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RESEARCH ARTICLE

Obesity, Diabetes and Energy Homeostasis

Consuming sucrose solution promotes leptin resistance and site specifically modifies hypothalamic leptin signaling in rats

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Abstract

Rats consuming 30% sucrose solution and a sucrose-free diet (LiqS) become leptin resistant, whereas rats consuming sucrose from a formulated diet (HS) remain leptin responsive. This study tested whether leptin resistance in LiqS rats extended beyond a failure to inhibit food intake and examined leptin responsiveness in the hypothalamus and hindbrain of rats offered HS, LiqS, or a sucrose-free diet (NS). Female LiqS Sprague-Dawley rats initially only partially compensated for the calories consumed as sucrose, but energy intake matched that of HS and NS rats when they were transferred to calorimetry cages. There was no effect of diet on energy expenditure, intrascapular brown fat tissue (IBAT) temperature, or fat pad weight. A peripheral injection of 2 mg of leptin/kg on *day 23* or *day 26* inhibited energy intake of HS and NS but not LiqS rats. Inhibition occurred earlier in HS rats than in NS rats and was associated with a smaller meal size. Leptin had no effect on energy expenditure but caused a transient rise in IBAT temperature of HS rats. Leptin increased the phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) in the hindbrain and ventromedial hypothalamus of all rats. There was a minimal effect of leptin in the arcuate nucleus, and only the dorsomedial hypothalamus showed a correlation between pSTAT3 and leptin responsiveness. These data suggest that the primary response to leptin is inhibition of food intake and the pattern of sucrose consumption, rather than calories consumed as sucrose, causes leptin resistance associated with site-specific differences in hypothalamic leptin signaling.

female rats; dietary sucrose; food intake; energy expenditure; pSTAT3

INTRODUCTION

Animals offered a palatable, high-calorie diet develop leptin resistance, defined as a failure of exogenous leptin to inhibit food intake or cause weight loss (1, 2). In previous studies, we found that rats offered a choice diet consisting of rat chow, lard, and a 30% sucrose solution rapidly became resistant to both peripherally and centrally administered leptin (3), and we subsequently demonstrated that consumption of sucrose solution and not the consumption of lard, total energy intake, or increase in body fat mass was responsible for this resistance (4). We have also reported that rats offered sucrose solution in addition to a sucrose-free diet become leptin resistant, whereas those that consume a similar amount of sucrose from a dry diet remain leptin responsive (5). The leptin resistance is present in the absence of a significant change in body fat mass or circulating leptin levels and is apparent in both male and female rats but is detected earlier in females than in males (5).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor activated by leptin and transgenic mouse studies have shown it to be essential for prevention of hyperphagia and obesity (6). For this reason, phosphorylation of STAT3 (pSTAT3) is frequently used as an indirect marker of leptin activity (7), and leptin resistance would be expected to be associated with a failure to phosphorylate STAT3 (8). Unexpectedly, the leptin resistance in sucrose-drinking rats was associated with a 30%–50% increase in pSTAT3 in the arcuate nucleus of the hypothalamus (Arc) in basal conditions (9). The observations on central STAT3 activation were made in the hypothalamus of male rats offered chow or chow plus sucrose solution, and we did not test whether the same response would be found in rats eating an equivalent amount of sucrose from a dry, formulated diet. In addition, leptin responsiveness in sucrose-drinking rats has only been tested by measuring food intake, but leptin has also been reported to stimulate energy expenditure and increase sympathetic outflow to brown adipose tissue (10), resulting in an increase in UCP1 protein expression and thermogenesis (11).

The objectives of this experiment were to evaluate the effect of leptin on different aspects of energy balance and determine which areas of the hypothalamus were associated with leptin resistance in rats drinking sucrose solution. Female rats consuming sucrose from either a dry formulated diet or as sucrose solution offered in addition to a dry,

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sucrose-free diet were compared, making it possible to test whether changes in hypothalamic leptin signaling were specific to drinking sucrose solution or were secondary to consuming sucrose, irrespective of whether it was part of a composite diet or was in solution form. During the experiment, the effect of a peripheral injection of leptin on energy expenditure, brown fat temperature, and energy intake was measured, and, at the end of the experiment, pSTAT3 in the hypothalamus and hindbrain was quantified in basal and leptin-stimulated conditions.

METHODS

Thirty-six female Sprague-Dawley rats (Envigo, Prattville, AL) were included in this experiment that was completed with three cohorts of twelve animals to accommodate housing in a 12-cage calorimeter for the last 11 days of the experiment. All of the animal procedures were approved by the Institutional Animal Care and Use Committee of Augusta University. The rats weighed 175–200 g at the start of the experiment and were initially housed individually in hanging wire-mesh cages with free access to food and water and a Nylabone (Neptune City, NJ) for enrichment. The room was maintained at 21°C–22°C, with lights on for 12 h a day from 6.00 AM. All of the rats were offered a sucrose-free diet (D 11724, Research Diets, New Brunswick, NJ) and after 3 days they were divided into three weight-matched groups. One group continued to be offered the sucrose-free (NS) diet and one group was offered a high-sucrose (HS) diet (DL 11725 Research Diets) that contained 66.6% kcal as sucrose. The final group of rats (LiqS) was offered the NS diet and a 30% sucrose solution. Both NS and HS diets contained 67.7% kcal as carbohydrate and had a gross energy content of 3.902 kcal/g.

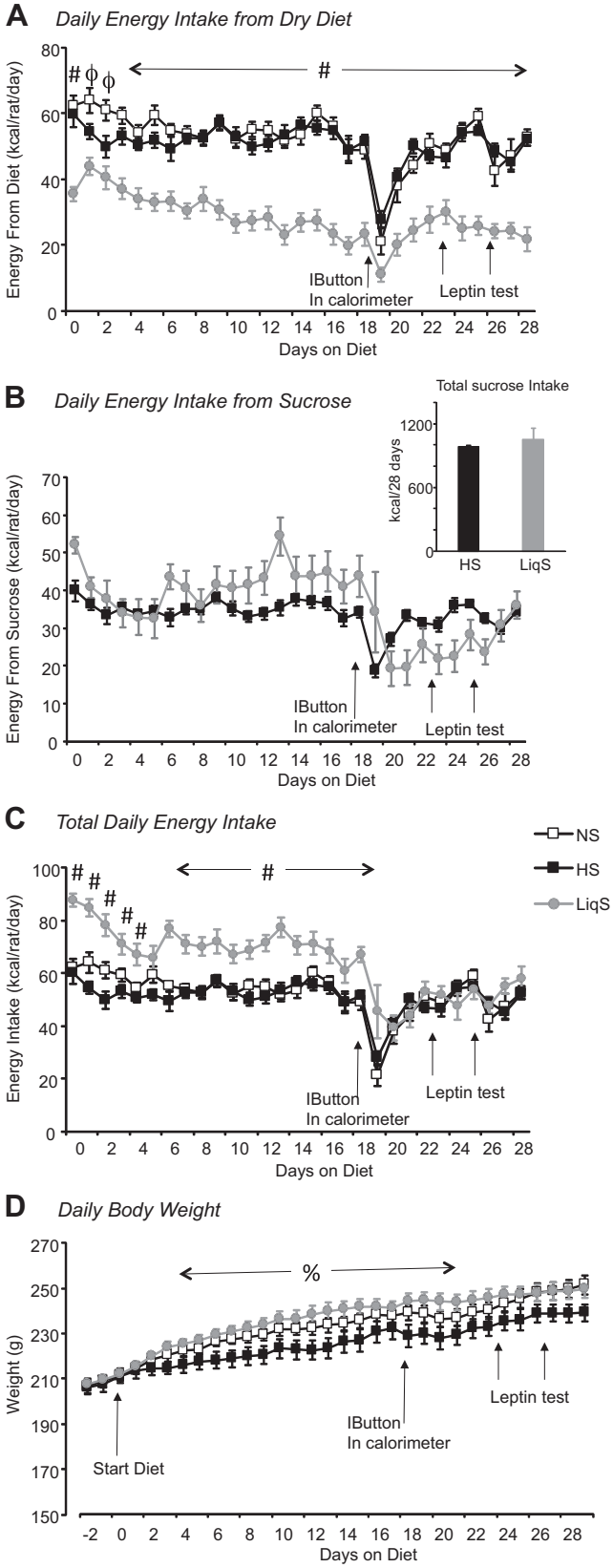
Rats were offered fresh diet and sucrose solution each day and daily food and sucrose consumption were recorded for 18 days. Food intake was corrected for spillage, but intake of sucrose solution was not because we previously found that the rats spill less than 1 mL each day. On *day 19* an iButton (Embedded Data Systems, Lawrenceburg, KY) was placed over intrascapular brown fat tissue (IBAT) with the rats under isoflurane anesthesia. They received a subcutaneous injection of ketoprofen analgesic (2 mg/kg; Ketofen, Fort Dodge Animal Health, Fort Dodge, IA) immediately before surgery and again 24 h later. The iButtons were programmed to record temperature every 30 min until the end of the experiment. The rats were transferred to calorimetry cages maintained at 21°C (TSE LabMaster, Metabolic Research Platform; TSE Systems International, Chesterfield, MO), which automatically recorded food, sucrose, and water intake every minute, but routinely reported intake every 39 min. The calorimeter sampled the gases from each cage for 3 min every 39 min, and the values recorded during the last of the 3 min of sampling were used to calculate energy expenditure (kcal/rat/h) and respiratory exchange ratio (RER). The calorimeter was stopped at 8.00 AM each day for 30 min to allow filling of food hoppers and sucrose and water bottles, weighing of the rats, and changing of cage bedding.

Leptin responsiveness of the rats was tested after 23 and 26 days on diet. Food and sucrose solution were removed from the cages at 8:00 AM, and starting at 5:00 PM, the rats received intraperitoneal injections of PBS or 2 mg of leptin/kg in a volume of 0.2 mL/100 g body wt (Recombinant rat leptin protein, CF. R&D Systems, Minneapolis, MN). Food and sucrose solution were returned to the cages at 6.00 PM. Each rat acted as its own control, receiving PBS or leptin in random order. On *day 29* food and sucrose were removed from the cages at 8:00 AM. Starting at 11:00 AM, half of the rats in each dietary group received an intraperitoneal injection of 1 mg of leptin/kg and the other half were injected with PBS. Exactly 1 h later, the rats were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg)-xylazine (10 mg/kg). One inguinal and one retroperitoneal (RP) white fat pad was dissected from each rat and weighed as an index of adiposity. Intrascapular brown adipose tissue (IBAT) was dissected and snap frozen for subsequent quantification of UCP1 protein by Western blot (primary antibody: ab23841, Abcam, Cambridge, MA, RRID:AB_2213764; secondary goat anti-rabbit antibody: 111-035-003 Jackson ImmunoResearch Laboratories, West Grove, PA, RRID:AB_2313567). The rats were perfused pericardially with 300 mL of cold heparinized saline followed by 500 mL of cold 4% paraformaldehyde solution. Brains were extracted and held in paraformaldehyde overnight at 4°C and then stored in 25% sucrose 0.1% sodium azide solution at 4°C. Thirty-micron sections were made through the hindbrain, midbrain, and hypothalamus. pSTAT3 was detected using free-floating immunohistochemistry (12) and pSTAT3 immunoreactivity (pSTAT3-IR) was detected using ABC reagents and diaminobenzidine (DAB; Vector Laboratories, Burlin-game, CA). Images of pSTAT3 positive nuclei were captured from an Olympus BX51 microscope and quantified in specific regions by manual counting. The areas quantified were the medial and lateral arcuate nucleus (ArcM and ArcL) and the dorsomedial region of the ventromedial nucleus of the hypothalamus (VMHdm) at bregma −3.14 mm and −3.30 mm [plates 32 and 33; Paxinos and Watson Rat Brain Atlas (13)], the dorsomedial hypothalamus (DMH) at bregma −3.14 mm, the dorsomedial hypothalamic nucleus compact part (DMC) at bregma −3.30 mm, and the nucleus of the solitary tract (NTS) at bregma −14.08 mm [plate 76; Paxinos and Watson Rat Brain Atlas (13)]. No pSTAT3 positive nuclei were found in the ventral tegmental area of any of the rats.

Comparisons between groups were determined using Statistica (StatSoft, Tulsa, OK). Measures of energy intake, body weight, IBAT temperature, RER, or energy expenditure were compared by repeated-measures analysis of variance. Rat number was used as a covariant for analysis of data following leptin or PBS injection. Data on meal patterns and pSTAT3-IR in the hypothalamus or brainstem and other single end-point measures were compared by two-way analysis of variance. Post hoc comparisons at specific time points were made using Tukey's honestly significant difference (HSD) test with rat number as a covariant when appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

LiqS rats consumed less dry diet than NS or HS rats (Fig. 1*A*) to compensate for the calories consumed from sucrose solution (Fig. 1*B*), but the compensation was incomplete resulting in a greater daily calorie intake while the animals were housed in wire-mesh cages (Fig. 1*C*). When the rats



were transferred to calorimetry cages, which were enclosed cages with corn cob bedding, the energy intake of LiqS rats decreased and matched that of the other two groups (diet, $P < 0.0001$; time, $P < 0.0001$; interaction, $P < 0.0001$). The decrease in energy intake was entirely due to a decrease in consumption of sucrose solution (Fig. 1*B*). Despite these changes in sucrose intake by LiqS rats, total sucrose intake during the entire experiment was not different between HS and LiqS rats (Fig. 1*B*, *inset*). Although total energy intake of LiqS rats was greater than that of the two other groups for the first part of the experiment, the weight of LiqS rats was not different from that of NS rats. By contrast, the energy intake of HS rats was the same as that of NS rats, but they weighed less than LiqS rats as early as *day 3* on experimental diets (Fig. 1*D*; diet, $P < 0.03$; time, $P < 0.0001$; interaction, $P < 0.001$) and must have had a lower efficiency of energy utilization than NS rats. The difference in weight between HS and LiqS rats was maintained until the rats were housed in the calorimeter and the LiqS rats reduced their energy intake.

Calorimetry data were examined on *day 22*. By this time, the rats had adapted to the calorimeter for 4 days, sucrose intake was stable, energy intakes were the same for all three groups of rats, and it was the day before the first leptin test. Because the calorimeter reported food intake and energy expenditure at 39-min intervals, it was possible to evaluate the diurnal pattern of food and sucrose intake of the rats. All of the rats consumed a similar percentage of their total energy intake during the light period (Fig. 2*A*); however, the LiqS rats consumed 40% of their daily sucrose but only 26% of their daily dry diet intake during the light period. There was no effect of diet on the amount of energy consumed during the light or the dark period.

LiqS rats had an increased energy expenditure and RER compared with NS and HS rats in the first 2 h after they had been weighed and the food, water, and sucrose solution refreshed (Fig. 3*A*; diet, $P < 0.05$; time, $P < 0.0001$<interaction, $P < 0.02$). Average energy expenditure was lower during the light period in HS rats than in LiqS rats, and it is possible that the difference was associated with repeated visits to the sucrose bottle by LiqS rats. All of the rats consumed a significant amount of energy during the first 39 min after food had been refreshed (Fig. 2*B*; NS: 4.1 ± 1.1 kcal, HS: 3.0 ± 1.1 kcal, and LiqS: 3.9 ± 0.6 kcal) but LiqS rats primarily consumed sucrose solution (2.8 ± 0.5 kcal). The elevated RER was likely associated with metabolism of the sucrose that would have been digested and absorbed faster than complex carbohydrate in the NS diet

Figure 1. Daily energy intake from dry diet (*A*), sucrose (*B*), and total energy intake (C) of groups of 12 female Sprague-Dawley rats offered a sucrose-free diet (NS), a 66.6% sucrose dry diet (HS), or NS diet plus a 30% solution of sucrose (LiqS). Total sucrose consumption is shown in the *inset* to *B*. Daily body weight is shown in *D*. Statistically significant differences ($P < 0.05$) between groups were determined by repeated-measures analysis of variance and post hoc comparison on each day using Tukey's honestly significant difference test. % Significant difference between HS and LiqS rats, #difference between LiqS and both HS and NS rats, and ϕdifference between all three groups. Rats were housed in wire-mesh cages until *day 18* when they were moved into a calorimeter. Leptin responsiveness was tested on *days 23* and *26*.

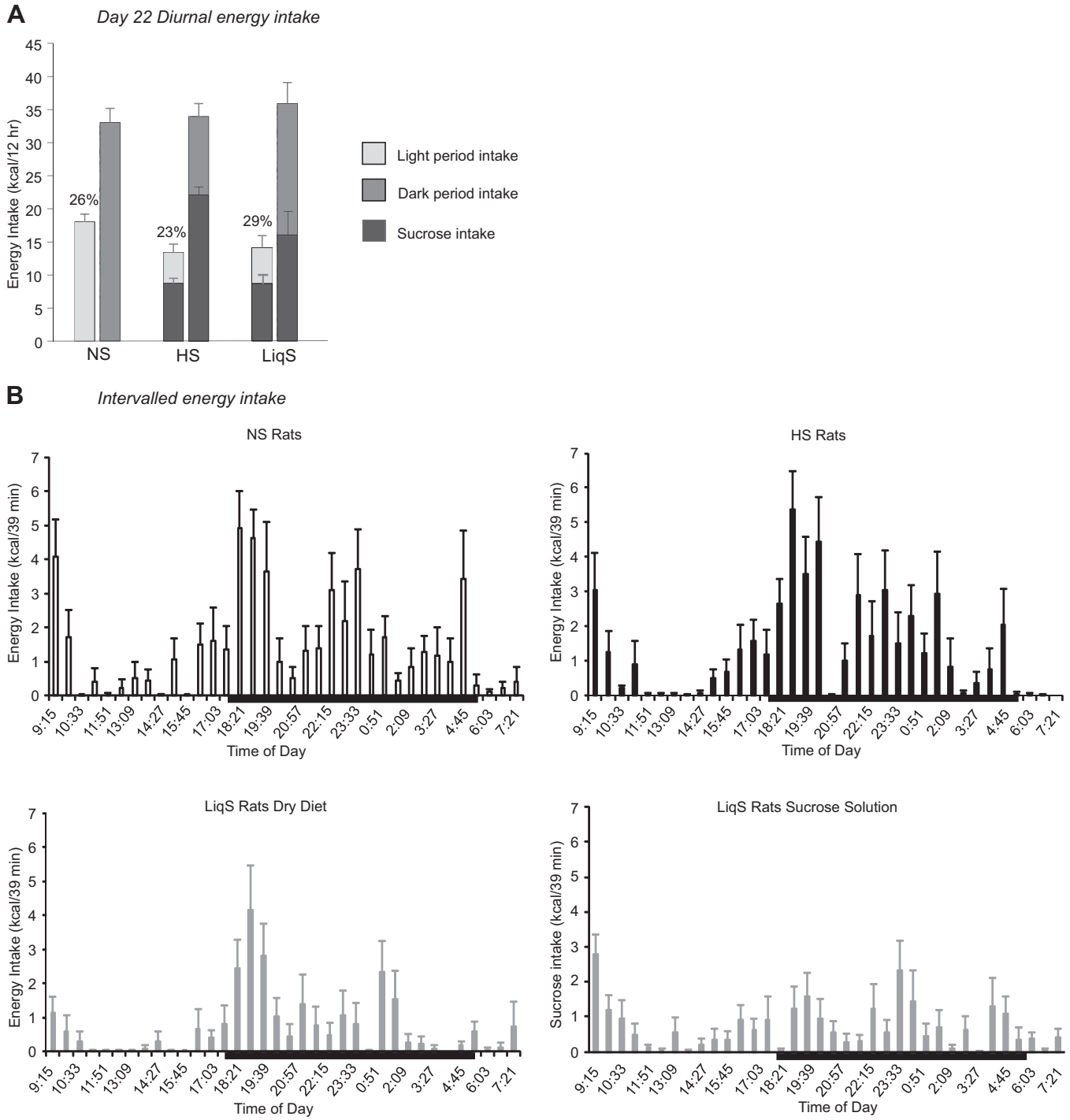


Figure 2. *A*: the percentage of daily energy intake consumed during the light and dark phase by rats offered diets of different sucrose content. There was no difference between groups for energy intake during the light period or during the dark period. *B*: the energy consumed every 39 min across 24 h for the three dietary groups. Data are means + SE for groups of 12 female Sprague-Dawley rats. The measurements were made on *day 22* when the rats had adapted to the calorimeter for 4 days. The dark bar on the *x*-axis in *B* indicates the dark period of the day.

or the sucrose that was combined with other nutrients in the HS diet. During the dark period, when all of the rats were eating, the RER of both LiqS and HS rats was higher than that of NS rats, even though the total carbohydrate content of NS and HS diets were the same (Fig. 3*B*; diet, NS; time, $P < 0.001$; interaction, $P < 0.009$). For the LiqS rats, RER increased for up to 2 h after drinking sucrose (Figs. 3*B* and 2*B*) and likely reflected the rapid availability of glucose as an energy substrate from sucrose compared with maltodextrin and cornstarch in the NS diet. This transient spike in RER did not translate into a significant difference in average RER during the light or dark period (diet, $P < 0.06$; light phase, $P < 0.0001$; interaction, NS). There was no effect of diet on IBAT temperature during either the light or dark period (Fig. 3*C*).

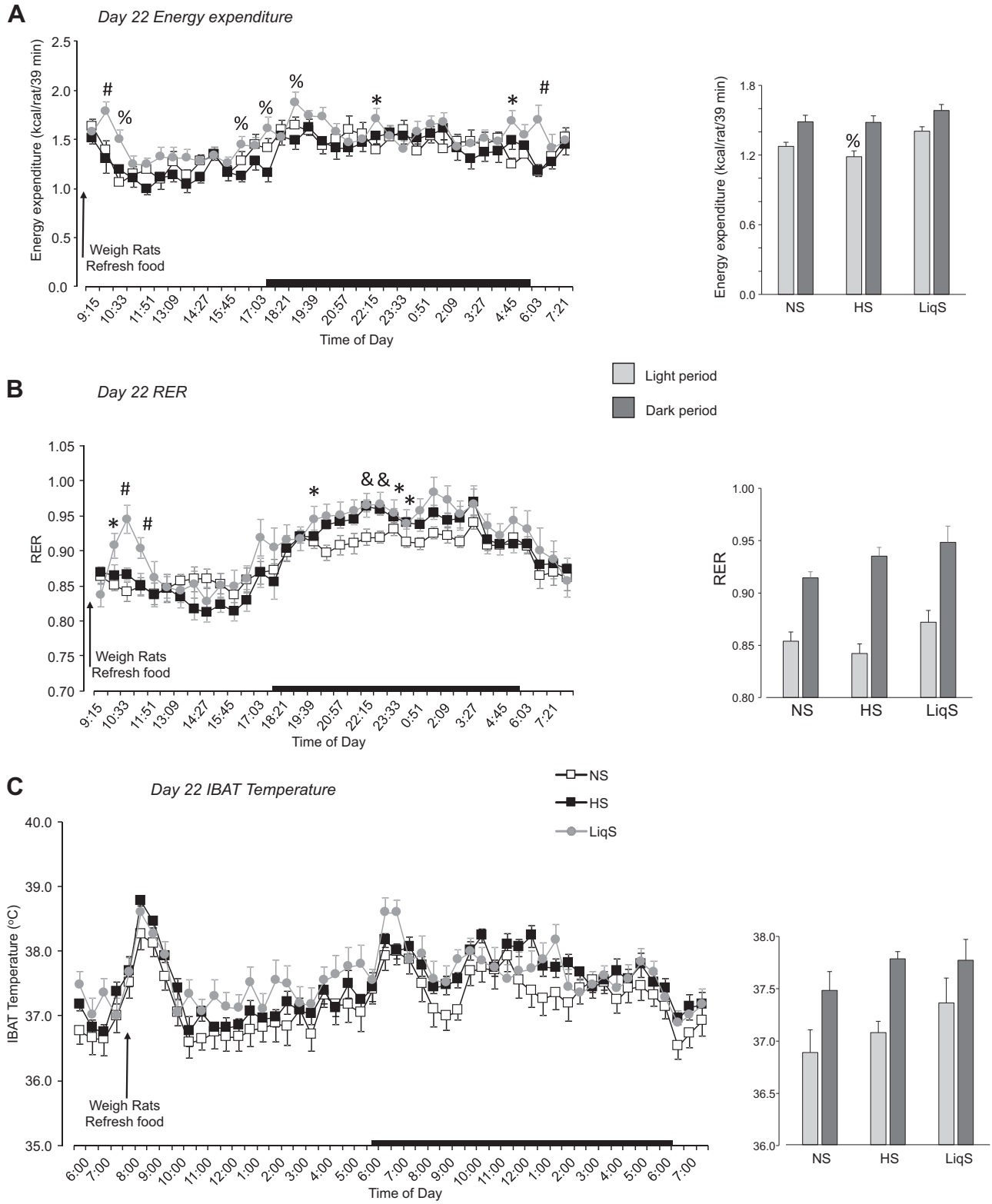


Figure 3. Energy expenditure, respiratory exchange ratio (RER), and intrascapular brown fat tissue (IBAT) temperature of female Sprague-Dawley rats on *day 22* of consuming diets of different sucrose content. Data are means ± SE for groups of 12 female Sprague-Dawley rats. Energy expenditure and RER were reported every 39 min, IBAT temperature was recorded every 30 min. In the charts on the left, the dark bar on the *x*-axis indicates the dark period. The bar graphs on the right illustrate averages for light and dark period for each parameter. Statistically significant differences ($P < 0.05$) between groups were determined by repeated-measures analysis of variance and pos hoc comparison at each time point using Tukey's honestly significant difference test. *Significant difference between NS and LiqS rats, %difference between HS and LiqS rats, #difference between LiqS and both HS and NS rats, &difference between NS and both HS and LiqS rats.

On *days 23* and *26*, the rats were tested for leptin responsiveness with each animal acting as its own control. Sucrose intake of HS and LiqS rats had stabilized during the 3 days before the first injection and although sucrose intake of LiqS rats was lower than that of HS rats (Fig. 1*B*), energy intake was the same for all three groups (Fig. 1*C*). Data were analyzed during the 12-h dark period following the intraperitoneal injection of PBS or 2 mg of leptin/kg. Leptin significantly inhibited energy intake of NS and HS rats but stimulated energy intake of LiqS rats (Fig. 4*A*; diet, NS; leptin, $P < 0.05$; time, $P < 0.001$; leptin × time, $P < 0.001$). The degree and timing of inhibition for HS and NS rats was different. Cumulative energy intake of HS rats was inhibited during the 2 h after leptin injection and there was a later, nonsignificant inhibition 10–12 h after injection (Fig. 4*B*), whereas leptin inhibited intake of NS rats 2–4 h after injection and again at the end of the 12-h period (Fig. 4*B*).

The minute-by-minute data on food and sucrose solution intake were used to analyze meal patterns during the first 2 h after leptin injection and again during the 4 h from 8 to 12 h after injection (Table 1). A meal was defined as consumption of 0.01 g or more and an intermeal interval was a period of ≥10 min between eating bouts. There was no effect of leptin on any aspect of sucrose solution intake during either of the time intervals considered. Analysis of dry diet intake confirmed that leptin only inhibited energy intake of HS rats during the first 2 h after injection. There was no effect of diet or leptin on meal number or intermeal interval but there was a significant effect of diet on meal size and duration (diet, $P < 0.005$; leptin, NS; interaction, NS). Post hoc analysis indicated that this was due to NS-fed rats eating larger meals than the LiqS PBS and LiqS lep rats. The analysis also indicated that leptin injection reduced meal size in HS rats. During the 4-h period starting 8 h after injection leptin inhibited intake of dry diet in all three dietary groups. There was no effect of diet or leptin on meal duration or meal number. A significant effect of both diet and leptin injection on meal size (diet, $P < 0.03$; leptin, $P < 0.04$; interaction, NS) was attributable to LiqS rats eating small meals and a nonsignificant leptin-induced reduction in meal size for all three dietary groups. There was also a significant effect of both diet and leptin on intermeal interval (diet, $P < 0.0001$; leptin, $P < 0.05$; interaction, NS), which was due to an extended interval between meals in LiqS lep rats.

There was no effect of diet or leptin on energy expenditure during the 12 h following injection (data not shown). Average RER of leptin-injected HS rats was significantly lower than that of HS PBS rats (0.920 ± 0.009 vs. 0.942 ± 0.006) during the 2 h following injection, when food intake was suppressed. There were no other effects of diet or leptin on RER (Fig. 4*C*). Comparison of IBAT temperatures for all rats showed a significant interaction between time and leptin injection (diet, NS; leptin, NS; time, $P < 0.0001$; time × leptin, $P < 0.003$). Comparisons of IBAT temperatures of rats within each dietary group following PBS or leptin injection showed a significant effect of leptin in HS but not in NS or LiqS rats (Fig. 4*D*; leptin, NS; time, $P < 0.001$; interaction, $P < 0.03$). Post hoc analysis revealed a transient increase of IBAT temperature ~4 h after leptin injection (Fig. 4*D*). When average IBAT temperature during the 6 h following injection was compared for all groups, leptin caused a significant increase in HS but not in NS or LiqS rats (Fig. 4*E*). Leptin reduced the amount of weight gained during the 14 h following injection (diet, NS; leptin, $P < 0.006$; interaction, NS), but post hoc analysis did not identify a significant difference between leptin and PBS treatment for any of the dietary groups (Fig. 4*F;* $P < 0.08$ for HS and LiqS rats, $P < 0.09$ for NS rats).

At the end of the experiment, there was no effect of diet on the size of RP or inguinal fat pads of the rats (inguinal fat: NS = 1.39 ± 0.07 g, HS = 1.23 ± 0.11 g, LiqS = 1.44 ± 0.11 g; RP fat: NS = 0.57 ± 0.04 g, HS = 0.50 ± 0.08 g, LiqS = 0.64 ± 0.06 g) and there was no effect of diet or leptin injection on brown adipose tissue UCP1 protein expression (Fig. 4*E*). Quantification of pSTAT3 in the hypothalamus and hindbrain showed a significant stimulation in response to leptin injection. In the hindbrain, leptin caused a substantial increase in NTS pSTAT3 but there was no effect of diet on the levels of pSTAT3 following PBS or leptin injection (Table 2). pSTAT3 was quantified at two different levels of the hypothalamus. At both levels and for all nuclei, there was a significant stimulation of pSTAT3 by leptin when data from all rats were analyzed (diet, NS;, leptin, $P < 0.01$; interaction, NS), however, post hoc analysis showed substantial differences in the degree of response by different nuclei. Basal levels of pSTAT3 tended to be higher in ArcM and ArcL than in the VMHdm, DMH, or DMC, but leptin caused a much greater stimulation of pSTAT3 in the VMHdm, DMH, and DMC than in the ArcM or ArcL (less than a doubling). At bregma −3.14 mm (Fig. 5), stimulation by leptin was significant in the VMHdm for all groups of rats, in the ArcM, stimulation was only significant in NS rats and there was no significant effect for any group in the ArcL. Leptin produced a significant increase in pSTAT3 in NS and HS but not in LiqS rats in the DMH. At bregma −3.30 mm, post hoc analysis did not identify a significant response to leptin in the ArcM or ArcL for any of the dietary groups. By contrast, leptin stimulated pSTAT3 in the VMHdm and DMC of all three groups (Table 2).

DISCUSSION

There were two primary objectives to this study, the first was to determine whether leptin resistance in rats given access to sucrose solution was applicable to any end points associated with energy balance beyond a failure to reduce energy intake. The results show that inhibition of food intake was the dominant response to a peripheral injection of 2 mg of leptin/kg and that the timing and degree of inhibition was changed by sucrose consumption. There was a small, transient increase in IBAT temperature of leptin-treated HS rats but there was no effect of leptin on energy expenditure of any of the groups. Others have reported that 7-day peripheral infusions of 1 mg of leptin/day from Alzet pumps (11) or single injections of 3 μg of leptin into the fourth ventricle (14) stimulate IBAT temperature and UCP1 expression due to increased sympathetic drive (10), but the results from this experiment are more consistent with a study in which much lower doses of peripheral infusions (40 μg/day) or third ventricle injections (1.5 μg twice a day) of leptin inhibited weight gain and food intake but did not increase norepinephrine turnover in IBAT (15). The second objective of this study was to examine the effect of diet on activation of the

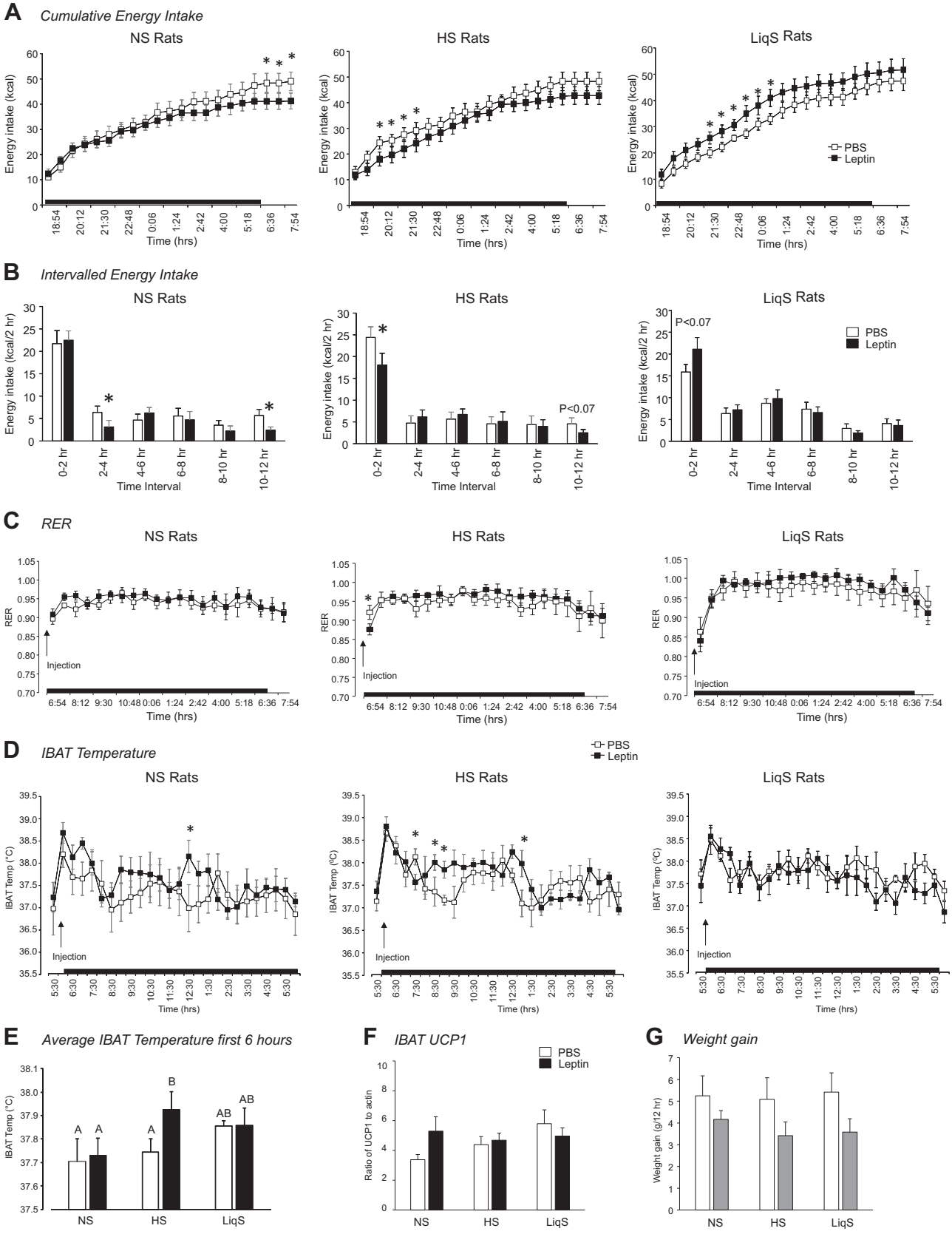


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Table 1. *Meal patterns following leptin injection*

	NS PBS	NS Leptin	HS PBS	HS Leptin	LiqS PBS Chow	LiqS Leptin Chow	LiqS PBS Sucrose	LiqS Leptin Sucrose
0–2 h postinjection								
Meal number	3.4 ± 0.4	2.5 ± 0.2	3 ± 0.4	3.5 ± 0.4	2.7 ± 0.2	2.8 ± 0.2	4.5 ± 0.3	4.4 ± 0.6
Meal size, kcal	7.9 ± 1.5^{C}	9.4 ± 1.1^{AC}	7.9 ± 0.8^{C}	6.7 ± 1.0^{B}	4.0 ± 0.7^{BC}	3.6 ± 0.6^{B}	1.6 ± 0.3	2.4 ± 0.6
Meal duration, min	13.3 ± 2.6^{AB}	16.1 ± 2.3^{A}	11.8 ± 2.0^{AB}	8.5 ± 1.1^{B}	6.8 ± 1.4^{B}	8.6 ± 1.2^{B}	8.8 ± 1.9	19.1 ± 7.9
Intermeal interval, min	25 ± 3	30 ± 2	29 ± 4	31 ± 8	39 ± 4	34 ± 2	23 ± 3	24 ± 4
Total intake, kcal/2 h	24.2 ± 2.5	24.1 ± 2.1	27.8 ± 2.0	20.2 ± 2.7*	10.9 ± 2.8	10.7 ± 2.7	5.0 ± 1.2	6.6 ± 0.8
8–12 h postinjection								
Meal number	2.2 ± 0.2	1.7 ± 0.3	1.7 ± 0.3	1.8 ± 0.3	1.6 ± 0.3	0.9 ± 0.5	7.6 ± 0.6	8.1 ± 0.8
Meal size, kcal	4.6 ± 1.1^{AB}	3.2 ± 0.9^{AB}	5.9 ± 1.3^{B}	3.0 ± 1.0^{AB}	2.5 ± 1.1^{AB}	1.4 ± 0.8^{A}	0.6 ± 0.2	0.5 ± 0.2
Meal duration, min	7.5 ± 1.1	5.0 ± 1.0	4.0 ± 0.9	3.1 ± 0.8	3.8 ± 1.3	3.9 ± 2.1	2.1 ± 0.3	2.7 ± 0.6
Intermeal interval, min	76 ± 6^{A}	92 ± 9^{A}	92 ± 10^{A}	94 ± 8^{A}	132 ± 23^{AB}	186 ± 23^{B}	29 ± 2	27 ± 4
Total intake, kcal/4 h	8.5 ± 1.5	4.9 ± 1.3*	9.4 ± 1.8	4.3 ± 0.9*	3.6 ± 1.2	1.8 ± 0.9*	3.9 ± 1.1	3.2 ± 1.3

Meal pattern data during the first 2 h following injection of 2 mg of leptin/kg or an equivalent volume of PBS or during the 4 h starting 8 h after injection. Injections were administered to rats that were food deprived from 8.00 AM to 6.00 PM following 23 or 26 days on experimental diet. Data are means ± SE for groups of 12 female rats offered a sucrose free diet (NS), a high-sucrose diet (HS), or NS diet plus access to 30% sucrose solution (LiqS). Values for a specific parameter related to meal patterns for dry diet that do not share a common superscript are significantly different at $P < 0.05$ determined by repeated-measures analysis of variance and post hoc Tukey's honestly significant difference (HSD) test with rat number as a cofactor. *Significant ($P < 0.05$) effect of leptin on total energy intake for a specific dietary group. Differences were determined by paired t test. Values for meals of sucrose solution were compared by paired Student's t test, but there were no significant differences between PBS- and leptin-injected condition.

transcription factor STAT3 in basal and stimulated conditions. We previously reported that basal levels of pSTAT3 in the arcuate nucleus were elevated in leptin-resistant male rats offered chow and sucrose solution (9), compared with those offered only chow, and this study allowed a comparison between rats consuming a sucrose-free diet (NS) and those consuming high level of sucrose from either a composite diet (HS) or as a sucrose solution (LiqS). The increase in activation of STAT3 following leptin injection was site specific, with a smaller response in the ArcM and ArcL than in other hypothalamic areas, and only the DMH showed a specific effect of consuming sucrose solution compared with sucrose from a composite diet.

Consistent with results from previous experiments (5, 16), LiqS rats reduced their intake of the sucrose-free diet when offered access to sucrose solution, but this only partially compensated for the calorie intake. LiqS rats had a significantly higher energy intake than NS and HS rats while they were housed in wire-mesh cages, but did not weigh significantly more than the NS controls, whereas HS rats ate the same as NS rats but gained less weight. This suggests that a high-sucrose diet results in a lower efficiency of energy utilization and implies that a thermogenic stress contributed to the differences in calorie intake or weight gain during the first part of the experiment when the rats were housed in wire-mesh cages, even though the animal room was maintained at the same temperature as the internal cage temperature for the calorimeter. Others have reported an increase in brown fat thermogenesis in male rats offered 10% sucrose solution in addition to chow for 3 wk (17). Although we did not observe a sustained elevation of IBAT temperature or an increase in IBAT UCP1 protein expression in HS or LiqS rats after they had been in the calorimeter, it is possible that IBAT thermogenesis was increased when they were housed in wire-mesh cages and that the decreased efficiency of energy utilization was compensated for by an increase in sucrose intake by LiqS rats, but that HS rats did not compensate, which led to a slower rate of weight gain. Once the rats were housed in the calorimeter, there were no longer any differences in energy intake or body weight of the rats, and, in these conditions, HS rats had a lower energy expenditure during the light period than LiqS rats, implying a decreased efficiency of energy utilization in the LiqS rats. However, there was no difference in average expenditure during the dark period, which would be expected if there was an inherent difference in nutrient metabolism, therefore it is possible that the difference was due to increased activity of LiqS rats during the light period when they continued to drink sucrose. It is difficult to determine the exact cause of the change in sucrose intake of LiqS rats when they were moved to the calorimetry cages because there were multiple differences in the housing conditions, including wire bottom versus enclosed cages with corn cob bedding, different types of bottles used for water and sucrose solution delivery, food in the bottom of the wire cage but in a hanging hopper in the calorimeter, and isolation from ambient

Figure 4. Cumulative energy intake over 14 h (*A*), intervaled energy intake over 12 h (*B*), intrascapular brown adipose tissue (IBAT) temperature over 12 h (*C*), respiratory exchange ratio (RER) over 12 h (*D*), or average IBAT temperature during 6 h (*E*), following an intraperitoneal injection of PBS or 2 mg of leptin/kg. *F*: IBAT UCP1 protein expression measured at the end of the experiment. *G*: weight change of the rats during the 12 h after injection. Data are means ± SE for groups of 12 female Sprague-Dawley rats offered diets of different sucrose content for 23 or 26 days. Each rat was its own control and injected with PBS or leptin in random order. Significant differences in cumulative intake and 12 h intrascapular brown fat tissue (IBAT) temperature were determined by repeated-measures analysis of variance and post hoc Tukey's honestly significant difference (HSD) test at each time point. Differences in interval intake were determined by paired t test at each time interval and differences in IBAT temperature averaged over 6 h were determined using two-way analysis of variance and post hoc Tukey's HSD test. Rat number was used as a covariate in all analyses except intervaled energy intake. *Significant difference between PBS- and leptin-injected conditions.

Table 2. *Hindbrain and hypothalamic pSTAT3*

	NS PBS	NS Leptin	HS PBS	HS Leptin	LiqS PBS	LiqS Leptin
NTS	3 ± 1^{A}	77 ± 15^{B}	5 ± 3^{A}	52 ± 12^{B}	12 ± 7^{A}	58 ± 12^{B}
Bregma −3.30 mm						
ArcM	78 ± 9	113 ± 25	94 ± 9	129 ± 24	68 ± 10	155 ± 28
ArcL	39 ± 6	45 ± 6	45 ± 7	47 ± 9	35 ± 6	72 ± 17
VMHdm	21 ± 5^{A}	135 ± 37^{B}	18 ± 5^{A}	180 ± 49^{B}	22 ± 8^{A}	154 ± 9^{B}
DMC	19 ± 7^{A}	119 ± 37^{B}	18 ± 4^{A}	139 ± 27^{B}	14 ± 3^{A}	177 ± 59^{B}

Number of pSTAT3 -positive nuclei measured 1 h after an ip injection of PBS or 1 mg of leptin/kg in female rats offered a sucrose-free diet (NS), a high-sucrose diet (HS) or NS diet plus access to 30% sucrose solution (LiqS). Data are means ± SE for six rats, except NTS for NS leptin, where $n = 4$ rats due to tissue damage during sectioning. Values for a specific nucleus that do not share a common superscript are significantly different at $P < 0.05$ determined by two-way analysis of variance and post hoc Tukey's honestly significant difference test. ArcL, lateral arcuate nucleus; ArcM, medial arcuate nucleus; DMC, dorsomedial nucleus of the hypothalamus, compact part; NTS, nucleus of the solitary tract; pSTAT3, phosphorylation of signal transducer and activator of transcription 3; VMHdm, dorsomedial ventromedial nucleus of the hypothalamus.

noise in the calorimetry cages compared with in the wire cages. However, it is unlikely that the drop in sucrose solution intake while LiqS rats were in the calorimeter was due to difficulty in obtaining the solution as bottles of water and 30% sucrose were both freely available in both housing conditions and the sucrose solution was replaced each day, which also ensured that the sippers were working and had not become blocked with sucrose crystals. It also is unlikely that the bottles on the wire-mesh cages were leaking and causing an overestimate of sucrose intake because sucrose would have been observed on the spill papers, which were changed daily, and leaking bottles would have resulted in large daily variations in apparent intake of sucrose solution, which was not the case for either cage system.

It was possible to measure the diurnal pattern of energy intake of the rats when they were housed in the calorimeter. All three dietary groups consumed less than a third of their total daily energy intake during the light period, but the LiqS rats consumed almost 40% of their sucrose intake as small meals throughout the light period with almost no consumption of dry diet. HS rats consumed more sucrose than LiqS rats when they were in the calorimeter and this difference was all attributable to night-time intake. Sucrose consumption had no effect on whole animal energy expenditure or brown fat thermogenesis while the rats were in the calorimeter, but RER of both LiqS and HS rats was higher than that of NS rats during the dark period, when the rats had their highest rate of food intake. The increased RER, which is indicative of increased utilization of carbohydrate as an energy substrate, most likely resulted from the rapid digestion and absorption of simple sugars from sucrose, compared with the slower digestion of the starch and maltodextrin in the NS diet.

A previous experiment demonstrated that leptin resistance in LiqS rats is due to consumption of carbohydrate and is not secondary to intake of a preferred sweet solution (16), and we have previously hypothesized that the frequent small meals of sucrose by LiqS rats throughout the light and dark period have a metabolic impact that contributes to the development of leptin resistance (9). A persistent elevation of blood glucose levels would potentially increase activity of the hexosamine biosynthetic pathway (HBP) (18), which results in *O*-GlcNAcylation of serine and threonine residues of multiple proteins (19), including STAT3 (20). This labile modification has the potential to impair bioactivity and has been implicated in the development of cancer (21), heart disease (22), and insulin resistance (23). Leptin resistance in rats offered chow plus sucrose solution is associated with increased basal levels of pSTAT3 that are not stimulated further by leptin and direct activation of the HBP with glucosamine infusion produces a similar change in hypothalamic pSTAT3 (9). Measurement of the enzymes responsible for glucose entry into the HBP [glutamine fructose-6-phosphate amidotransferase (GFAT)], addition of a single *O*-GlcNAc moiety to a serine or threonine [*O*-linked *N*-acetylglucosamine transferase (OGT)], or reversal of *O*-GlcNAc protein modification [*O*-linked *N*-acetylglucosamine transferase (*O*-GlcNAcase)] showed increased basal levels of GFAT and OGT in the liver of sucrose-drinking rats and a decrease in *O*-GlcNAcase in the hypothalamus (9). In vitro studies with HepG2 cells confirmed that elevated concentrations of glucose in the media stimulated the HBP and raised basal levels of pSTAT3 that could not be stimulated further by leptin (24). More focused investigation of HBP activity in LiqS and HS rats is needed first to determine whether HBP activity is increased in both HS and LiqS rats and, if not, whether there is an association between *O*-GlcNAcylation of STAT3 and leptin resistance in LiqS rats. Because the rats in this experiment were perfused to quantify hypothalamic and brainstem STAT3 activation, it was not possible to also investigate the levels of *O*-GlcNAcylation.

Unexpectedly, not only did leptin fail to inhibit energy intake of LiqS rats but it caused a significant increase in cumulative energy intake, which appeared to be due to a nonsignificant but uncompensated stimulation of energy intake during the first 2 h after injection. We have reported a stimulation of energy intake by leptin in a number of different conditions in both mice (25) and rats (26–29), but have not identified the mechanism of response. By contrast, leptin injection caused a relatively small, but significant reduction in 12-h intake of both LS and HS rats. The small response to leptin may be attributable to the mode of administration of leptin, which has a half-life of 24–40 min (30, 31). Leptin injections cause a very large but transient increase in circulating leptin, whereas leptin is normally released continuously into the circulation from adipose tissue. We have found that 2 mg/kg is the lowest injected dose that reliably inhibits food intake even though it increases circulating leptin concentrations 500-fold (7), whereas as little as 50 μg/kg stimulates hypothalamic pSTAT3 (32). Lower doses of leptin

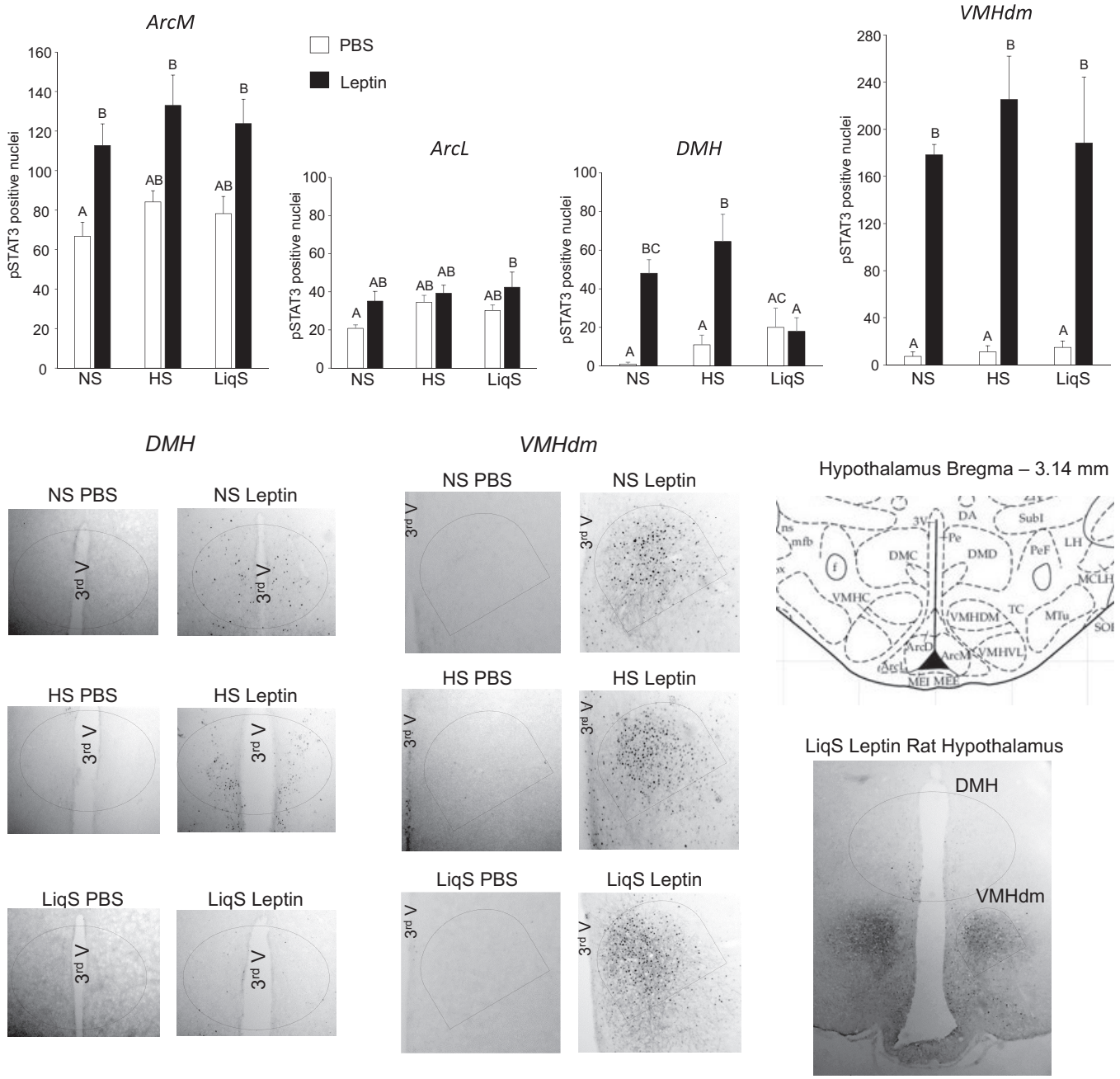

Figure 5. Number of phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) -positive nuclei measured 1 h after an intraperitoneal injection of PBS or 1 mg of leptin/kg in female rats offered a sucrose-free diet (NS), a high-sucrose diet (HS), or NS diet plus access to 30% sucrose solution. Data are means ± SE for groups of four to six rats measured at bregma −3.14 mm according to the Paxinos and Watson Rat Brain Atlas. Values for a specific nucleus that do not share a common superscript are significantly different at $P < 0.05$ determined by two-way analysis of variance and post hoc Tukey's honestly significant difference test. ArcM, medial arcuate nucleus; ArcL, lateral arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus (incorporates DMH and DMC identified in atlas); VMHdm, dorsomedial ventromedial nucleus of the hypothalamus. Images are representative of pSTAT3 expression in the DMH and VMHdh of rats from the different dietary groups. The DMH and VMHdm image are from the same rat for each treatment group. Dotted lines outline the nuclei based on the schematic for the hypothalamic nucleus from the Paxinos and Watson atlas of the rat brain (13), with permission.

can be used with peripheral infusions from an Alzet pump, but it is more difficult to detect leptin resistance because there is an initial hypophagia and weight loss followed by normalization of food intake and maintenance of a reduced weight without any significant change in circulating leptin levels (15, 33). Another alternative would be to administer leptin centrally. In a previous study, we found that a third ventricle injection of leptin inhibited food intake of control rats, but not of rats offered a choice of chow, sucrose solution, and lard. However, there was no effect of leptin on energy expenditure of either group (3).

The inhibitory effect of leptin on food intake of HS rats but not LiqS rats confirms that the resistance is due to some, as yet undefined, aspect of drinking sucrose and is not

simply due to consumption of calories as sucrose. Although both NS and HS rats responded to leptin in this experiment by reducing their food intake, the timing of inhibition was different for the dietary groups. The energy intake of HS rats was inhibited immediately after leptin injection due to a decreased meal size, implying early satiation. This would be consistent with reports that leptin synergizes with gastrointestinal short-term signals of satiety, such as CCK (34) and gastric distention (35). It is possible that 10 h of food deprivation before injection caused hunger signals to override the early impact of leptin on intake in NS rats that did not respond to leptin until 4 h after injection. The HS rats were also food deprived but rapid digestion of sucrose and absorption of the simple carbohydrate could have corrected the energy deficit enough to make the rats sensitive to leptin as soon as the first 2 h after injection. The interaction between leptin and CCK has been attributed to vagal afferent neurons (36) and leptin resistance in these neurons contributes to the development of obesity (37), thus an insensitivity to leptin in vagal afferents may contribute to leptin resistance in LiqS rats.

There was also a delayed inhibition of energy intake that had a significant impact on cumulative intake of NS rats but not HS rats. The data collected for analysis of meal patterns indicated that the delayed inhibition of intake of dry food was significant for all groups of rats, but because this made only a small contribution to total energy intake of LiqS rats during the later time period, there was no effect of leptin on total energy intake of these rats. This delayed inhibition was not associated with a reduced meal size or number, implying a different mechanism of response than the early inhibition observed exclusively in HS rats. However, because the rats were eating much less food 8 h after leptin injection than immediately after injection, it may have been impossible to detect changes in meal pattern. Meal size and duration were decreased and intermeal interval was extended for dry food for all rats at the later time point, compared with the 2 h immediately after injection. By contrast, intermeal interval did not change for consumption of sucrose solution, although the size of each meal was much smaller during the later time interval. These data illustrate that LiqS rats maintain a steady pattern of frequent meals of sucrose solution, even when their calorie intake is reduced. Further studies are needed to determine whether the metabolic impact of this pattern of intake contributes to leptin resistance.

At the end of the study, STAT3 activation was measured 1 h following a leptin injection. This transcription factor has been shown to be essential for leptin's inhibition of food intake (6) and phosphorylation peaks 1–2 h following leptin administration (7). Leptin injection caused a substantial increase in pSTAT3 in the hindbrain and VMHdm of all rats. Others have reported that activation of VMH leptin receptors attenuates the development of diet-induced obesity (38, 39), primarily due to a failure to increase thermogenesis (40), but in this study leptin had no effect on energy expenditure of any of the rats, including those consuming a significant proportion of their energy from sucrose solution. The studies reporting that VMH leptin receptors inhibit development of diet-induced obesity used mice in which leptin receptors (38, 39) or leptin-signaling proteins (40) were selectively deleted from the VMH. Therefore, there was a chronic, total loss of leptin activity in this nucleus, which could have a different effect on feeding behavior than acute changes in receptor activity. Alternatively, activation of VMH leptin receptors could cause very small, but chronic changes in energy intake or expenditure that would influence energy balance over the long term, but could not be detected during the short time periods evaluated in this experiment.

The arcuate nucleus of the hypothalamus is generally identified as the primary site of control of food intake by leptin (41) and selective replacement of leptin receptors in the arcuate nucleus of leptin-receptor-deficient rats produces a significant inhibition of food intake and weight loss (42). In this study, leptin injection increased pSTAT3 in both the medial and lateral areas of the arcuate nucleus, but the increase reached significance only for NS rats at bregma −3.14 mm. Therefore, although it is well established that leptin action in the arcuate nucleus is required for maintenance of a normal food intake and body composition in basal conditions (42), the degree of response of the arcuate nucleus to leptin administration was less than that of the hindbrain and other areas of the hypothalamus that were quantified in this study. These results are consistent with the outcome of an experiment measuring pSTAT3 in different nuclei of rats injected with increasing doses of leptin (32). The arcuate nucleus was the most sensitive to an increase in leptin, but also reached a maximal level of activation at lower doses of leptin than are required to induce an inhibition of food intake. As noted above, we previously identified an increase in basal pSTAT3 in the arcuate nucleus of chow-fed male rats drinking sucrose (9). That observation was not confirmed in this experiment using female rats consuming purified diets. Leptin produced a significant increase in Arc pSTAT3 only in NS rats, but it is worth noting that leptin-stimulated levels of pSTAT3 were the same for all dietary groups and it was a nonsignificant increase in basal pSTAT3 in HS and LiqS rats that prevented the response from reaching significance and this increase was equivalent in magnitude to that reported for chow-fed rats (9). It is also worth noting that this increase was dependent on sucrose consumption, irrespective of whether it was part of a dry diet or offered as a solution.

The dorsomedial hypothalamus was the only area quantified that showed a differential response to leptin based on diet. At bregma −3.14 mm, DMH pSTAT3 was increased at least fourfold in NS and HS rats but did not change in LiqS rats, whereas leptin increased pSTAT3 for all rats in the DMC at bregma −3.30 mm. Zhang et al. (43) used transynaptic tract tracing to demonstrate that leptin receptor-expressing neurons in the DMH project to brown adipose tissue of mice, and Rezai-Zadeh et al. (44) demonstrated that stimulation of leptin receptors in the DMH of mice contribute to the control of body temperature and IBAT thermogenesis potentially via projections to the parvicellular paraventricular nucleus of the hypothalamus (PVN) that contributes to the control of the autonomic nervous system. In the rats in this study, leptin increased IBAT temperature only in HS rats, despite stimulating DMH pSTAT3 in both NS and HS rats. Activation of leptin receptors in the DMH has also been reported to increase physical activity, reduce food intake, and cause weight loss (45) through local inhibition of neuropeptide Y (46). Physical activity of the rats was not measured in this experiment and a single injection of leptin did not cause

significant weight loss, however, pSTAT3 levels in the DMH correlated with inhibition of food intake, even though the timing of inhibition differed between NS and HS rats. Interestingly, chemical lesions of the DMH in rats results in a loss of the circadian variation in feeding, sleep, and corticosterone release (47), and it is possible that reduced activation of the DMH in LiqS rats contributed to the lack of circadian variability in sucrose intake.

PERSPECTIVES AND SIGNIFICANCE

Leptin resistance has been identified as a causative factor in the development of obesity (48), and many animal studies have focused on the impact of high-fat diets on leptin responsiveness (1). In this study, rats offered sucrose solution developed leptin resistance, whereas those consuming large amounts of sucrose from a dry diet did not. The resistance developed faster than has been reported for consumption of a formulated high-fat diet and in the absence of a change in body fat mass, implying that activation of specific metabolic pathways by different nutrients has the potential to influence sensitivity to hormones that contribute to the control of energy balance. Although it has been reported in other studies that leptin stimulates energy expenditure and IBAT thermogenesis, we found that inhibition of food intake was the primary response to the dose of leptin injected in this study. Evaluation of pSTAT3 in the hypothalamus suggested that, although the Arc is required for maintenance of a normal body weight and composition, other hypothalamic sites were more responsive to elevations of leptin above basal levels. The DMH, which has been implicated in the stimulation of thermogenesis by leptin, was the only nucleus in which activation of STAT3 correlated with inhibition of feeding by leptin. These results highlight the need to obtain a better understanding of the role of each hypothalamic nucleus as a mediator of leptin action.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

R.B.S.H. conceived and designed research; performed experiments; analyzed data; interpreted results of experiments; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript.

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