (at 30–60 min) just preceding the test meal hyperphagia were the reverse. Along with lower levels of glucose, they included markedly elevated serum and CSF levels of corticosterone and mRNA levels of NPY and AgRP in the ARC. In addition to establishing an animal model for sucrose-induced hyperphagia, these results demonstrate peripheral and central mechanisms that may mediate this behavioral phenomenon.

1. Introduction

Consumption of carbohydrates, largely in the form of added sugar, has increased dramatically over the past 20 years (Harrington, 2008). This greater consumption of sugar-rich foods is believed to be a key contributor to the current epidemic of increased body weight and obesity, by virtue of their low satiety impact (Harrington, 2008; Malik et al., 2006). There is extensive clinical and animal evidence indicating that high-sucrose compared to high-starch diet, when consumed chronically, leads to various physiological abnormalities, including disturbances in glucose homeostasis, insulin insensitivity, and hypertriglyceridemia (Allison et al., 1999; Must et al., 1999). Clinical studies also show high-sucrose diets to produce overeating (Harrington, 2008; Raben et al., 1997). Whereas this effect has yet to be demonstrated in rats chronically consuming high-sucrose diets (Chicco et al., 2003; Lombardo et al., 1983; Toida et al., 1996), studies involving acute dietary manipulations show that they eat larger sucrose-rich meals than starch-rich meals (Glass et al., 1997; Glass et al., 2001) and that rats consuming a high-sucrose compared to high-starch meal may eat a larger subsequent meal that is rich in sucrose or starch, respectively (Glass et al., 1997).

Generally, carbohydrates are thought to exert their effect on ingestion through the post-prandial glucose response or glycemic index (GI). Although the results are mixed, there is some evidence in both human and animal studies showing chronic consumption of high-GI foods, such as those rich in sucrose, to decrease satiety and increase hunger when compared to low-GI products, such as those rich in starch (Aston et al., 2008; Kabir et al., 1998; Lerer-Metzger et al., 1996; Raben, 2002; Roberts, 2000; Scribner et al., 2008; Vermunt et al., 2003). Whereas acute investigations in rats have yet to be performed, there are acute human studies that, while again yielding mixed results on the effect of a high-GI meal on subsequent intake, provide some evidence for an increase in hunger and overeating (Alfenas and Mattes, 2005; Holt et al., 1992; Lavin and Read, 1995). The lack of conclusive evidence regarding the effect of high-GI foods stems from various dietary factors, such as fiber content, caloric density, texture, palatability and duration of access, which vary widely across the experimental paradigms and are known to influence eating behavior (Ramirez and Friedman, 1990; Sclafani, 1989). Despite these confounding factors and variability of results, there are both acute and chronic studies that provide some support for the idea that high-GI foods like sucrose may be less satiating and promote greater caloric intake as compared to low-GI foods rich in starch.

* Corresponding author. Tel.: +1 212 327 8378; fax: +1 212 327 8447.
E-mail address: leibow@rockefeller.edu (S.F. Leibowitz).

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Mechanisms possibly mediating the stimulatory effect of sucrose on food intake may involve specific peptide systems in the hypothalamus, such as neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus (ARC), which potentiate feeding. In contrast to other orexigenic peptides that are more potent in stimulating intake of a fat-rich diet (Leibowitz and Wortley, 2004), both NPY and AgRP when centrally injected are found to preferentially stimulate consumption of carbohydrates, such as sucrose over fat (Badia-Elder et al., 2003; Baird et al., 2006; Lynch et al., 1993; Stanley et al., 1985; Welch et al., 1994; Wirth and Giraudo, 2000). These endogenous peptides, in turn, are highly responsive to changes in glucose metabolism, markedly stimulated under conditions of glucose deficiency, such as before onset of the natural feeding cycle, during food deprivation or after injection of the glucocorticoid, corticosterone (CORT), or of 2-deoxy-D-glucose, which enhances the release of CORT while producing glucoprivation (Akabayashi et al., 1994; Bi et al., 2003; Chee and Colmers, 2008; Leibowitz and Wortley, 2004; Mizuno and Mobbs, 1999; Sahu et al., 1988; Savontaus et al., 2002). This increase in NPY and AgRP is also produced by the acute consumption of sucrose or injection of glucose, although after a significant delay of 1–2 h when glucose levels are declining from their initial peak and CORT levels are rising (Chang et al., 2005; Lynch et al., 1993; Wang et al., 1999). Preceding this peptide stimulation and shortly after glucose injection, the reverse pattern is evident, which includes a decrease in NPY and AgRP expressions in association with high levels of glucose (Chang et al., 2005). These studies suggest a close, biphasic relationship between glucose, CORT and the orexigenic peptides, NPY and AgRP, which is evident after sucrose intake or glucose injection and may be involved in promoting overeating.

Building on this evidence, the present study was designed to elucidate the mechanisms underlying the greater caloric intake induced by consumption of a sucrose-rich diet. The first objective was to establish an acute feeding paradigm that can consistently demonstrate the phenomenon of increased food intake subsequent to the consumption of sucrose. With no studies in rats directly addressing the effect of sucrose compared to starch on subsequent intake, we chose for these experiments a "preload-to-test meal" paradigm, which has been used in studies of hyperphagia induced by a high-fat diet (Gaysinskaya et al., 2007; Warwick, 2003; Warwick et al., 2000) and allows one to compare equicaloric diets, a sucrose preload to a starch preload, in terms of their effects on the size of a subsequent test meal. In contrast to chronic *ad libitum* feeding paradigms, this acute paradigm controls for dietary variables, related to palatability, energy density and caloric intake, and allows the animals to be maintained on lab chow and tested while at normal body weight. After firmly establishing the phenomenon of sucrose-induced hyperphagia with these dietary controls, we then examined physiological and neurochemical changes that may produce this overeating. With measurements of circulating and cerebrospinal fluid (CSF) levels of glucose and CORT together with expressions of NPY and AgRP in the ARC, the results revealed marked changes in these variables that immediately precede the post-sucrose hyperphagia and thus possibly contribute to this behavioral phenomenon.

2. Materials and methods

2.1. Subjects

Adult, male Sprague–Dawley rats (Charles River Breeding Labs, Kingston, NY) were single housed in wire mesh cages, in a fully accredited AAALAC facility (22 °C, with a 12:12-h light–dark cycle with lights off at 2 pm), according to institutionally approved protocols as specified in the NIH Guide to the Use and Care of Animals and also with the approval of the Rockefeller University Animal Care and Use Committee. All animals were given 1 week to acclimate to lab conditions, during which time they were maintained *ad libitum* on laboratory chow and water. The experiments, including the period of adaptation to the feeding paradigm, lasted approximately 6–7 weeks, with the rats weighing 250–270 g at the start of the adaptation, 310–330 g at the start of the testing, and 420–450 g by the end of the experiment. Standard rodent chow (LabDiet Rodent Chow 5001, St. Louis, MO) and water were available *ad libitum*, except for brief periods in the test paradigm when there was no food or only preloads available (see later discussion). The tests were conducted mostly during weekdays in the home cages 3 h before dark onset, with lab chow removed 90 min before the test.

2.2. Diet

The liquid preload diets were prepared using sucrose (table sugar, Domino, Yonkers, and NY) or starch (ICN Nutritional Biochemicals, Cleveland, OH) dissolved in tap water (wt/vol), and they were presented in 50 cc plastic tubes (PETCO Animal Supplies, Inc, San Diego, CA). The preloads, which were equal in caloric density (1.6 kcal/ml), consisted of either 30% sucrose compared to 30% starch (Experiments 1 and 2a) or 30% sucrose compared to 25% starch mixed with 5% sucrose (Experiments 2b and 3–6). While the sucrose dissolved easily in water, the starch needed to be vigorously shaken immediately before use, to minimize any precipitation during the test. The test meal after the preload in Experiments 1–2 consisted of solid rodent chow, which contains 12% fat (3.3 kcal/g).

2.3. Test procedures

Using the diets and procedures outlined in Table 1, six experiments were conducted to test the effects of a sucrose preload in rats (n = 16–40/experiment) on different measures of caloric intake, hormones, metabolites and hypothalamic peptides. For all experiments, the rats were maintained in their home cages and tested around the onset of the dark cycle, just prior to the start of spontaneous feeding. In each

Table 1
Outline of experimental designs.

Experiments		Preloads	Time after preload (min)	Measures	Time measured (min)
1	Group 1	30% sucrose vs 30% starch	0	Chow intake	60,120,180
	Group 2	30% sucrose vs 30% starch	60	Chow intake	120,180
2	Group 1	30% sucrose vs 30% starch	0.60	Chow intake	60,120,180
	Group 2	30% sucrose vs 25% starch (+5% sucrose)	0.60	Chow intake	60,120,180
3		30% sucrose vs	n/a	Serum: glucose,CORT insulin, leptin	5,30,60
		25% starch (+5% sucrose)	n/a		
4		30% sucrose vs	n/a	CSF: glucose, CORT	10,30,60
		25% starch (+5% sucrose)	n/a		
5		30% sucrose vs	n/a	NPY, AgRP mRNA (qRT-PCR)	10,30,60
		25% starch (+5% sucrose)	n/a		
6		30% sucrose vs	n/a	NPY, AgRP mRNA (ISH)	30
		25% starch (+5% sucrose)	n/a		

Abbreviations: CORT, corticosterone; NPY, neuropeptide Y; AgRP, agouti-related peptide; qRT-PCR, real-time quantitative PCR; and ISH, *in situ* hybridization.

experiment, a 3-week period of adaptation to the diets and test paradigm preceded the actual tests. The adaptation period involved 2 weeks of daily exposure before dark onset to a 30-min preload of sucrose or starch in counterbalanced order and then 1 week of daily exposure to these preloads followed 60 min later by a test meal of lab chow at dark onset lasting 60 min. After receiving these daily exposures to the liquid preload diets followed by a test meal, the rats learned to consume approximately 9.3 ml (15 kcal) of the preload within 30 min and at least 1 kcal of the test meal within 60 min. The few rats (<4% of total group) that consumed <13.5 kcal during the preload and/or <1.0 kcal during the test meal were eliminated from the study.

The preload-to-test meal paradigm used in these experiments involved, specifically, a 30-min preload 0.5 h before dark onset, followed by either 0 min or 60 min with no food and the chow test meal, respectively, at dark onset or 1 h into the dark period. To minimize random eating before the start of this paradigm, food was removed 90 min prior to the preload, 2 h before dark onset. The tests, with either a 0-min or 60-min interval of no food between the preload and test meal, were conducted in counterbalanced order over a period of 8 consecutive days, resulting in four tests with both preloads that were subsequently averaged. In Experiments 1 and 2, this preload-to-test meal paradigm was used to measure the impact of a sucrose compared to starch preload on caloric intake during the subsequent test meal. To determine the relative palatability of the sucrose and starch preloads, preference tests were also conducted in which the sucrose and starch diets were given *ad libitum* for 30 min, either separately in randomized order over 2 days (1-bottle test) or together in the same test with their positions in the cage alternated over the 2 test days (2-bottle test). In Experiments 3–6, the same procedures were used, except that rather than providing the chow test meal, the rats were sacrificed and their blood, CSF or brain collected to measure levels of hormones, metabolites, or NPY and AgRP mRNA in the ARC using real-time quantitative PCR (qRT-PCR) or radiolabeled *in situ* hydridization (ISH). The specific procedures and rationale for each experiment, outlined in Table 1, were as follows.

In *Experiment* 1, the effect of a liquid preload diet on the intake of a subsequent chow test meal was examined in a set of rats (N = 40). After being adapted to the diets and experimental paradigm, rats were divided into two groups (n = 20/group). Each group was given 8 tests with the 15-kcal sucrose (30%) or starch (30%) preloads, which were equal in caloric density. These preloads were administered in counterbalanced order and followed by either 0 min (Group 1) or 60 min (Group 2) with no food and then the chow test meal. In Group 1, with chow made available immediately after the preload, chow intake measurements were taken at the end of the 60-, 120- and 180-min intervals, whereas in Group 2 with no food for 60 min after the preload, chow intake was measured at the 120- and 180-min intervals. After these behavioral tests involving the preload-to-test meal paradigm, 1-bottle and 2-bottle palatability tests were carried out in a manner described above.

In *Experiment* 2, two groups of rats (n = 20/group) were tested using the same preload-to-test meal paradigm and preference tests as in Experiment 1. In Group 1, the rats were examined with the 15-kcal sucrose (30%) vs starch (30%) preloads, to confirm the findings of Experiment 1. They were first tested with the experimental paradigm that made chow available immediately after the preload and then with the paradigm that delayed chow presentation until 60 min after the preload. Since the results of Experiment 1 showed these preloads to be different in palatability, we further tested in Group 2 a 30% sucrose preload compared to a different starch preload, an equicaloric preload with 25% starch and 5% sucrose added to increase its palatability. After conducting 1-bottle and 2-bottle preference tests (see Experiment 1) that showed these two diets to be equal in palatability, the effect of these preloads on chow intake during the test meal was measured.

In *Experiment* 3, tests were conducted to examine possible physiological processes, involving changes in blood glucose levels, which precede and may contribute to the hyperphagia induced by a sucrose preload compared to an equally palatable starch preload. Blood was collected from rats (N = 20) via tail vein puncture at 5, 10, 30 and 60 min after the 15-kcal preload of 30% sucrose (n = 10) or 25% starch with 5% sucrose (n = 10), and serum was assayed for levels of glucose. After these tail vein collections, the rats were given one final test with the preloads and then sacrificed after 5, 30 or 60 min to collect trunk blood for measurements of CORT, in addition to leptin and insulin.

In *Experiment* 4, levels of glucose and CORT after the 30% sucrose compared to 25% starch preload (with 5% sucrose) were measured in the brain. Every other day, CSF was collected via a cisterna magna cannula (see later discussion) in rats (n = 7/diet/time period) at 10, 30 and 60 min after the 30-min preload.

In *Experiment* 5, tests were conducted to measure changes in NPY and AgRP mRNA levels in the ARC that precede the hyperphagia induced by a sucrose preload. Rats (n = 8/group) were first trained to consume 15 kcal of 30% sucrose or 25% starch preload and were then sacrificed by rapid decapitation at 10, 30 and 60 min after the preload. Rat brains were rapidly removed and dissected for analysis of NPY and AgRP gene expression in the ARC using qRT-PCR.

In *Experiment* 6, rats (n = 8/group) were sacrificed by rapid decapitation at 30 min after consuming the preload of 30% sucrose or 25% starch. Whole brains were removed and placed in a 4% paraformaldehyde solution for measurements of NPY and AgRP mRNA using radiolabeled ISH.

2.4. Blood sampling procedures

In Experiment 3, blood was collected during testing using a tail vein puncture technique, as previously described (Gaysinskaya et al., 2007). The collections were conducted in a cross-over design, with two tail vein procedures performed one day apart. After all tests were performed, the rats were sacrificed 30 min after consuming the preloads and trunk blood collected for further analysis.

2.5. CSF sampling procedure

In rats anesthetized with ketamine/xylazine (10 mg/80 mg) a stainless steel cannula was stereotaxically implanted in the cisterna magnum, according to methods described elsewhere (Consiglio and Lucion, 2000). Rats were allowed to recover for one week after surgery, and then were adapted to the preloads before the CSF collections were initiated.

2.6. Hormone and metabolite determinations

Serum from tail vein or trunk blood was assayed for insulin (Catalog #RI-13K) and leptin (Catalog #RL-83K) using commercially available RIA kits (Linco Research Inc, MO). Serum and CSF levels of CORT were measured using RIA kit (Catalog #0712002, MP Biomedicals, NY) and of glucose were measured with an E-Max Microplate Reader using a Glucose liquid reagent (G520-480**,** TECO Diagnostic, CA).

2.7. Brain dissection

Immediately after sacrifice, the brain was placed in a matrix slicing guide with the ventral surface facing up, and three 1.0 mm coronal sections were made, with the middle optic chiasm as the anterior boundary. The second section was placed on a glass slide, and the ARC (Bregma −2.6 to −3.3 mm), the area adjacent to the bottom of the third ventricle, was dissected parallel to the border of the ventricle, with the width of 0.1 mm at the top gradually widening to 0.2 mm at the bottom (Chang et al., 2004).

2.8. Real-time quantitative PCR analysis

As previously described (Chang et al., 2004), total RNA from pooled microdissected samples was extracted with TRIzol reagent.

RNA was treated with RNase-free DNase I before RT. For qRT-PCR, cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II Reverse Transcriptase. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA) was used, with cyclophilin as an endogenous control. qRT-PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems). This was done on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems), under the condition of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each study consisted of 4 independent runs of qRT-PCR in triplicate, and each run included a standard curve, non-template control, and negative RT control. The levels of target gene expression were quantified relative to the level of cyclophilin, using the standard curve method. The primers, designed with ABI Primer Express V.1.5a software from published sequences, were: (1) cyclophilin: 5′-GTGTTCTTCGACATCACGGCT-3' (forward) and 5′-CTGTCTTTGGAACTTTGTCTGCA-3′ (reverse); (2) NPY: 5′-CACAGAAAATGCCCCCAGAA-3′(forward) and 5′-GTCAGGAGAG-CAAGTTTCATTTCC-3′ (reverse); and (3) AgRP: 5′-GCAGAGGTGCTAGATC-CACAGAA-3′ (forward) and 5′-x AGGACTCGTGCAGCCTTACAC-3′ (reverse). The concentrations of primers were 100 nM. All reagents, unless indicated, were from Invitrogen (Carlsbad, CA).

2.9. Radiolabeled in situ hybridization histochemistry

Besides qRT-PCR, mRNA levels of NPY and AgRP were measured using radiolabeled ISH, which allows for more anatomically precise quantification of changes in gene expression than qRT-PCR. Antisense and sense RNA probes were labeled with ^{35}S-UTP (Amersham Biosciences, Piscataway, NJ), as previously described (Chang et al., 2008). Alternate free-floating coronal sections were consecutively processed as follows: 10 min in 0.001% proteinase K, 5 min in 4% paraformaldehyde, and 10 min each in 0.2 N HCl and acetylation solution, with a 10-min wash in PB between each step. After the wash, the sections were hybridized with a ^{35}S-labeled probe (10^3 cpm/ml) at 55 °C for 18 h. Following hybridization, the sections were washed in 5× sodium chloride and sodium citrate (SSC), and the nonspecifically bound probe was removed by RNase (Sigma) treatment for 30 min at 37 °C. Sections were then run through further stringency washes with 0.1 M dithiothreitol (Sigma-Aldrich, St, Louis, MO) in 2× SSC and 1× SSC and 0.1× SSC at 55 °C. Sections were finally mounted, air-dried, and exposed to a Kodak BioMax MR film for 18 to 24 h at −80 °C, when films were developed and microscopically analyzed. The sense probe control was performed in the same tissue, and no signal was found.

Computer-assisted microdensitometry of autoradiographic images was determined, as described (Reagan et al., 2004), on the MCID image analysis system (Image Research Inc., St. Catherines, ON, Canada). Microscale ^{14}C standards (Amersham Biosciences) were exposed on the same Kodak film with the sections and digitized. Gray-level/optical density calibrations were performed with a calibrated film-strip ladder (Imaging Research Inc.) for optical density. This was plotted as a function of microscale calibration values. All subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. The values obtained represent the average of measurements taken from 10 sections per animal. Within each section, the optical density for the nucleus was recorded, from which the background optical density from a same-size area in the corpus callosum was subtracted. The mean value of the sucrose-drinking group in each experiment is reported as a percentage of the starch-drinking control group.

2.10. Data analysis

The data in the figures and tables, for food intake, diet palatability, circulating and CSF glucose, hormones and peptides, are expressed as mean ± SEM. Statistical analyses of these data were performed using a 2-way repeated measures ANOVA followed by Tukey's HSD post-hoc tests for multiple comparisons between groups (Experiments 1–4), a 2-way ANOVA with Tukey's HSD post-hoc analysis (Experiment 5), or a paired *t*-test to compare the intake of sucrose and starch preloads during the palatability tests (Experiments 1 and 2) and changes in NPY and AgRP gene expressions measured by radiolabeled ISH (Experiment 6).

3. Results

As outlined in Table 1, six experiments were conducted to establish a model of sucrose-induced hyperphagia and characterize possible mechanisms that mediate this behavioral phenomenon.

3.1. Experiment 1: hyperphagia after sucrose compared to starch preload of unequal palatability

Building on prior studies that have generally tested chronic conditions or compared a sucrose solution to a non-caloric control (see Introduction), this experiment used an acute, preload-to-test meal paradigm to examine the effect of a 30% sucrose preload, compared to an equicaloric 30% starch preload, on subsequent food intake during a chow test meal. Two groups of rats (n = 20/group) were tested. In Group 1, chow was provided immediately after the preload, and intake was measured across the 3 time intervals, 0–60 min, 60–120 min and 120–180 min. In Group 2, however, there was a 60-min delay before the presentation of chow, and intake was measured across 2 intervals, from 60 to 120 min and 120–180 min. In both groups, these measurements revealed a larger test meal after consumption of the sucrose preload compared to the starch preload (Fig. 1), as indicated by a significant main effect of preload in Group 1 [$F(1,9) = 6.7$, $p<0.05$] and Group 2 [$F(1,9) = 6.4$, $p<0.05$]. While not evident during the first 60 min, this effect was seen subsequently during the 60–120 min time interval in both Group 1 (Fig. 1, left panel) and Group 2 (Fig. 1, right panel). The results in the latter group demonstrate that this overeating can occur even with the higher baseline intake after the 60 min of no food and that the consumption of food during the 0–60 time interval allowed in Group 1 was not essential to the phenomenon. After these tests with the preload-to-test meal paradigm, 1-bottle and 2-bottle preference tests were

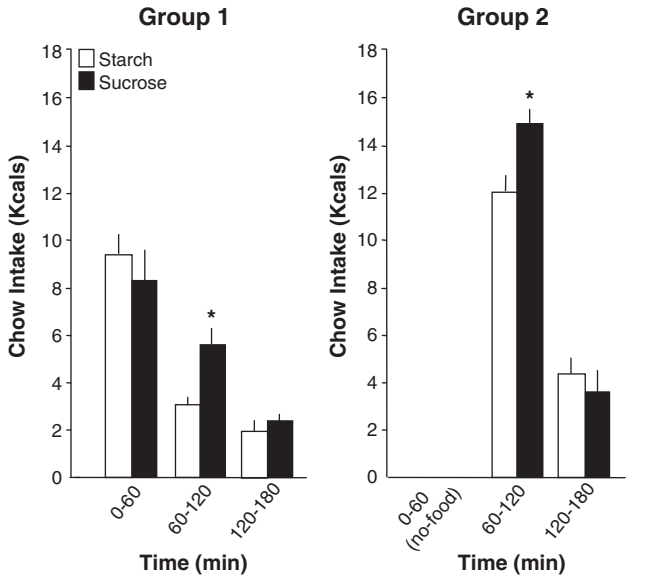

Fig. 1. Effect of sucrose preload on subsequent intake of chow (Experiment 1). Rats were given a 30-min preload of 30% sucrose compared to 30% starch, followed by a chow test meal and measurements of intake across the 3 time intervals, 0–60 min, 60–120 min and 120–180 min in Group 1 and 60–120 min and 120–180 min in Group 2. The sucrose preload compared to starch preload significantly increased chow intake during 60–180 min time interval in both Groups 1 and 2. * $p<0.05$ for comparisons between the sucrose and starch groups.

conducted to determine the relative palatability of the two preloads. These acute tests revealed a significant difference between the 30% sucrose and 30% starch solutions, with the rats in both the 1-bottle and 2-bottle tests showing a stronger preference for the sucrose solution (Table 2). Thus, while the sucrose preload compared to the equicaloric starch preload produced hyperphagia in a subsequent chow meal, it is not clear whether this hyperphagia was due to the greater palatability of the sucrose solution, leading us to examine in Experiment 2 another set of solutions that are similar in palatability.

3.2. Experiment 2: hyperphagia after sucrose compared to starch preload of equal palatability

The purpose of this experiment was two-fold, to confirm the results obtained in Experiment 1 and determine whether sucrose-induced hyperphagia can occur after a sucrose preload that is equal in palatability to a starch preload. Two groups of rats (n = 20/group) were used. In Group 1, the animals were tested with a 30% sucrose solution compared to an equicaloric 30% starch solution, in the same manner as described in Experiment 1. Consistent with the results of Experiment 1, there was a significant main effect of the unequally palatable preloads, both when chow was made available immediately after the preload [$F(1,9) = 7.8$, $p<0.05$] and also when there was a 60-min delay of no food after the preload [$F(1,9) = 8.5$, $p<0.05$]. In both paradigms, the data once again revealed a larger meal during the 60–120 min interval after the sucrose compared to the starch preload (Table 3). In Group 2, the rats were tested with a 30% sucrose solution compared to an equicaloric, 25% starch solution mixed with 5% sucrose to enhance palatability. Preference tests using the 1-bottle and 2-bottle paradigms were first conducted and showed this 25% starch preload to be equally palatable to the 30% sucrose preload (Table 2). After these tests, the rats were then examined to determine whether the 30% sucrose preload promotes hyperphagia when compared to the 25% starch solution of equal palatability. A significant main effect of these preloads on subsequent chow intake was again observed when chow was made available right after the preload [$F(1,9) = 9.2$, $p<0.01$], as well as when there was a 60-min delay in food presentation [$F(1,9) = 8.4$, $p<0.01$] (Table 3). This effect was apparent at 60–120 min post-preload, similar to that observed with the 30% sucrose compared to the 30% starch preload. These results substantiate the phenomenon of sucrose-induced overeating during the 2nd hour after the preload and demonstrate that palatability is not an essential factor in revealing this effect.

3.3. Experiment 3: pre-hyperphagia levels of circulating glucose and CORT after a sucrose preload

With hyperphagia during the test meal evident during the 60–120 min interval after the sucrose preload, this experiment in a separate group of rats (n = 20) examined changes in circulating levels of glucose and different hormones that precede and possibly contribute to the overeating observed in Experiments 1 and 2. Blood from the tail vein was collected on separate days (see Materials and methods), first, at 10 min before the preload to obtain baseline measurements and, then, at 5, 10, 30 and 60 min after consumption of the 30% sucrose compared to the 25% starch preload (with 5% sucrose). A significant difference in glucose levels after the preloads was observed, as indicated by the main effect [$F(1,9) = 21.5$, $p<0.001$] that varied across time [$F(3,27) = 13.3$, $p<0.001$]. Compared to the baseline measures, glucose levels at 5–30 min after the preload were significantly elevated by both sucrose and starch. While reaching their peak at 30 min and descending toward baseline by 60 min after the sucrose preload, glucose levels after the starch preload continued to rise and remain high. When comparing sucrose to starch, glucose levels after the sucrose preload were initially elevated at the 5-min measurement interval (+12%, $p<0.05$) but then became significantly lower (−13–34%) at the 30- and 60-min time intervals (Fig. 2). In addition to these changes in glucose, measurements in trunk blood revealed a significant difference in levels of CORT [$F(1,6) = 29.3$, $p<0.001$] that again was moderated by time [$F(2,12) = 4.04$, $p<0.05$]. With a tendency towards higher levels at 30 min after consumption of the sucrose preload, this effect became statistically significant at 60 min, with CORT levels 75% higher after sucrose compared to starch (Fig. 3). Unlike glucose and CORT, little change in levels of insulin [$F(1,6) = 1.1$, ns] and leptin [$F(1,6) = 3.0$, ns] was evident in this acute paradigm (Fig. 3). Together, these results show that compared to a starch preload, a sucrose preload leads to lower glucose and higher

Table 2
Palatability of sucrose vs starch preload solutions in 30-min tests.

Solutions	Test	Starch (kcal)	Sucrose (kcal)
Experiment 1: 30% sucrose vs 30% starch	1-bottle	18.4 ± 1.6	24.8 ± 1.8[a]
	2-bottle	7.3 ± 1.2	18.8 ± 1.9[a]
Experiment 2B: 30% sucrose vs 25% starch (+5% sucrose)	1-bottle	19.4 ± 3.3	24.5 ± 2.3
	2-bottle	18.8 ± 3.1	13.9 ± 2.9

[a] $p<0.05$, comparison between sucrose and starch preload solutions.

Table 3
Food intake (kcal) after sucrose vs starch in Groups 1 and 2 (Experiment 2).

	Group 1		Group 2	
	30% starch	30% sucrose	25% starch[b]	30% sucrose
Food available in hours 1–3				
Hour 1	11.2 ± 0.62	9.3 ± 0.73	12.6 ± 0.62	12.4 ± 0.75
Hour 2	2.8 ± 0.11	5.1 ± 0.31[a]	1.5 ± 0.09	3.7 ± 0.26[a]
Hour 3	5.4 ± 0.39	4.7 ± 0.42	3.8 ± 0.22	4.9 ± 0.59
Food available in hours 2 and 3				
Hour 1	–	–	–	–
Hour 2	10.6 ± 0.89	13.8 ± 1.10[a]	9.4 ± 0.55	12.6 ± 0.84[a]
Hour 3	3.7 ± 0.12	6.6 ± 0.49	3.2 ± 0.18	4.5 ± 0.33

[a] $p<0.05$, comparison between sucrose and starch preload groups.
[b] 25% starch solution also contains 5% sucrose.

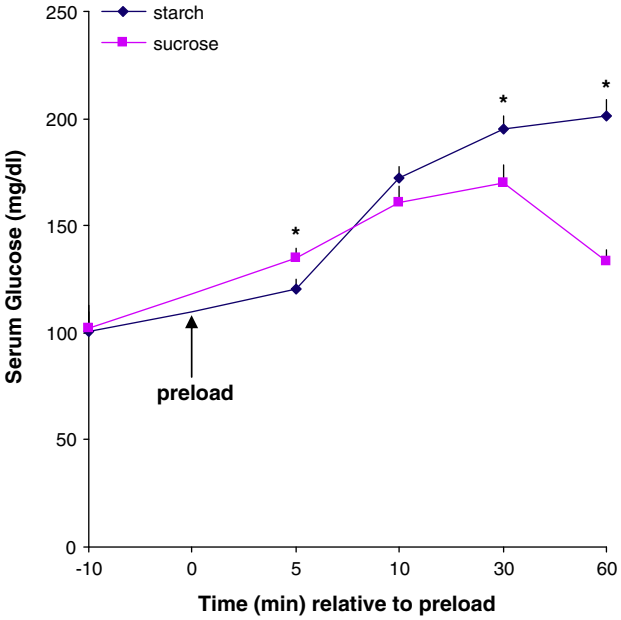

Fig. 2. Effect of sucrose preload on serum levels of glucose (Experiment 3). Rats were given a 30-min preload of 30% sucrose compared to 25% starch (with 5% sucrose), followed by blood collections via tail vein puncture. Serum levels of glucose after the sucrose (vs starch) preload were significantly higher at 5 min while lower at 30 and 60 min. * $p<0.05$ for comparisons between the sucrose and starch groups.

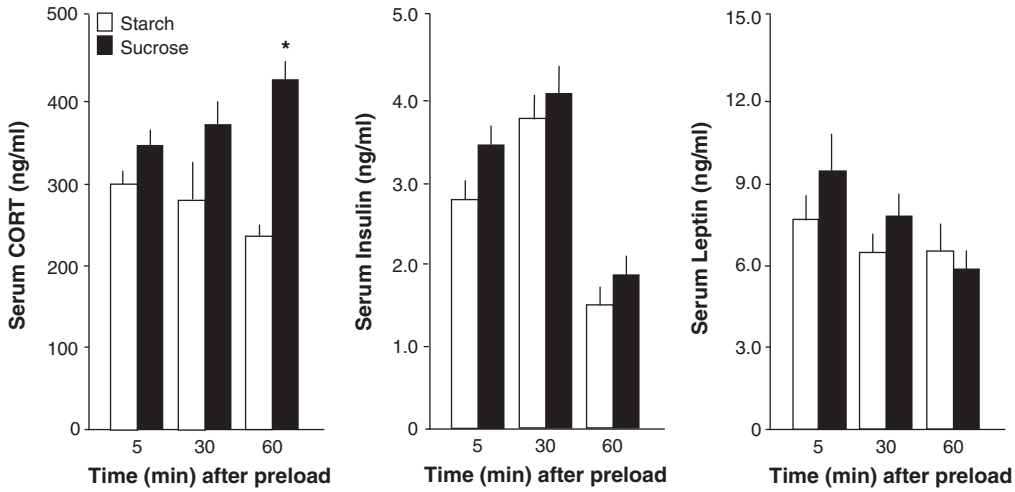

Fig. 3. Effect of sucrose preload on serum levels of CORT, insulin and leptin (Experiment 3). Rats were given a 30-min preload of 30% sucrose compared to 25% starch (with 5% sucrose) and then sacrificed for collection of trunk blood at 5, 30 and 60 min after the preload. Serum levels of CORT were significantly elevated at 60 min, while insulin and leptin levels were unaltered at all 3 time points. * $p<0.05$ for comparisons between the sucrose and starch groups.

CORT levels from 30 to 60 min post-preload, just before the episode of hyperphagia that occurs between 60 and 120 min post-preload.

3.4. Experiment 4: pre-hyperphagia levels of glucose and CORT in CSF after a sucrose preload

Building on the results of Experiment 3 that revealed time-dependent changes in circulating glucose and CORT during the pre-hyperphagia hour, this experiment examined whether similar changes can be detected in the CSF. Samples of CSF were collected from a cisterna magna cannula on separate days, at 10, 30 and 60 min after consumption of the preload. Similar to circulating glucose, the sucrose preload had a significant overall effect on CSF levels of glucose [$F(1,5)=14.9$, $p<0.05$] that shifted over time [$F(2,10)=10.0$, $p<0.04$]. Compared to the starch preload, they were significantly increased (+19%) at 10 min after sucrose, while reduced at 30 and 60 min (−20%) (Fig. 4). Measurements of CORT levels in the CSF also revealed a significant main effect that was similar to that seen in the circulation [$F(1,6)=7.7$, $p<0.05$], although this difference did not change as a function of time [$F(2,12)=0.6$, ns]. With no effect at 10 and 30 min, CORT levels were significantly higher at 60 min (+48%) after the sucrose preload. These results show changes in CSF preceding the hyperphagia, which mirror those detected in the blood and possibly contribute to this behavioral phenomenon.

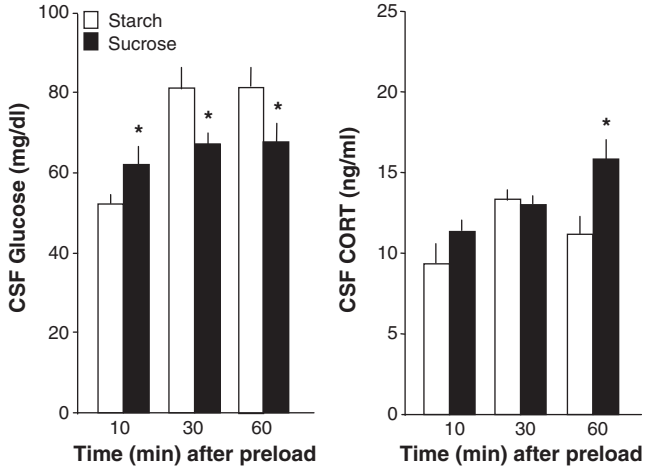

Fig. 4. Effect of sucrose preload on CSF levels of glucose and CORT (Experiment 4). Rats were given a 30-min preload of 30% sucrose compared to 25% starch (with 5% sucrose), followed by CSF collections from cisterna magna cannula. Levels of glucose were significantly elevated at 10 min after the sucrose preload while reduced at 30 and 60 min, and levels of CORT were significantly increased at 60 min. * $p<0.05$ for comparisons between the sucrose and starch groups.

3.5. Experiment 5: pre-hyperphagia expression of NPY and AgRP after sucrose as measured by qRT-PCR

This next experiment examined, prior to the onset of sucrose-induced hyperphagia, the mRNA expression of orexigenic peptides NPY and AgRP in the ARC, both of which are known to be responsive to changes in glucose and CORT levels. Rats were allowed to consume 15 kcal of either the 30% sucrose or 25% starch preload and were sacrificed at 10, 30 or 60 min later. The results of this analysis, similar to the measurements of glucose and CORT, revealed a significant overall effect of the sucrose preload on NPY [$F(1, 29)=7.4$, $p<0.05$], which was moderated as a function of time [$F(2,29)=78.5$, $p<0.001$], and also on AgRP [$F(1,29)=18.2$, $p<0.001$], which similarly changed with time [$F(2,29)=57.2$, $p<0.001$]. After the sucrose compared to starch preload, mRNA levels of both NPY and AgRP in the ARC were reduced at 10 min by approximately 40% (Fig. 5), just after the increase in glucose shown in the circulation and CSF. At 30 and 60 min, in contrast, their expression was significantly increased, by 40–70% (Fig. 5), at the same time that glucose was lower and CORT levels higher after the sucrose preload. Together, these results demonstrate that orexigenic peptides NPY and AgRP are elevated at 30–60 min after the sucrose preload, just prior to the onset of the hyperphagia during the 60–120 min time interval, as described in Experiments 1 and 2.

3.6. Experiment 6: pre-hyperphagia expression of NPY and AgRP after sucrose as measured by radiolabeled ISH

To confirm the results of Experiment 5, this experiment used radiolabeled ISH to more precisely quantify the changes in NPY and AgRP gene expressions after a preload of 30% sucrose compared to 25% starch ($n=5$/group). The results revealed a significant 30% increase ($p<0.001$) in NPY and AgRP mRNA levels at 30 min after completion of the preload (Fig. 6), consistent with the qRT-PCR results obtained in Experiment 5. This effect is illustrated in the photomicrograph showing the expression of NPY in the ARC (Fig. 7). Together, these

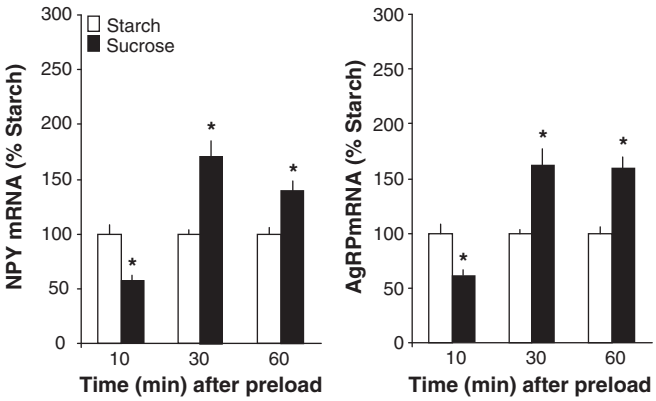

Fig. 5. Effect of sucrose preload on NPY and AgRP mRNA in ARC (Experiment 5). Rats were given a 30-min preload of 30% sucrose compared to 25% starch (with 5% sucrose) and then sacrificed at 10, 30 and 60 min for measurements of NPY and AgRP mRNA using qRT-PCR. Compared to the starch preload, the sucrose preload produced an initial decrease in peptide expression at 10 min, followed by an increase at 30 and 60 min. * $p<0.05$ for comparisons between the sucrose and starch groups.

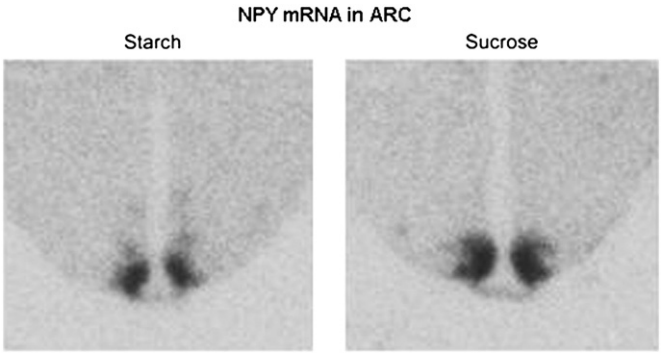

Fig. 7. Photomicrograph illustrating the effect of sucrose preload on NPY mRNA in the ARC at 30 min after the sucrose vs starch preload (Experiment 6). Data for NPY mRNA are presented in Fig. 6.

findings support a possible role of these potent, orexigenic peptides in mediating the increase in food intake induced by the sucrose preload.

4. Discussion

With no existing animal models to date studying the acute effect of different carbohydrates on food intake, the first goal of this study was to establish a paradigm that reliably reveals hyperphagia associated specifically with sugar. The first two experiments of this report performed a variety of tests using a modified version of the preload-to-test meal paradigm, which was originally described by Warwick and colleagues (Warwick, 2003; Warwick et al., 2000) and subsequently used in our laboratory to study hyperphagia induced by a fat-rich diet (Gaysinskaya et al., 2007). This standard protocol involved tests with: 1) rats in their home cages and during the last few hours of the light cycle, just prior to the start of spontaneous feeding at dark onset; 2) a 90-min, food-free interval prior to the start of the test to minimize random *ad libitum* feeding and thus variability in the intake scores during the test; 3) a 3-week period of adaptation to the diets and test paradigm preceding the actual test meal, which involved 2 weeks of daily exposure to a preload of sucrose or starch in a counterbalanced order and then 1 week of daily exposure to these preloads followed 60 min later by a chow test meal; and 4) a 30-min

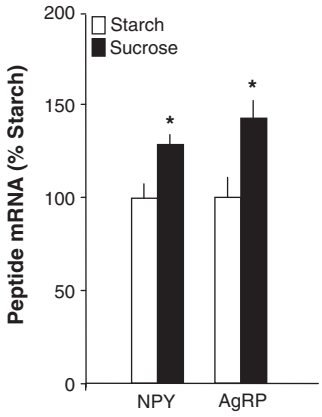

Fig. 6. Effect of sucrose preload on NPY and AgRP mRNA in ARC (Experiment 6). Rats were given a 30-min preload of 30% sucrose compared to 25% starch (with 5% sucrose) and then sacrificed after 30 min for measurements of NPY and AgRP mRNA using *in situ* hybridization. Compared to the starch preload, the sucrose preload produced an increase in peptide expression. * $p<0.05$ for comparisons between the sucrose and starch groups.

preload, during which most rats (>95%) learn to consume approximately 9.3 ml (15 kcal) of the sucrose or starch solutions, and a 60-min test meal, during which most rats (>95%) consume at least 1 kcal of lab chow, with the 4% of rats not meeting these criteria eliminated from the study. After the 3-week training period, the actual tests were begun, with the sucrose or starch preloads presented in counterbalanced order over a period of 8 consecutive days and a 0-min or 60-min interval with no food between the preload and test meal. Taken together, the present study describes a well-defined model, which allows one to measure the number of calories consumed after a sucrose vs starch preload and characterize mechanisms that may contribute to differences in the consummatory behavior.

There are only a few studies that have investigated the dietary determinants of sucrose-induced hyperphagia, and those that exist compared the amount consumed of the sucrose diet to that of the starch diet. One investigation in rats given chronic access to the diets showed daily caloric intake to increase by 15% on a high-sucrose compared to high-starch diet (Raben et al., 1997). Also, two reports in rats given acute access showed a meal high in sucrose content to be larger than a corn-starch meal (Glass et al., 1997; Glass et al., 2001). When measuring caloric intake subsequent to diet exposure, this latter report provided additional evidence indicating that the sucrose preload was also followed by a larger meal than that seen after the starch preload, when this subsequent meal was high in sucrose or starch, respectively (Glass et al., 2001). Using the preload-to-test meal paradigm described above, the present study confirmed the phenomenon of sucrose-induced hyperphagia and established an experimental paradigm that allows one to reveal this effect following a brief, 30-min period of sucrose consumption. After being given the small (15 kcal) preload of 30% sucrose, the animals exhibited greater, subsequent intake of lab chow compared to what they ingested after an equicaloric, 30% starch preload. Since the sucrose and starch preloads in the present paradigm were similar in caloric density, this factor does not appear to be involved in the overeating observed after sucrose consumption. Although sucrose is generally accepted to be more palatable then starch (Nissenbaum and Sclafani, 1987a,b; Weldon et al., 1996), the present results showing overeating to occur with equally palatable preloads, 30% sucrose compared to 25% starch (with 5% sucrose), suggest that this variable is also not an essential factor. Together, these findings establish the phenomenon of overeating subsequent to a small sucrose preload and show it to be independent of caloric intake, energy density and palatability of the preload. The occurrence of the sucrose-induced hyperphagia between 60 and 120 min after the preload, whether or not food is available during the first 60 min, suggests that the physiological and neurochemical factors that may be promoting this increase in consummatory behavior require time to exert their control.

As described in the Introduction, the potent feeding-stimulatory peptides, NPY and AgRP, are highly responsive to changes in glucose

homeostasis, both levels and utilization, and thus may have a role in the phenomenon of sucrose-induced overeating. Whereas there are no prior investigations examining the effects of sucrose compared to starch on these peptides, there are some that have tested a sucrose solution compared to a non-caloric control. An acute period of drinking 20% sucrose compared to water produces an increase in NPY mRNA levels in the ARC after 90 min (Wang et al., 1999). A similar increase in AgRP as well as NPY mRNA is also seen at 90 min after intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) injection of 10% glucose solution compared to saline (Chang et al., 2005). Results presented here also show an increase in NPY and AgRP mRNA in the ARC, specifically at 30 and 60 min after sucrose consumption, indicating that sucrose compared to an equicaloric, starch preload has similar neurochemical as well as physiological effects as those seen when compared to a non-caloric control. At 10 min after the sucrose compared to the starch preload, the reverse effect was observed, a significant reduction in NPY and AgRP expressions in the ARC. This effect is similarly consistent with previous findings, showing acute i.p. or i.c.v. injections of glucose vs saline to initially suppress NPY and AgRP expressions (Chang et al., 2005) and chronic central infusion of glucose compared to saline to reduce NPY mRNA (Fekete et al., 2006). Whereas this transient reduction in the NPY and AgRP appears from our results to have little effect on food intake during the first 60 min, the stimulation of these peptides prior to the onset of sucrose-induced hyperphagia after 60 min suggests the involvement of these peptides in this phenomenon.

The measurements of glucose in serum and CSF taken after the sucrose preload also revealed time-dependent changes relative to the starch preload, which were diametrically opposite to and possibly related to the changes in NPY and AgRP. Compared to the starch preload, sucrose produced a small (12%) but significant increase in circulating glucose at 5 min after the preload, similar in magnitude to the change in glucose levels following i.p. injection of 10% glucose compared to saline (Chang et al., 2005). Consistent with other studies (Chang et al., 2005; Daly et al., 1998; Hallfrisch et al., 1981), this increase was transient, with no difference detected by 10 min after the preload, possibly explaining why it had no apparent effect on food intake during the first hour. Whereas glucose levels after the sucrose preload reached a peak at 30 min relative to baseline measures and started to descend toward baseline by 60 min, glucose levels after the starch preload continued to rise, resulting in lower glucose levels after sucrose at the 30- and 60-min intervals. This time-dependent change in glucose levels is consistent with studies showing sucrose, which is readily digested, to produce a rapid and transient rise in circulating glucose, in contrast to starch, a complex polysaccharide, which is metabolized more slowly and takes more time to elevate glucose levels while producing a more sustained rise (Hallfrisch et al., 1981). Of particular interest is the finding that the changes in circulating glucose were mirrored by changes in CSF glucose, which was elevated at 10 min and lower at 30 and 60 min after the sucrose preload. This is consistent with changes in CSF glucose observed in response to food intake in food-deprived rats or glucose infusion in fed rats (Mayer et al., 2006; Steffens et al., 1988). These time-dependent changes in glucose following the sucrose preload may be causally related to the changes in NPY and AgRP, with their expressions reduced by an initial rise and then stimulated by a subsequent decline in glucose. This relationship is supported by published studies, comparing the effects of peripheral or central injection of glucose to a non-caloric control, which demonstrate an initial suppression of NPY and AgRP expressions at a time when circulating glucose levels are high (Chang et al., 2005; Fekete et al., 2006; Mayer et al., 2006; Steffens et al., 1988). Whereas no direct evidence exists regarding the effect of relative changes in glucose levels on NPY and AgRP neurons, studies show these neurons may participate in the process of sensing physiological changes in circulating or brain glucose via glucokinase, which is found on the majority of NPY neurons in the ARC (Lynch et al., 2000) and may also be responsive to changes in glucose metabolism (Yang et al., 2004). Furthermore, investigations show that glucose has an inhibitory effect on the firing of NPY neurons (Dunn-Meynell et al., 1997; Funahashi et al., 1999; Mountjoy et al., 2007; Muroya et al., 1999) and that these neurons are excited in the state of hypoglycemia (Murphy et al., 2009; Wang et al., 2004), indicative of a close relationship between glucose and the functioning of NPY neurons. Thus, the present results provide evidence to suggest that the consumption of sucrose compared to starch, by virtue of their differential effects on circulating and CSF glucose, modulate the expression of the feeding-stimulatory peptides, NPY and AgRP, in the ARC.

A decline in glucose is invariably associated with a rise in CORT, which has a primary function in maintaining glucose homeostasis while having a stimulatory effect on food intake (Strack et al., 1995; Tempel and Leibowitz, 1994; Udden et al., 2003). This inverse relationship was observed in the present study, with serum CORT levels elevated at 30 and 60 min after the sucrose compared to the starch preload. This delayed increase in CORT agrees with published reports, showing elevated CORT levels at 30–90 min after acute, peripheral injections of glucose compared to saline (Chang et al., 2005) or after one time consumption of a glucose solution compared to water (Payne et al., 2003; Wang et al., 1999). The elevated CORT occurred precisely at the time when serum glucose levels were lower, consistent with previous studies involving glucose injection or sucrose consumption (Chang et al., 2005; Wang et al., 1999) and with the inverse relationship between CORT and glucose that is evident shortly before the nocturnal cycle and initiation of feeding (Tempel and Leibowitz, 1994). As with glucose, the effect of sucrose consumption on circulating CORT was mirrored by the changes in CSF CORT. While unaffected at 10 min, its levels were elevated at 30 and 60 min, suggesting that CORT in the brain may also directly affect peptide expression. As with the lower levels of glucose, this rise in CORT may have an important role in the subsequent, stimulatory effect of the sucrose preload on NPY and AgRP. The expressions of NPY and AgRP are known to be stimulated an hour after the administration of CORT (Akabayashi et al., 1994; Savontaus et al., 2002; Tempel and Leibowitz, 1994) and also in association with a rise in CORT induced by food deprivation (Bi et al., 2003; Leibowitz and Wortley, 2004; Mizuno et al., 1999; Sahu et al., 1988). Thus, the lower levels of glucose at 60 min after the sucrose preload may act, in part, through a stimulation of CORT that, in turn, enhances peptide gene expression. In contrast to glucose and CORT, the measurements of insulin and leptin failed to reveal any changes in the acute paradigm tested in the present study. This indicates that these two hormones, in normal-weight rats, are unlikely to play a major role in the behavioral and peptide effects induced by the sucrose preload.

The results of this study show that the hyperphagia induced by sucrose compared to starch is immediately preceded by significantly lower levels of glucose and elevated CORT in the circulation and CSF and also by the increased expressions of NPY and AgRP in the ARC. Based on published evidence, each of these changes would be expected to stimulate food intake. There are several studies suggesting that circulating glucose and food intake are inversely related (Grossman, 1986; Singer and Ritter, 1996; Smith and Epstein, 1969), with a small, 6–10% drop in circulating glucose that produces a 0.2–0.3 mM change in brain glucose (Levin, 2002) generally preceding the initiation of spontaneous meals (Campfield and Smith, 2003). The greater caloric intake induced by the sucrose compared to the starch preload may result, in part, from the higher GI as well as increased metabolism and absorption of sucrose (Anderson and Woodend, 2003; Foster-Powell et al., 2002; Roberts, 2000) and may also reflect a less potent satiety effect of sucrose in addition to a stronger stimulatory effect on food intake. In addition to glucose, CORT may also be involved in the hyperphagia following the sucrose preload. This is supported by the evidence that CORT, when administered systemically in humans or directly into the PVN of rats, significantly stimulates food intake (Strack et al., 1995; Tempel and Leibowitz, 1993; Udden et al., 2003) and also

increases the expressions of NPY and AgRP (Akabayashi et al., 1994; Savontaus et al., 2002; Tempel and Leibowitz, 1994). These peptides both have potent, stimulatory effects on food intake (Hagan et al., 2000; Leibowitz and Wortley, 2004; Stanley and Leibowitz, 1984), with their strongest effect occurring on diets rich in carbohydrate or sucrose (Badia-Elder et al., 2003; Baird et al., 2006; Lynch et al., 1993; Stanley et al., 1985; Welch et al., 1994; Wirth and Giraudo, 2000). Building on this evidence, the results of the present study suggest that NPY and AgRP functions in concert with glucose and CORT to promote the hyperphagia induced by a small, sucrose preload. A direct test of this hypothesis could be provided by measurements of the effect of central injections of glucose and CORT on peptide expression and neuronal activity in the ARC.

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