Research report

Glucose injection reduces neuropeptide Y and agouti-related protein expression in the arcuate nucleus: A possible physiological role in eating behavior

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Accepted 5 December 2004
Available online 8 February 2005

Abstract

Evidence suggests that neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus (ARC) are modulated by glucoregulatory hormones and involved in maintaining normal eating patterns and glucose homeostasis in states of energy deficiency. This study investigated whether these peptides respond to glucose itself under conditions, e.g., before the nocturnal feeding cycle, when carbohydrate stores are low. After removal of food 3 h before dark onset, Sprague–Dawley rats were given a single, intraperitoneal (i.p.) injection of saline or 10% glucose (0.13 g/kg) and were sacrificed at different intervals, from 3.5 to 90 min later, for measurements of circulating hormones and metabolites or of NPY and AgRP mRNA in the ARC. With no change in insulin, leptin, or triglycerides, glucose injection produced a 1.8-mM rise in circulating glucose during the first 15 min, followed by a 30–60% reduction in NPY and AgRP mRNA at 30 and 60 min post-injection. A similar effect was observed with intraventricular administration of 5% glucose. At 90 min, however, this suppressive effect of i.p. glucose relative to saline was lost and actually reversed into a 50% increase in NPY and AgRP, possibly attributed to a decline in circulating glucose followed by a 50% rise in corticosterone at 60 min. These biphasic shifts over a 90-min period may reflect mechanisms underlying normal eating patterns at the onset of the nocturnal cycle, when spontaneous meals are approximately 90 min apart and rich in carbohydrate, glucose levels are low, and corticosterone and ARC peptides naturally peak.

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1. Introduction

The peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP), are known to have an important role in eating and body weight regulation. These peptides, which co-exist in neurons of the arcuate nucleus (ARC) [8,25], stimulate feeding, inhibit energy expenditure, and produce obesity when administered chronically [15,24,29,33,66]. To understand the role of these peptides in normal feeding patterns and weight control, numerous studies have

examined factors that control their expression and production in the ARC. Evidence demonstrates that NPY and AgRP mRNA and peptide immunoreactivity are suppressed by adrenalectomy and stimulated by replacement of the glucocorticoid, corticosterone (CORT) [3,57,67]. In contrast, NPY and AgRP are inhibited by both insulin and leptin, which rise with obesity [15,45,51,59,70], and they are over-expressed in insulin-deficient diabetic rats or leptin-deficient ob/ob mice [15,45,51,59,76]. Further, these peptides together with CORT are stimulated by 2-deoxy-D-glucose (2-DG), which produces glucoprivation [1,60], and also in physiological states of energy deficiency or increased energy demand, including at the onset of the natural feeding cycle or after periods of food deprivation [15,33,42,45,56].
Additional evidence obtained from pharmacological and biochemical studies suggests that NPY may be related to dietary carbohydrate, which is most effective in replenishing carbohydrate stores and promoting de novo lipogenesis in states of energy insufficiency [30,33]. The injection of NPY can preferentially enhance the consumption of carbohydrate [65,75], particularly when the carbohydrate is sweet [22], and it can stimulate the consumption of a sucrose solution [5,41]. Conversely, the expression of NPY in the ARC and levels of peptide in the paraventricular nucleus (PVN) are stimulated, together with circulating CORT levels, by the consumption of a high-carbohydrate (65%) compared to moderate-carbohydrate (45%) diet [21,71,74] and are positively correlated specifically with the amount of carbohydrate consumed by rats given a choice of dietary macronutrients [28,67]. Also, measurements across the light–dark cycle reveal a sharp rise in levels of NPY and CORT at the onset of the nocturnal feeding period, when energy stores are at their nadir and carbohydrate is the preferred macronutrient [32,67]. These levels are even further enhanced, along with circulating glucose, when the first meal of the dark is particularly rich in carbohydrate [73]. Together with evidence that NPY stimulates glucose uptake and metabolism in peripheral tissues [43,69], these findings linking NPY to circulating CORT and carbohydrate intake suggest a role for this peptide in glucose homeostasis, specifically under conditions when carbohydrate stores are low [33,67]. Evidence that AgRP may be similarly responsive to hormones and glucoprivation and is also stimulated by a high-carbohydrate compared to low-carbohydrate diet [15,45,51,60,74] suggests a similar function for this peptide as well.

This proposed relationship of NPY and AgRP to glucose homeostasis in states of energy deficiency leads one to question whether these hypothalamic peptides are responsive to glucose itself. Glucose is transported across the blood–brain barrier and taken up by neurons and astrocytes [68], and glucose-sensing neurons have been identified that alter their firing rate in response to glucose administration [35,48]. Those that increase their firing are referred to as glucose-excited (GE, formerly “glucose-responsive”), while those that decrease their firing are referred to as glucose-inhibited (GI, formerly “glucose-sensitive”). These glucosensing neurons exist in the ARC, as well as the paraventricular and ventromedial nuclei and lateral hypothalamus [36,46], and evidence suggests that NPY-expressing neurons in the ARC, which are likely to co-express AgRP, are GI-type neurons. They are inhibited by a rise in glucose in vitro [18,46], and they colocalize the enzyme, glucokinase, as well as KIR6.2, the pore-forming subunit of the ATP-sensitive K+ channel, both of which are involved in the glucose-sensing process [40]. There is only one in vivo study, however, indicating that glucose may actually modulate NPY. This report demonstrates that, prior to the onset of the natural feeding cycle, NPY gene expression and peptide immunoreactivity in the ARC, together with circulating CORT, are stimulated 90 min after injection or ingestion of glucose [73]. This effect does not agree with the existing evidence suggesting that ARC NPY neurons are inhibited by glucose, thus leading us to perform a more in-depth analysis of this phenomenon.

In this study, five experiments were performed to assess in vivo the impact of glucose injection, both peripheral and central, on gene expression of NPY and its co-existing peptide, AgRP, in the ARC. This was examined a few hours prior to the onset of the natural feeding cycle, as a function of time after glucose injection and also in relation to changes in circulating levels of glucose, in addition to CORT, insulin, leptin, and triglycerides. The results of these experiments demonstrate reproducible, time-dependent changes in the NPY and AGRP peptide systems that occur in close association with changes in circulating glucose and CORT. These effects suggest that ARC neurons expressing both NPY and AgRP can, in fact, respond to transient changes in circulating or brain glucose within a physiologic range, specifically under conditions, e.g., at the onset of the natural feeding cycle, when carbohydrate stores are low.

2. Materials and methods

2.1. Animals

Adult, male Sprague–Dawley rats (Charles River Breeding Labs, Kingston, NY) were individually housed (22 °C, with lights off at 3:30 p.m. for 12 h), in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility, 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 Committee. All protocols fully conformed to the Guiding Principles for Research Involving Animals and Human Beings [4]. The rats were given 1 week to acclimate to lab conditions and were maintained ad libitum on standard lab chow and water. They were of normal body weight (275–300 g) and were normophagic throughout the experiment, consuming 25–30 g/day.

2.2. Test procedures

Following the 1-week period of adaptation to the lab conditions and chow diet, the rats were handled daily. Food intake measurements were taken twice weekly and body weight once a week. In Experiments 1–4, the rats were adapted to the procedures of intraperitoneal (i.p.) injection and for the actual experiment received a single i.p. injection of saline or 10% glucose (0.5 ml) 3 h before onset of the dark cycle. (Similar procedures were used in preliminary tests in which 20% i.p. glucose was examined.) In Experiment 5, the rats received cannula implants aimed at the third ventricle and for the actual test were given a single i.c.v. injection of artificial cerebral spinal fluid (aCSF) or 5% glucose solution.
(8 μl) also 3 h before dark onset. In these rats, a 22-gauge stainless steel guide cannula was stereotaxically implanted, under pentobarbital anesthesia (50 mg/kg i.p.), into the 3rd ventricle using the following coordinates: relative to bregma, AP −0.8 mm, ML 0.0 mm, and DV −7.0 mm, with nose bar at −3.3°. A stainless steel wire obturator (28-gauge) was inserted to prevent leakage of CSF, and the guide cannula was fixed to the skull with dental cement. After 7 days of recovery, the rats were adapted to the test procedures with two i.c.v. injections of aCSF.

Each experiment tested multiple groups, with a range of 4–10 rats/group. At different time intervals after vehicle or glucose injection, some rats (Experiment 3) were given tail vein punctures, and blood was collected for measurements of circulating glucose levels. Other groups (Experiments 1, 2, 4, and 5) were sacrificed by decapitation, and their brains were rapidly removed and trunk blood collected for analysis of hormones and metabolites. For in situ hybridization histochemistry, the brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 3 days and cryoprotected in 25% sucrose-phosphate buffer at 4 °C for a further 48 h, then frozen at −80 °C until day of use. For real-time quantitative PCR, the brains were placed in a brain matrix with the ventral surface facing up, and two 1.0-mm coronal sections were made, with the middle optic chiasma as the anterior boundary. The sections were placed on a glass slide, and the ARC (Bregma −2.56 to −3.3 mm) was rapidly microdissected under a microscope as described [10] and immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Hormone and metabolite determinations

In some experiments, blood was collected via tail vein puncture, whereas in others, trunk blood was collected at sacrifice. Serum was analyzed for the hormones, leptin and insulin, using assay kits from Linco Research Inc, MO, while CORT was measured using an assay kit from MP Biomedicals, NY. Serum metabolites, glucose and triglycerides, were measured with an E-Max Microplate Reader using glucose Trinder Reagent Kit or Triglyceride Assay Kit, respectively (Sigma, St. Louis, MO).

2.4. Real-time quantitative PCR

As previously described [10], total RNA from pooled microdissected ARC samples was extracted with TRIZol reagent. RNA was treated with RNase-free DNase I to remove any contaminating genomic DNA before RT. 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, CA) was used, with β-actin as endogenous control. PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection system (Applied Biosystems), with the condition of 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each study consisted of 4 independent runs of PCR in triplicate, and each run included a standard curve, non-template control, and negative RT control. The levels of NPY and AgRP gene expression were quantified relative to the level of β-actin, using standard curve method. The primers, designed with ABI Primer Express V1.5a software on published sequences, were: (1) β-actin: 5'-GGCCAACCCGTGAAAGATGA-3' (forward) and 5'-CACACGCTTGATGCTACGT-3' (reverse); (2) NPY: 5'-CACAGAAGATGCCCCAGAA-3' (forward) and 5'-GTCAAGGAGCAATTTCTATTCC-3' (reverse); and (3) AgRP: 5'-GCAGAGGTGCTAGATCCACAGAA-3' (forward) and 5'-AGGACTGTGCGACCTTA-CAC-3' (reverse). The concentrations of primers were 100 to 200 nM, and all reagents, unless indicated, were from Invitrogen.

2.5. In situ hybridization histochemistry with digoxigenin-labeled probe for AgRP

Brains were cut into 30-μm-thick sections using a cryostat. A consistent angle of cut was maintained by examining the shape of the 3rd ventricle. Brains within a given experiment were always processed at the same time to maintain stringent tissue preparation and staining conditions. A digoxigenin-labeled antisense RNA probe was in vitro transcribed, as described [6]. Free-floating, coronal sections were consecutively treated as follows: 10 min in 0.001% protease K, 5 min in 4% paraformaldehyde, 10 min each in 0.2 N HCl and acetylation solution, and 18 h in hybridization buffer (at 55 °C), between each step 2 × 5 min wash in PB (0.1M, pH7.2). This was followed by 20 min in 5 × SSC, 30 min in 50% formamide, both at 60 °C, 30 min in RNase A (1 μg/ml) at 37 °C. Then, the sections were incubated in AP-conjugated sheep anti-digoxigenin Fab fragments (1:500, Roche) for 16 h and developed in freshly prepared color developer [50 μl 4-nitroblue tetrazotium chloride solution, 37.5 μl 5-bromo-4-chloro-3-indolyl-phosphate solution in 10 ml Tris buffer, pH 9.5 (Roche)]. Finally, the sections were fixed for 10 min in 4% paraformaldehyde, then mounted, air-dried, dehydrated, cleared, and coverslipped. The sense probe control was performed in the same tissue, and no signal was found. All procedures were conducted at room temperature, unless otherwise indicated.

Sections were viewed using a Leitz microscope with a 10× illumination objective, and images were captured with a Nikon DXM 1200 digital camera. Captured image was analyzed using Image-Pro Plus software (Version 4.5, Media Cybernetics Inc., Silver Spring, MD 20910, USA) on a gray-value scale from 1 to 255. The coordinates of the ARC were Bregma −2.8 mm according to the atlas of Paxinos and Watson [49]. Ten sections at the same level in this nucleus were examined in each animal. The area of interest was outlined, and the number of AgRP neurons in
this area was counted and expressed as cell density (AgRP cells/mm²) and integrated optical density (area × average intensity). Average density in the ARC for the different groups was compared and statistically analyzed. Before measurement, a threshold was first established. Using 10 randomly selected sections from the experiment, this threshold was set by matching the number of cells counted by the software in a defined area of each section with the number of cells counted manually in that same area. The value of 100 was obtained for mRNA. When these numbers of cells were found to match in all 10 sections, this threshold value, which varied across areas, was kept constant and used for all sections viewed within a given experiment. The analyses were performed by an observer blind to the identity of the animals.

2.6. Radioactive in situ hybridization histochemistry

Besides quantitative real-time PCR, mRNA levels of NPY and AGRP in ARC were measured with radioactive in situ hybridization histochemistry in rats sacrificed at 30 or 90 min after i.p. injection of 10% glucose or saline. Antisense RNA probes and sense probes were labeled with 35S-UTP (Amersham Biosciences), as described [6,73]. Alternative 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 10 min wash in PB between each step. After wash, the sections were hybridized with 10³ cpm/µl 35S-labeled probe at 55 °C for 18 h. Following hybridization, the sections were washed in 4× SSC, and nonspecifically bound probe was removed by RNase (Sigma) treatment for 30 min at 37 °C. Then, sections were run through further stringency washes with 0.1 M dithiothreitol (Sigma) in 2× SSC and 1× SSC and 0.1× SSC at 55 °C. Finally, sections were mounted, air-dried and exposed to Kodak BioMax MR film for 48–72 h at −80 °C, when films were developed and macroscopically 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 [39,52] on the MCID image analysis system (Image Research, Inc., St. Catherines, Canada). Microscale 14C standards (Amersham Biosciences) were exposed on the same Kodak film with the sections and digitized. Gray level/optical density calibrations were performed by using a calibrated film strip ladder (Imaging Research, St. Catherines, ON, Canada) for optical density. Optical density was plotted as a function of microscale calibration values. It was determined that 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 to 12 sections per animal. In each section, optical density for the ARC was recorded, from which the background optical density from a same size area in the thalamus was subtracted. Mean values of glucose-injected groups were reported as percentage of saline-injected, control group.

2.7. Data analysis

All values were expressed as mean ± SEM. Hypotheses regarding group and brain area differences in different measures were tested using either a one-way or two-way ANOVA, followed by a Bonferroni post hoc test for multiple comparisons between groups, or an unpaired t test, where appropriate. For quantitative PCR, the ratio of the relative concentration of peptide to β-actin mRNA in the experimental vs. control samples was analyzed by an unpaired t test. The criterion for the use of the term ‘significant’ in the text was that the probability value (P) for a given test be <0.05.

3. Results

3.1. Experiment 1: effect of peripheral glucose injection on NPY and AgRP at 90 min

To reexamine the finding that i.p. glucose after a 90-min period stimulates NPY in the ARC [73], this experiment performed a similar experiment in two sets of rats with measurements of AgRP as well as NPY. In Group 1 (n = 20), AgRP mRNA was measured via in situ hybridization histochemistry with a digoxigenin-labeled probe, as previously reported for NPY [73], and in Group 2 (n = 14), both NPY mRNA and AgRP mRNA were measured using real-time quantitative PCR. With food absent, rats were given a single i.p. injection of saline or 10% glucose (0.13 g/kg) 3 h before dark onset and were sacrificed 90 min later. With both techniques, measurements of mRNA levels at 90 min showed a statistically significant, 20–40% increase (P < 0.01) in peptide gene expression after glucose injection compared to saline, thus confirming the original finding [73]. This was demonstrated for measurements of AgRP mRNA (cell density and integrated optical density) using in situ hybridization histochemistry in Group 1 (Fig. 1) and of both NPY and AgRP (ratio of peptide mRNA/β-actin mRNA) via quantitative PCR in Group 2 (Fig. 2). Assays of circulating metabolites and hormones in trunk blood of both groups revealed little change at this 90-min test interval, in levels of glucose, CORT, insulin, leptin, or triglycerides, as shown for Group 1 (Table 1).

3.2. Experiment 2: impact of peripheral glucose injection on NPY and AgRP as a function of time

To determine more precisely the temporal nature of the relationship between glucose and peptides, rats were sacrificed at different time intervals after peripheral glucose injection, and their peptide gene expression in the ARC was
measured via real-time quantitative PCR. Using similar procedures as in Experiment 1, the rats \((n = 45)\) were given i.p. injection of saline or 10% glucose \((0.13 \text{ g/kg})\) and were sacrificed at 30, 60, or 90 min after injection. The measurements of gene expression in glucose-injected compared to saline-injected rats revealed significant changes in both NPY \((F_{1,21} = 71.8, P < 0.001)\) and AgRP \((F_{1,21} = 27.3, P < 0.001)\). The 90-min measure confirmed the findings of Experiment 1, exhibiting a significant increase after glucose injection in both NPY and AgRP mRNA in the ARC (Fig. 3). This effect, however, was not observed at the earlier time periods, which actually showed the opposite pattern. Glucose compared to saline injection caused a significant suppression of NPY and AgRP mRNA at 30 min \((-30\%)\) and also at 60 min \((-62\%)\) (Fig. 3). Whereas measurements of circulating glucose, insulin, leptin and triglycerides exhibited no change at any test interval, levels of CORT showed a statistically significant change \((F_{2,42} = 6.23, P < 0.001)\) after glucose injection (Table 2). This was characterized by a 50% rise at 60 min \((P < 0.01)\) and an insignificant trend towards higher levels \((+22\%)\) at 90 min, as previously described [50,73].

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>177 ± 7.1</td>
<td>179 ± 7.6</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>213 ± 19</td>
<td>218 ± 17</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.68 ± 0.17</td>
<td>1.71 ± 0.19</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.30 ± 0.13</td>
<td>1.31 ± 0.16</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>90 ± 16</td>
<td>92 ± 29</td>
</tr>
</tbody>
</table>

Given are means ± SEM.

3.3. Experiment 3: effect of peripheral glucose injection on circulating glucose levels as a function of time

To perform a more precise, temporal analysis of the changes in circulating glucose that precede the glucose-induced shifts in peptide expression, this experiment tested...
an additional set of rats (n = 14) with multiple blood collections, on separate days, via tail vein puncture at different intervals after injection. After removal of food 3 h before dark onset, the rats were given i.p. injection of saline or 10% glucose (0.13 g/kg), and blood samples were collected at 3.5, 7, 15, 30, 60, and 90 min after injection, in random order. Glucose administration significantly increased circulating glucose levels (F_{1,72} = 7.06, P < 0.01), specifically at two test intervals (Fig. 4). With no change at 3.5 min, glucose was elevated by 13% (+1.0 mM) at 7 min and by 25% (+1.8 mM) at 15 min compared to this group’s 3.5-min baseline (P < 0.01). The saline control baseline, in contrast, remained fairly stable over time. This effect of glucose was no longer apparent at 30 min. Thus, between 15 and 30 min, glucose levels significantly declined (P < 0.01) from a peak of 161 mg/dl (9.0 mM) to a baseline level of 132 mg/dl (7.3 mM), which is equivalent to that of the same rats at 3.5 min after injection and also of the saline-injected rats at 30 min after injection (Fig. 4).

3.4. Experiment 4: effect of peripheral glucose injection on NPY and AgRP at 30 or 90 min

This experiment used radioactive in situ hybridization histochemistry to firmly establish the biphasic effects, demonstrated in Experiment 2, of i.p. glucose injection on peptide gene expression. Using the same procedures, two groups of rats (n = 10/group) were administered i.p. injections of saline or 10% glucose (0.13 g/kg) and were sacrificed at one of two intervals after injection. The first interval was at 30 min, which followed the peak in circulating glucose levels observed at 15 min in Experiment 3, and the second interval was at 90 min, which was associated with elevated levels of CORT, as shown above in Experiment 2 (Table 2). In situ hybridization in glucose-injected vs. saline-injected rats revealed significant changes in mRNA expression of both NPY (F_{1,16} = 14.9, P < 0.001) and AgRP (F_{1,16} = 17.3, P < 0.001). Relative to saline baseline scores which were similar for the two intervals and thus averaged in Fig. 5, glucose injection produced at 30 min a significant, 22–24% decrease in both NPY and AgRP mRNA levels. Once again, this suppressive effect was reversed to an enhancement, a 45–65% increase in peptide expression at 90 min after glucose administration (Fig. 5). This effect is illustrated in the representative autoradiographs of Fig. 6, showing NPY-expressing neurons in the ARC at 30 or 90 min after injection compared to saline control. Consistent with Experiment 2, circulating glucose levels in the saline-injected rats (124 mg/dl) were unaltered by glucose injection at 30 min (136 mg/dl) and 90 min (119 mg/dl), while CORT levels after saline injection (246 ng/ml) tended to be elevated, but not significantly, at both 30 min (306 ng/ml) and 90 min (278 ng/ml) after glucose injection.

3.5. Experiment 5: effect of central glucose administration on NPY and AgRP at 60 min

This experiment tested whether glucose administered directly into the brain is similar to peripheral glucose in its
suppress effect on peptide gene expression, as shown in Experiments 2 and 4, and whether this effect can occur in the absence of changes in circulating glucose or hormone levels. Using similar procedures except for mode of injection, two groups of rats with ventricular cannula implants received i.c.v. injection of aCSF or 5% glucose. In Group 1, the rats (n = 7) were sacrificed at 60 min after injection, when i.p. glucose produced its strongest suppressive effect on NPY and AgRP in Experiment 2 (Fig. 3). Their trunk blood was collected for hormone and metabolite measures, and their brains were dissected for measurements of NPY and AgRP mRNA in the ARC using real-time quantitative PCR. In Group 2, rats (n = 13) received multiple blood collections via tail vein puncture, for measurements of metabolites and hormones at earlier post-injection intervals (3.5, 7, and 15 min), when i.p. glucose administration significantly elevated circulating glucose levels in Experiment 3 (Fig. 4). Consistent with Experiment 2, central 5% glucose compared to aCSF caused a significant suppression of both NPY (−28%, P < 0.001) and AgRP (−42%, P < 0.001) mRNA levels in the ARC (Fig. 7). This effect of central glucose, however, occurred in the absence of any detectable changes in circulating levels of glucose or CORT, as well as insulin and triglycerides (Table 3), in contrast to the effects seen with peripheral glucose administration (Table 2 and Fig. 4).

4. Discussion

4.1. Time-dependent changes in NPY and AgRP after glucose injection

The results demonstrated a reproducible, time-dependent change in both NPY and AgRP after peripheral or central glucose injection. As shown by real-time quantitative PCR and both radioactive and non-radioactive in situ hybridization histochemistry, glucose administration produced a significant suppression, ranging from 30% to 60%, of both NPY and AgRP in the ARC. This suppressive effect was detected at 30 and 60 min after peripheral administration of 10% glucose. It was also seen at 60 min after central administration of 5% glucose. These results reveal a robust phenomenon, showing glucose to have an inhibitory effect on peptide-expressing neurons in the ARC. They also show that AgRP responds similarly to NPY, consistent with evidence that these peptides colocalize in neurons of the ARC [8,25] and are similarly regulated under...
Given are abbreviations: aCSF, artificial cerebral spinal fluid; CORT, corticosterone; TG, triglycerides.

In addition to this biphasic change in NPY and AgRP mRNA, there was a time-dependent shift in circulating glucose levels after peripheral glucose administration. In prior studies with higher concentrations of glucose, i.p. injection of 40% glucose (4.7 g/kg) increased glucose levels by 300% at 10 min [31], while intravenous administration of 0.5–1.0 g/kg glucose produced a more prolonged elevation of glucose levels lasting up to 60 min [11]. In contrast, intracarotid injection of glucose at a comparable dose (0.10 g/kg) to that used here caused a more transient rise at 2 min after administration that returned to baseline by 30 min [34]. With blood samples collected at different intervals from the same subjects, the results here after i.p. 10% glucose administration (0.13 g/kg) compared to saline revealed a clear, time-related shift in circulating levels of glucose. Whereas no change was detected at 3.5 min, there was a significant rise in glucose levels at 7 and 15 min after injection. This constituted a 25% increase (+1.8 mM) to 161 mg/dl, which lasted for at least 15 min. By 30 min after injection, this effect was lost, and glucose declined to baseline levels of approximately 130 mg/dl. These shifts in glucose at the onset of the dark cycle, between 7.3 mM and 8.9 mM, fall within a physiological range of between 4 and 10 mM [35,36]. Interestingly, they are also comparable to the changes in glucose levels, between 7.6 and 8.6 mM, that occur in response to a carbohydrate-rich meal at the start of the natural feeding cycle [73], suggesting that they may have a physiological function in relation to carbohydrate ingestion during this particular phase of the circadian rhythm.

### 4.3. NPY and AgRP in relation to changes in glucose and CORT

In light of these time-dependent changes, it is possible that the initial, 1.8-mM rise in circulating glucose levels between 7 and 15 min after peripheral glucose administration may be causally related to the initial suppression of NPY and AgRP gene expression observed at 30 min and, then, at 60 min after injection. Based on evidence that extracellular brain glucose is approximately 30% of circulating glucose levels [62], a 1.8-mM rise in peripheral glucose would be expected to raise brain glucose by at least 0.5 mM. The suppressive effect of glucose on peptide gene expression in the ARC was confirmed in the experiment.

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Table 3

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glucose (mg/dl)</th>
<th>CORT (ng/ml)</th>
<th>Insulin (ng/ml)</th>
<th>TG (mg/dl)</th>
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<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>aCSF 186 ± 2.3</td>
<td>296 ± 12</td>
<td>2.7 ± 0.53</td>
<td>86 ± 19</td>
</tr>
<tr>
<td></td>
<td>Glucose 185 ± 4.4</td>
<td>213 ± 95</td>
<td>2.4 ± 0.24</td>
<td>92 ± 7</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>aCSF 162 ± 6.3</td>
<td>248 ± 29</td>
<td>1.4 ± 0.26</td>
<td>82 ± 14</td>
</tr>
<tr>
<td></td>
<td>Glucose 157 ± 9.9</td>
<td>313 ± 32</td>
<td>1.0 ± 0.43</td>
<td>76 ± 19</td>
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<tr>
<td>5.0</td>
<td>aCSF 169 ± 8.5</td>
<td>293 ± 30</td>
<td>1.3 ± 0.32</td>
<td>76 ± 11</td>
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<td></td>
<td>Glucose 156 ± 7.2</td>
<td>209 ± 48</td>
<td>1.1 ± 0.48</td>
<td>83 ± 10</td>
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<tr>
<td>15</td>
<td>aCSF 165 ± 6.9</td>
<td>208 ± 31</td>
<td>1.5 ± 0.25</td>
<td>87 ± 7</td>
</tr>
<tr>
<td></td>
<td>Glucose 169 ± 11.4</td>
<td>248 ± 59</td>
<td>1.2 ± 0.36</td>
<td>82 ± 17</td>
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</tbody>
</table>

Abbreviations: aCSF, artificial cerebral spinal fluid; CORT, corticosterone; TG, triglycerides. Given are ± SEM.

a variety of conditions [33,45,74,78]. With this suppressive effect of glucose lost at the 90-min test interval, these findings establish the first 60 min after elevated glucose as a critical time for observing a negative, glucose–peptide interaction at the onset of the nocturnal feeding cycle. At 90 min after glucose administration, the reverse effect actually occurred, a significant increase in NPY and AgRP mRNA after i.p. injection of 10% glucose compared to saline. This subsequent response is clearly a robust phenomenon. It was detected in each of the 4 experiments conducted here, using either real-time quantitative PCR or in situ hybridization histochemistry, and it has also been described in an earlier study of NPY [73]. Fig. 3 provides some insight into this phenomenon, by revealing in the control animals (open bars) a decline in both NPY and AgRP from the 30-min to the 90-min time period after saline injection. With the rats at the 90-min interval sacrificed an hour later than those at the 30-min interval, this shift in the control baseline may reflect a circadian-related change shown to occur at dark onset, as the natural feeding cycle is initiated. While starting to rise in the middle of the light period and peaking 2 h before dark onset, NPY expression exhibits a significant decline by dark onset in association with a rise in spontaneous feeding [2]. A similar although somewhat delayed pattern is seen for AgRP [38]. Thus, rather than stimulating NPY and AgRP at this 90-min period, the glucose injection may actually be preventing or postponing the circadian drop in these peptides evident in the saline control animals. As described below, this secondary phenomenon may be more directly related to changes in levels of CORT, which occur as a consequence of shifts in circulating glucose itself.

4.2. Time-dependent shifts in circulating glucose levels

In light of these time-dependent changes, it is possible that the initial, 1.8-mM rise in circulating glucose levels between 7 and 15 min after peripheral glucose administration may be causally related to the initial suppression of NPY and AgRP gene expression observed at 30 min and, then, at 60 min after injection. Based on evidence that extracellular brain glucose is approximately 30% of circulating glucose levels [62], a 1.8-mM rise in peripheral glucose would be expected to raise brain glucose by at least 0.5 mM. The suppressive effect of glucose on peptide gene expression in the ARC was confirmed in the experiment.
with central glucose administration. Although brain glucose was not measured in the present study, a similar report with i.c.v. 5% glucose injection showed a 1.1-mM rise in CSF glucose 30 min after injection [17]. Thus, with either mode of administration, central glucose levels may rise between 0.5 and 1.1 mM and have direct effects in the brain. This increase in glucose is sufficient to elicit a neuronal response [63] and is temporally associated with the 30–60% reduction in NPY and AgRP gene expression in the ARC at 30 and 60 min. Consistent with published studies using higher 10–25% concentrations of glucose [17,58], the present experiment with i.c.v. 5% glucose administration demonstrated significant effects on peptide expression without changes in circulating glucose levels. This further strengthens the idea that glucose may be acting directly within the brain. Tests involving step-wise glucose clamping would provide more specific information on the nature of this glucose-induced suppression of peptide gene expression, including the latency and threshold of the effect.

Following this transient rise in glucose lasting approximately 15 min, there was a significant decline in glucose from peak levels of 161 mg/dl and a return to baseline of 130 mg/dl by about 30 min after injection. We propose that this secondary event has its own consequences, perhaps affecting circulating CORT levels that, in turn, modulate NPY and AgRP. A decline in circulating glucose is invariably associated with a rise in CORT, which has a primary function in maintaining glucose homeostasis [67]. This function is most clearly reflected in the circadian-related rise in CORT, which occurs during the second half of the sleep cycle when glucose levels and carbohydrate stores are at their nadir. Whereas levels of CORT in the present study were unaffected during the first 30 min after i.p. glucose injection, they increased significantly at 60 min by 50%, from 160 to 241 ng/ml, and showed a tendency to remain elevated at the 90-min interval (Table 2). This is consistent with published reports showing elevated CORT between 30 and 90 min after peripheral glucose injection, as well as following consumption of a glucose solution [50,73]. This rise in CORT may be a primary stimulus for the secondary stimulatory effect of glucose injection on NPY and AgRP at 90 min. Acute or chronic administration of CORT increases the expression of both NPY and AgRP in the ARC [3,57,67]. Further, stimulation of these peptides induced by food deprivation, in insulin-deficient diabetic [44] and leptin-insensitive db/db [45] mice, is suggested to be due to the deprivation-induced rise in CORT [27]. Thus, together with the drop in glucose itself, it may also be the rise in CORT at 60 min that contributes to the secondary increase in NPY and AgRP at 90 min, possibly reflecting a delay in the circadian-related decline in these peptides. A similar relationship has been suggested for CORT and NPY measured 90 min after the consumption of a glucose solution or a carbohydrate-rich meal at dark onset [73].

Although insulin and leptin are known to have a potent inhibitory effect on NPY and AgRP mRNA [15,45,51,59,70], the results obtained with peripheral as well as central glucose administration failed to reveal any change in circulating levels of these hormones, suggesting that they are unlikely to exert major effects under the present test conditions. Although chronic, peripheral glucose administration can elevate leptin levels [37,72], this was not seen with acute injections of glucose [54]. Also, while higher concentrations of intravenous glucose (40–50%, 0.5–1.0 g/kg) can increase insulin for 30–60 min [11,34], an effect seen in our preliminary tests with 20% i.p. glucose (see Materials and methods), the results obtained with the lower, 10% glucose concentration used in the present experiments showed no change in insulin at any time point. Since our measurements may have missed an early rise in circulating insulin, like the transient effect observed from 2 to 5 min after intracarotid glucose (0.1 g/kg) administration [34], this hormone may still contribute to the initial reduction in NPY and AgRP mRNA seen at 30–60 min, perhaps acting through insulin receptors on NPY neurons in the ARC [7,26]. However, our measurements of circulating hormones during the first 15 min after centrally administered glucose (Table 3) indicate that this suppressive effect on peptide gene expression can occur in the absence of any detectable change in circulating insulin. One must consider the possibility that insulin synthesized within the brain may have a role, although the receptors for brain insulin and the factors that regulate hypothalamic insulin production remain to be characterized [19]. Since circulating triglyceride levels were unaffected by glucose administration and appear to have little impact on NPY or AgRP [10], it is unlikely that circulating lipids are involved in the glucose-induced change in peptide expression. Thus, from the measures taken so far, it appears that glucose and possibly CORT are key regulators of peptide mRNA under the present test conditions, at the onset of the nocturnal feeding cycle.

4.4. Involvement of glucose-inhibited neurons in the effects on NPY and AgRP

The biphasic changes in peptide mRNA in the ARC may involve direct effects of glucose on glucose-inhibited or GI neurons. This is suggested by in vitro evidence that NPY-expressing neurons in this nucleus are inhibited by a rise in glucose levels [18,46] and that GI neurons also respond to and are excited by low levels of glucose [36,48]. Further, in contrast to other hypothalamic areas, the ARC shows no change in c-Fos immunostaining in the first 60 min after glucose administration, supporting the inhibitory effect of glucose on neurons in this nucleus [13,14]. Thus, the initial rise in brain glucose, in the range of 0.5–1.1 mM, during the first 15–30 min after peripheral or central glucose administration is suggested to inhibit GI neurons and, thereby, reduce NPY and AgRP mRNA and presumably peptide release over the next 30–60 min. Further, the subsequent decline in glucose, by activating GI neurons, may contribute to the stimulation of peptide mRNA
observed at 90 min. These glucose-sensing neurons may also respond to the delayed rise in CORT, which stimulates NPY and AgRP gene expression, and also to changes in glucose metabolism [77] and malonyl Co-enzyme A, which is increased by glucose to enhance glucose oxidation [53,55]. In addition, the majority of NPY neurons in the ARC also colocalize glucokinase, which participates in the process of sensing physiological changes in circulating or brain glucose [40]. Thus, NPY- and AgRP-expressing neurons are well-designed to process signals directly from glucose. The present study provides the first in vivo evidence that these neurons prior to the onset of the nocturnal feeding cycle are inhibited by a rise in glucose. Although they may also be stimulated by a decline in glucose, this phenomenon is likely to involve intermediary, glucoregulatory processes that include the release of CORT.

4.5. Involvement of glucose-sensing NPY and AgRP neurons in normal eating behavior

These effects of glucose on GI neurons and peptide gene expression may have functional consequences in the control of eating behavior. The administration of glucose, whether peripherally or centrally, has a suppressive effect on food intake and delays the initiation of meals in animals [9,17,23]. Also, glucose administration reduces eating and delays expected requests for food in humans [9,16,20,47]. The present results suggest that the mechanisms underlying this phenomenon are reflected in the initial, direct effects of glucose administration. These include a transient rise in circulating glucose levels, the inhibition of GI neurons in the ARC, and the suppression of NPY and AgRP, two potent stimulants of feeding [12,15,24,64]. The expected consequence of this sequence of events is a reduction in food intake and delay in subsequent meal initiation.

Rats normally initiate a meal approximately every 90 min in the nocturnal feeding cycle [9,61]. Moreover, under physiological conditions, a small, 6–10% drop in circulating glucose, resulting in about 0.2–0.3 mM change in brain glucose [35], generally precedes the initiation of spontaneous meals [9]. Thus, the secondary effects observed here after glucose injection, involving a decline in glucose at 30 min followed by a rise in CORT at 60 min and peptide expression at 90 min, may reflect physiological processes that mediate the natural sequence of post-prandial events. With the increase in NPY and AgRP in the ARC and possible activation of GI neurons, the next meal is likely to occur, leading to the normalization of glucose levels in the blood and brain.

This biphasic sequence of events, requiring particular sensitivity of the hypothalamic neurons to shifts in circulating glucose, is likely to be functionally active under specific conditions when carbohydrate stores and circulating glucose levels are at their nadir. This occurs naturally during the initial hours of the natural feeding cycle, when carbohydrate is the preferred macronutrient, and there is a spontaneous peak in CORT levels and peptide gene expression and production [32,67]. In fact, the changes in circulating glucose levels observed here after glucose administration are very similar to the changes, between 7.6 and 8.6 mM, that occur in relation to a carbohydrate-rich meal at the beginning of the natural feeding cycle [73].

Acknowledgments

This research was supported by NIMH grant MH43422 and NIAAA grant AA12882. We thank Dr. Steven Sabol (NIH, Bethesda, MD) and Dr. Ira Gantz (University of Michigan, Ann Arbor, MI) for providing the NPY and AgRP probes, respectively. We also greatly appreciate the help of Dr. Louis Lucas (Rockefeller University) for his help with the autoradiographic film analysis and Ms. Kate Sepiashvili for her help in the preparation of the manuscript.

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