Hypothalamic Orexin Expression

Modulation by Blood Glucose and Feeding

Xue J. Cai, Peter S. Widdowson, Joanne Harrold, Shelagh Wilson, Robin E. Buckingham, Jonathan R.S. Arch, Mohammad Tadayyon, John C. Clapham, John Wilding, and Gareth Williams

Orexins (hypocretins), novel peptides expressed in specific neurons of the lateral hypothalamic area (LHA), stimulate feeding when injected intracerebroventricularly. We investigated their role in feeding in the rat by measuring hypothalamic prepro-orexin mRNA levels under contrasting conditions of increased hunger. Prepro-orexin mRNA levels increased significantly after 48 h of fasting (by 90–170%; $P < 0.05$) and after acute (6 h) hypoglycemia when food was withheld (by 90%; $P < 0.02$). By contrast, levels were unchanged during chronic food restriction, streptozotocin-induced diabetes, hypoglycemia when food was available, voluntary overconsumption of palatable food, or glucoprivation induced by systemic 2-deoxy-D-glucose. Orexin expression was not obviously related to changes in body weight, insulin, or leptin, but was stimulated under conditions of low plasma glucose in the absence of food. Orexins may participate in the short-term regulation of energy homeostasis by initiating feeding in response to falls in glucose and terminating it after food ingestion. The LHA is known to contain neurons that are stimulated by falls in circulating glucose but inhibited by feeding-related signals from the viscera; orexin neurons may correspond to this neuronal population. ***Diabetes*** **48:2132–2137, 1999**

Orexin-A and -B are, respectively, 33- and 28-residue peptides recently isolated from rat hypothalamus (1). Their common precursor, prepro-orexin, is expressed in a specific population of neurons in the lateral hypothalamic area (LHA) that project to numerous hypothalamic and extrahypothalamic sites where the orexin OX-1 and -2 receptors are widely expressed (2–5). The orexins are related to the hypocretins, which are previously described hypothalamic neuropeptides (6). Alignment of their nucleotide sequences shows that hypocretin-1 and -2 have sequences in common with orexin-A and -B, respectively, but additional amino acids were deduced to be present in both hypocretins: six extra amino acids in hypocretin-1 (five at the NH_2-terminus and one at the COOH-terminus) and one extra in hypocretin-2 (at the COOH-terminus) (1,6).

The physiological roles of the orexins remain uncertain, but evidence points to involvement in feeding behavior. Orexin-A stimulates feeding when injected intracerebroventricularly (1,7,8) and at lower dosages into the LHA (9); orexin-B may also stimulate feeding (1), although this has been disputed (8). In addition, hypothalamic prepro-orexin mRNA levels rise in rats fasted for 48 h (1), suggesting activation of these neurons under conditions of hunger.

The LHA, where the orexin cell bodies are situated, has long been implicated in the control of feeding and body weight. Classical experiments showed that electrical stimulation of the LHA induced feeding, whereas LHA lesions decreased spontaneous eating and the hyperphagia caused by specific stimuli, including insulin-induced hypoglycemia and neuroglycopenia provoked by 2-deoxy-D-glucose (2-DOG) (10). The LHA receives neural inputs from other hypothalamic areas involved in energy homeostasis and from extrahypothalamic centers, notably the nucleus of the solitary tract (NTS), which relays vagally transmitted afferent signals from the gut that are related to feeding (10). LHA neurons have been identified that respond to feeding-related signals including changes in glucose and insulin and the presence of food in the gut (11–14), but their neurochemical identity has not been firmly established.

The orexin neurons are therefore strategically situated to integrate a variety of signals related to nutritional state and, through their projections to specific sites involved in controlling feeding behavior, to play a role in the regulation of energy homeostasis. Moreover, the appetite-stimulating actions of the orexins (1,7–9), together with the increased expression of their precursor during fasting (1), suggest that orexins released in the LHA or other sites may drive feeding in response to energy deprivation. However, the specificity, importance, and regulation of such an action are unknown.

Here, we investigated further the relationship between hunger, nutritional signals, and prepro-orexin expression in the rat. We selected a range of physiological and pharmacological manipulations that all stimulate feeding but through different mechanisms. In fasting, food restriction, and

From the Diabetes and Endocrinology Research Group (X.J.C., P.S.W., J.H., J.W., G.W.), Department of Medicine, the University of Liverpool, Liverpool; and SmithKline Beecham Pharmaceuticals (S.W., R.E.B., J.R.S.A., M.T., J.C.C.), Harlow, Essex, U.K.

Address correspondence and reprint requests to Professor Gareth Williams, Diabetes and Endocrinology Research Group, Department of Medicine, the University of Liverpool, Liverpool L69 3GA, U.K. E-mail: garethw@liverpool.ac.uk.

Received for publication 30 April 1999 and accepted in revised form 4 August 1999.

S.W., R.E.B., J.R.S.A., M.T., and J.C.C. hold stock in SmithKline Beecham Pharmaceuticals. G.W. has been a paid consultant for SmithKline Beecham Pharmaceuticals and has received lecture fees from them as well as laboratory funding.

CNS, central nervous system; 2-DOG, 2-deoxy-D-glucose; LHA, lateral hypothalamic area; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; RIA, radioimmunoassay; STZ, streptozotocin.

insulin-deficient diabetes, hunger may be stimulated by decreased circulating levels of leptin and insulin, which both act via the central nervous system (CNS) to inhibit feeding (15–17). Voluntary overeating of palatable food is due to its hedonic properties, acting through unknown CNS pathways to override normal set-point regulation (18). The intense hyperphagia caused by insulin-induced hypoglycemia and glucose antimetabolites such as 2-DOG has been attributed to reduced availability of glucose, the principal CNS energy substrate under normal conditions, at specific glucoreceptors in the CNS and periphery (19–22).

To investigate the nutritional factors that might regulate orexin expression, we also measured insulin, leptin, and glucose, circulating signals that are known to influence feeding and the activity of other appetite-regulating neuronal pathways. The possible role of decreased leptin levels in food restriction was further studied by giving exogenous leptin to maintain the levels found in freely fed control animals.

RESEARCH DESIGN AND METHODS

Animals and procedures. Adult male Wistar or Sprague-Dawley rats (initially 150–200 g) were maintained at 22 ± 2°C under a 12:12-h light:dark schedule (lights on at 0700) and habituated to frequent handling and daily measurements of weight and food intake. The strains used for each study are indicated below; because fasting and chronic food restriction unexpectedly showed divergent effects on prepro-orexin expression, these studies were repeated in both strains. Water was freely available, with standard laboratory food (CRM; Biosure, Cambridge, U.K.; 13.1 kcal/g) or a palatable high-fat diet (18) given as below.

At the end of each study, rats were killed by CO_2 inhalation and immediately exsanguinated by cardiac puncture, and plasma was stored for assay of glucose (Yellow Springs autoanalyzer; Yellow Springs, OH; glucose oxidase method), insulin (radioimmunoassay [RIA] kit; Shield Diagnostics, Washington, Tyne and Wear, U.K.), and leptin (RIA; Biogenesis, Poole, Dorset, U.K.). The epididymal fat pad was dissected and weighed.

Starvation. In separate experiments, groups of Wistar and Sprague-Dawley rats were fasted for 48 h, while control animals were freely fed throughout (n = 8 for each group).

Chronic food restriction with effects of leptin replacement and refeeding. Three groups of Sprague-Dawley rats (n = 8 for each group) were restricted to 50% of their usual food intake for 6 days, while another group fed freely. One food-restricted group carried subcutaneously implanted osmotic minipumps (AL2001; flow rate, 1 µl/h; Alzet, Palo Alto, CA) delivering leptin (1.3 mg/ml) to maintain circulating leptin concentrations comparable to those in freely fed control animals (Table 1). The two other food-restricted groups carried minipumps delivering saline; one of these groups was allowed to feed freely for 24 h after the 6 days of food restriction. The freely fed control animals also carried minipumps delivering saline. Pumps were placed into the interscapular region through a stab incision under brief general anesthesia (Hypnorm/midazolam, 3.3 ml/kg i.p.). Food intake was slightly lower on the day of surgery but returned to normal the following day with no impact on body weight.

In a further study, Wistar rats (n = 8) were given 50% of their usual daily food intake (17 g/day) for 6 days, while control animals were freely fed. In this experiment, leptin replacement was not given.

Insulin-deficient diabetes. Insulin-deficient diabetes was induced in Wistar rats by injecting streptozotocin (STZ) (55 mg/kg in saline) into a tail vein (23). This dosage induced sustained hyperglycemia (blood glucose >20 mmol/l) within 24 h, but the rats remained active and in good condition without insulin replacement. Blood glucose concentrations were measured in daily tail-prick samples (~20 µl) using an electrochemical meter (Exactech; Medisense, Oxford, U.K.).

Voluntary hyperphagia. Voluntary hyperphagia was induced by feeding Wistar rats a highly palatable high-fat diet consisting of powdered food (33% by weight), condensed milk (33%), sucrose (7%), and water. Energy content was 10.2 kcal/g (18). This was given for 8 weeks, during which daily food intake increased by 60% in weight, representing a 30% increase in daily energy intake.

Insulin-induced hypoglycemia. Acute effects of hypoglycemia with and without feeding were examined in two groups of Wistar rats up to 6 h after a single subcutaneous injection of Insulatard insulin (60 U/kg; Novo-Nordisk, Baegsverd, Denmark) at 0900. One group of insulin-treated rats was allowed to eat freely, while the other was food-deprived after injection. Control animals received a saline injection and had free access to food. Because control animals ate <1 g during this period, an additional control group (food-deprived, saline-injected) was judged unnecessary. Tail-prick blood glucose levels were monitored every 2 h.

Sustained hypoglycemia was induced for 6 days in other Wistar rats (n = 8) by once-daily Insulatard injections (20–60 U/kg s.c.) at doses adjusted individually to maintain 0900 blood glucose levels at <3.0 mmol/l. These rats and saline-injected control animals (n = 8) were allowed to eat freely.

2-DOG glucoprivation. Preliminary experiments in Wistar rats showed that intraperitoneal 2-DOG injection (100–300 mg/kg) stimulated feeding severalfold for 1–2 h but that compensatory hypophagia followed, such that 24-h food intake did not differ from control animals. We therefore used a schedule with 2-DOG (300 mg/kg) injected intraperitoneally at 0, 2, and 4 h that produced sustained hyperphagia whose time-course closely mimicked the 6-h feeding profile during insulin-induced hypoglycemia (Fig. 2*B*). As with the study of acute hypoglycemia, an additional group of rats was treated with 2-DOG but was food-deprived. Tail-prick blood glucose levels were monitored every 2 h.

Hypothalamic prepro-orexin mRNA measurements. The hypothalamus was dissected under a binocular microscope, using landmarks (23) that enclosed the population of orexin neurons (1), and snap-frozen in liquid nitrogen. Total RNA was extracted in Trizol (Gibco, Paisley, U.K.), and nominal 20-µg aliquots were electrophoresed on 1% agarose gel containing 2% formaldehyde and transferred overnight to a nylon membrane (Boehringer Mannheim, Lewes, U.K.). RNA was cross-linked to the membrane by UV radiation and prehybridized for 3 h in Easy-hyb (Boehringer Mannheim) at 50°C. The cDNA probe for orexin mRNA was prepared by polymerase chain reaction and digoxigenin labeling using a standard kit and a purified Bluescript SK (+) M13 vector containing the 0.29 kb prepro-orexin sequence (nucleotides 187–474, AF041241), which was grown in *Escherichia coli* (JM 109) (Wizard Plus Minipreps; Promega, Southampton, U.K.).

Northern blots were probed with the digoxigenin-labeled cDNA orexin probes at 50°C and washed at 50°C in 2× and 0.5× standard sodium citrate with 0.1% SDS. Orexin mRNA bands were detected using anti-digoxigenin antibodies (Boehringer Mannheim) and an enhanced chemiluminescence kit (Boehringer Mannheim). Autoradiograms were quantitated by image analysis (AIS system; Imaging Associates, Brock University, St. Catherine's, Canada). To standardize RNA loading concentrations, membranes were stripped at 68°C and reprobed with a digoxigenin-labeled oligonucleotide probe against 18S ribosomal RNA, whose levels did not show significant group differences in any of these studies. Orexin mRNA concentrations were therefore expressed as the ratio of densities of orexin mRNA band/18S RNA band.

Statistical analyses. Data are shown as means ± SE. Comparisons between groups were made using unpaired Student's t test preceded by two-way analysis of variance in studies that included more than two groups.

RESULTS

Hypothalamic prepro-orexin mRNA levels

Starvation. Wistar rats fasted for 48 h lost 14% of body weight compared with freely fed control animals, with a 40% decrease in fat-pad mass and significant falls in plasma glucose, insulin, and leptin (Table 1). Hypothalamic prepro-orexin mRNA levels were increased by 90% ($P < 0.05$) (Fig. 1).

Similarly, Sprague-Dawley rats fasted for 48 h (n = 8) showed comparable falls in body weight (15%), fat-pad mass (34%), and in plasma glucose (28%), insulin (66%), and leptin (74%) (data not shown). Prepro-orexin mRNA levels were again increased by 170% over those of freely fed control animals (n = 8) (2.7 ± 0.8 vs. 1.0 ± 0.6 arbitrary units; $P < 0.05$).

Chronic food restriction. Saline-treated food-restricted Sprague-Dawley rats lost 16% of weight and 32% of fat-pad mass compared with freely fed control animals and showed broadly similar changes in plasma analytes to 48-h fasted rats (Table 1). The saline-treated rats allowed to refeed partly normalized their body weight and other metabolic parameters. Leptin-treated food-restricted rats ate all the food presented to them and had weight, fat-pad mass, and glucose levels similar to the saline-treated food-restricted group; terminal plasma leptin concentrations were comparable to those in freely fed control animals (Table 1). None of the three experimental groups showed significant alterations in prepro-orexin mRNA levels compared with freely fed control animals (Fig. 1).

The same food restriction schedule in Wistar rats (n = 8) led to losses of 18% in weight and 40% in fat-pad mass com-

TABLE 1
Body weight, epididymal fat-pad mass, food intake, and plasma hormones and glucose

	n	Body weight (g) Initial	Body weight (g) Final	Fat-pad mass (g)	Food intake (g)	Plasma Insulin (ng/ml)	Plasma Leptin (µg/ml)	Plasma Glucose (mmol/l)
Fasting (48 h)								
Fasted	8	346 ± 8	323 ± 7‡	2.5 ± 0.3†	0	12.0 ± 2.3‡	0.20 ± 0.01‡	5.2 ± 0.6†
Freely fed	8	348 ± 5	374 ± 7	4.2 ± 0.3	28.0 ± 1.0	30.5 ± 3.6	4.40 ± 0.50	8.2 ± 0.6
Food restriction (6 days)								
Restricted	8	329 ± 4	331 ± 4‡	1.9 ± 0.1*	17 ± 0‡	12.1 ± 1.3‡	1.82 ± 0.28‡	8.8 ± 0.1†
Restricted + leptin	8	328 ± 5	336 ± 5‡	1.7 ± 0.1‡	17 ± 0†	21.9 ± 3.1*§	3.89 ± 0.25‖	7.5 ± 0.1†
Restricted + refed	8	327 ± 4	364 ± 6‡	2.0 ± 0.1†	(17 ± 0)‡	48.6 ± 4.9‡¶	3.84 ± 0.67‖	8.7 ± 0.7
Freely fed	8	330 ± 6	393 ± 7	2.8 ± 0.1	34.2 ± 0.81	30.8 ± 3.9	4.74 ± 0.34	10.6 ± 0.6
STZ-induced diabetes (3 weeks)								
Diabetic	10	195 ± 2	260 ± 6†	0.29 ± 0.05‡	53.9 ± 2.52‡	9.9 ± 1.2	0.16 ± 0.08‡	35.6 ± 2.1‡
Control	10	194 ± 3	288 ± 6	1.79 ± 0.09	30.3 ± 0.47	25.0 ± 2.7‡	2.84 ± 0.34	10.4 ± 0.5
Palatable diet (6 weeks)								
Palatable diet	8	112 ± 4	362 ± 6	6.47 ± 0.48‡	41.9 ± 0.90‡	15.1 ± 1.7	5.05 ± 0.33‡	11.9 ± 0.7
Standard-fed control	8	114 ± 4	317 ± 19	3.27 ± 0.31	25.3 ± 1.27	18.9 ± 2.4	1.42 ± 0.29	10.1 ± 0.9
Acute hypoglycemia (6 h)								
Hypoglycemic fed	10	260 ± 3	—	2.19 ± 0.10	4.69 ± 0.45‡	391.6 ± 19.3‡	5.04 ± 0.29‡	4.3 ± 0.3‡
Hypoglycemic fasted	10	262 ± 2	—	2.05 ± 0.06	0	300.4 ± 31.5‡	5.02 ± 0.28‡	1.5 ± 0.2‡
Control	10	262 ± 6	—	2.15 ± 0.08	0.06 ± 0.02	17.9 ± 2.3	2.19 ± 0.24	8.8 ± 0.5
Chronic hypoglycemia (6 days)								
Hypoglycemic	10	354 ± 10	418 ± 8†	4.78 ± 0.46*	40.8 ± 1.01†	463.5 ± 32.8‡	7.54 ± 0.41‡	4.8 ± 0.4‡
Control	10	336 ± 13	366 ± 10	3.5 ± 0.28	27.0 ± 0.84	25.8 ± 2.7	2.61 ± 0.15	10.2 ± 0.5
Acute 2-deoxyglucose (6 h)								
2-DOG, fed	8	238 ± 11	—	1.57 ± 0.13	4.71 ± 0.25‡	28.5 ± 2.3‡	4.14 ± 0.37‡	18.9 ± 2.1†
2-DOG, fasted	8	233.9 ± 9.3	—	1.03 ± 0.03	0	18.3 ± 1.1	2.38 ± 0.19	18.1 ± 1.0†
Control	8	240 ± 9	—	1.25 ± 0.11	1.25 ± 0.21	17.9 ± 0.9	2.19 ± 0.21	10.9 ± 0.5

Data are means ± SE or n. Differences vs. control rats: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$. Differences vs. untreated food-restricted rats: §$P < 0.05$, ‖$P < 0.01$, ¶$P < 0.001$.

pared with freely fed control animals ($n = 8$), while plasma glucose, insulin, and leptin levels fell significantly and by degrees similar to those in the Sprague-Dawley rats (data not shown). Prepro-orexin mRNA levels were again not significantly different from those of control animals (0.78 ± 0.22 vs. 1.00 ± 0.20 arbitrary units, $P > 0.1$).

STZ. STZ-induced diabetic rats weighed 10% less than non-diabetic control animals after 3 weeks, with fat-pad mass decreased by 84%. They had severe polyuria and polydipsia, and food intake was 80% greater than that of control animals. Plasma glucose concentrations were 30–35 mmol/l, while insulin and leptin were reduced by 60 and 94%, respectively. Prepro-orexin mRNA levels did not differ significantly from those of control animals (Fig. 1).

Palatable-diet feeding. Palatable-diet feeding for 8 weeks increased daily energy intake by 30% and resulted in dietary obesity, with 20 and 98% increases in body weight and fat-pad mass, respectively, over standard feeding. Plasma leptin levels were significantly raised 2.5-fold, but there were no significant changes in either insulin or glucose (Table 1). Prepro-orexin mRNA levels were comparable to those of control animals (Fig. 1).

Insulin-induced hypoglycemia. In the acute study, insulin-treated rats with access to food became hypoglycemic after 2 h (Fig. 2*A*) and ate consistently more than control animals after this time; the total 6-h food intake was 5 vs. 0.6 g in control animals ($P < 0.001$) (Fig. 2*B*). Insulin-treated rats that were also food-deprived suffered more profound hypoglycemia, with significantly lower terminal values (Table 1); the values recorded in tail-prick samples during the experiment were close to the lower limit of accuracy of the Exactech meter and so could not be reliably distinguished between the two hypoglycemic groups. Predictably, insulin

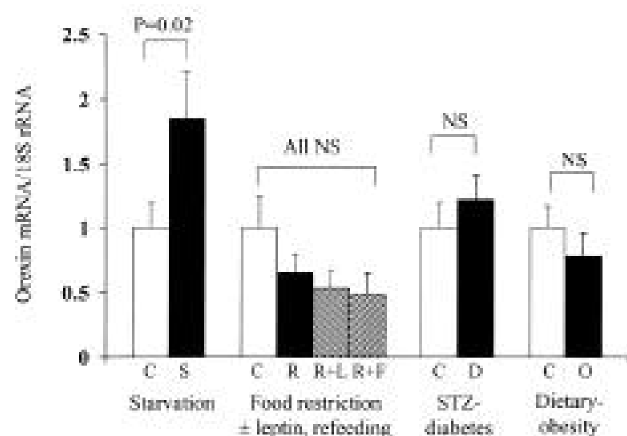

FIG. 1. Hypothalamic prepro-orexin mRNA levels in rats with various conditions of increased hunger compared with their respective control rats. Prepro-orexin mRNA levels were normalized against 18S rRNA. C, control; S, starved; R, food restricted; R+L, food restricted plus leptin; R+F, food restricted plus refed; D, STZ-induced diabetic rat; O, obese rat. Data are means ± SE. $P > 0.05$.

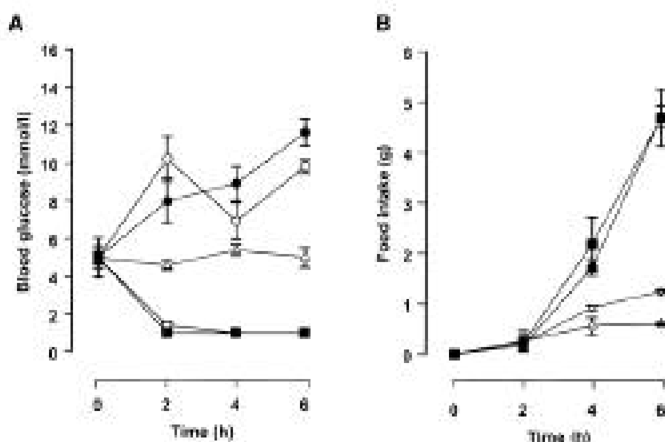

FIG. 2. Blood glucose (*A*) and cumulative food intake (*B*) in rats made hypoglycemic with insulin or in rats with glucoprivation induced by 2-DOG. Data are means ± SE. To minimize interference with the animals, blood glucose at 0, 2, and 4 h was measured in 20-μl tail-prick samples using an electrochemical meter, which is less accurate for severe hypoglycemia (<2.0 mmol/l). Terminal plasma glucose values (measured by autoanalyzer) are given in Table 1 and confirm that hypoglycemia was more profound in insulin-treated than in food-deprived rats. △, control hypoglycemic rats; □, fasted hypoglycemic rats; ■, fed hypoglycemic rats; ▽, control 2-DOG rats; ○, fasted 2-DOG rats; ●, fed 2-DOG rats.

levels were grossly elevated in both insulin-treated groups, while plasma leptin was raised over twofold (Table 1). Prepro-orexin mRNA levels in the food-deprived hypoglycemic rats were significantly raised by 90% above those of normoglycemic control animals ($P < 0.02$), but they were not significantly altered in the freely fed hypoglycemic group (Fig. 3).

Chronic hypoglycemia, induced by daily insulin injections, maintained food intake consistently 50% higher than that in saline-injected control animals, and after 6 days of treatment, body weight and fat-pad mass were increased by 14 and 36%, respectively. Plasma insulin and leptin concentrations were raised 18- and 2-fold (Table 1). Prepro-orexin mRNA levels were 59% higher than those in normoglycemic control animals, but the difference fell short of statistical significance ($P = 0.08$) (Fig. 3).

2-DOG–induced glucoprivation. Both 2-DOG–treated groups were hyperglycemic throughout (Fig. 2*A*), confirming sustained activation of neuroendocrine counterregulatory mechanisms in response to neuroglycopenia. 2-DOG–treated rats allowed to eat were hyperphagic after 2 h, showing a food intake curve that closely matched that of the insulin-induced hypoglycemic group and reached 5 g at 6 h (Fig. 2*B*). However, despite the similar time-course and magnitude of these feeding curves, there was no increase in hypothalamic prepro-orexin mRNA levels above those of control animals in 2-DOG–treated rats that were either fed or food-deprived (Fig. 3).

DISCUSSION

The orexin peptides were named for their experimental action of stimulating food intake in the rat (1). This observation, together with the localized expression of prepro-orexin in the LHA—the classical "feeding center"—and its upregulation in fasted rats has focused attention on the peptides' possible involvement in the control of feeding. However, the LHA serves many other functions including arousal, cardiovascular and gastrointestinal regulation, and electrolyte and water homeostasis (10), and these are also affected by fasting. Moreover, the hyperphagic effect of the orexins is less robust than that of certain other hypothalamic peptides, notably neuropeptide Y (NPY) (7,8,16). Thus, the physiological functions of these peptides may extend into areas other than energy homeostasis.

Our data support involvement of orexin neurons in feeding, but only under specific circumstances. Among the several conditions of increased hunger that we examined, we found significantly increased prepro-orexin mRNA levels in only two of them: *1*) prolonged (48 h) fasting and *2*) acute (6 h) hypoglycemia, but only if food was withheld.

The increased expression in fasting (in two different strains of rat) is in agreement with the original observation of Sakurai et al. (1), although the rises in prepro-orexin mRNA in our studies were not as pronounced. Very recently, Griffond et al. (24) have also reported increased prepro-orexin expression in rats with acute insulin-induced hypoglycemia, but not if glucose was also given to maintain euglycemia.

Intriguingly, no increases were seen after prolonged (6 days) food restriction that produced weight and metabolic changes similar to fasting, or when rats with acute or chronic hypoglycemia were allowed to feed, or after acute glucoprivation with 2-DOG that induced comparable hunger to hypoglycemia. Neither did prepro-orexin mRNA levels increase in rats with increased appetite due to insulin-deficient diabetes

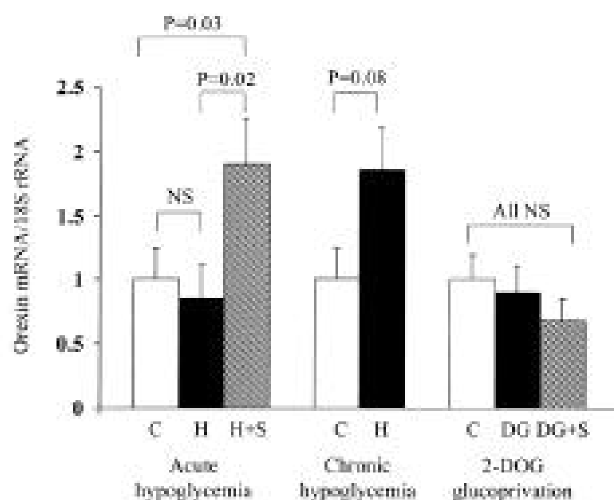

FIG. 3. Prepro-orexin mRNA levels in rats with acute (6 h) or chronic (6 days) hypoglycemia, or acute glucoprivation induced by 2-DOG, with or without access to food. C, control; H, hypoglycemia; H+S, hypoglycemia and starvation; DG, 2-DOG; DG+S, 2-DOG and starvation.

or presentation of palatable food. We cannot exclude involvement of orexins in stimulating appetite in these other conditions, since orexin release in discrete sites could be enhanced without a concomitant increase in whole hypothalamic prepro-orexin mRNA. Nevertheless, our data suggest that orexins do not stimulate hunger under all conditions, and that these neurons are regulated by a highly specific combination of nutritional signals. These findings are in striking contrast to the marked upregulation of NPY mRNA found in chronic food restriction as well as in fasting (25,26) and in STZ-induced diabetes (27). Moreover, hypothalamic NPY neurons are inhibited by both leptin (15,28,29) and insulin (16,30) and are stimulated under conditions of negative energy balance, probably signaled by falls in circulating levels of both hormones (15,17). By contrast, prepro-orexin expression bore no obvious relationship to insulin, leptin, or body fat mass. The apparent independence from leptin is interesting, since the orexin neurons have recently been shown to express leptin receptors, although the particular subtype, and therefore the signaling competence of these receptors, is uncertain (3).

Our findings may provide clues to the regulation and role of the orexin neurons. Factors common to 48-h fasting and hypoglycemia during food deprivation, but not to the other states of increased appetite that we studied, are subnormal plasma glucose levels and the absence of food intake. Both glucose and eating have long been recognized to have an impact on the LHA and to influence specific populations of LHA neurons. Some (~25%) LHA neurons are thought to be glucose-sensitive, being inhibited by increases in circulating glucose and stimulated by falls in glucose within the normoglycemic and hypoglycemic range (10–13). Some glucose-sensitive neurons are also stimulated by insulin and by 2-DOG (10,31). These neurons' responses to changes in glucose are mostly indirectly mediated, apparently relayed via the NTS and parabrachial nucleus from glucoreceptors in extrahypothalamic sites, including the NTS itself and the gut and the liver, from which signals are carried via vagal afferents to the NTS (14,32,33). In particular, certain glucose-sensitive neurons are inhibited specifically by a rise in glucose levels in the hepatic portal vein or by gastric distension (14). These neurons—which have not been characterized neurochemically—therefore have the capacity to respond in the way predicted by our studies of the orexin neurons.

We therefore suggest that the orexin neurons belong to the glucose-sensitive subpopulation of LHA neurons that are stimulated by falls in circulating glucose and inhibited by signals related to feeding, such as gastric distension and/or a rise in portal glucose concentrations. The known properties of LHA glucose-sensitive neurons point to two plausible explanations for the absence of prepro-orexin mRNA increases in rats that were chronically food-restricted rather than acutely fasted, and in rats with acute or chronic hypoglycemia that were allowed to overeat. First, the neuroglycopenic stimulus may have been less intense. In chronic under-feeding, CNS metabolism may adapt to allow neurons to utilize ketones (34), which are also known to inhibit some glucose-sensitive neurons (10). In chronic hypoglycemia, other compensatory mechanisms come into operation, with enhanced glucose transport across the blood-brain barrier (35). Second, the stimulatory effect of low glucose levels may have been counteracted in animals allowed to eat by inhibitory prandial signals, such as a rise in portal-vein glucose or gastric distension (14). The absence of any increase in prepro-orexin mRNA in acutely hypoglycemic rats allowed to eat may suggest that the orexin neurons are extremely sensitive to rapid inhibition by visceral signals.

The failure of 2-DOG to increase prepro-orexin mRNA is striking, because the dosage schedule we used mimicked closely the feeding profile with acute hypoglycemia. Moreover, both 2-DOG and hypoglycemia are presumed to interfere with neuronal glucose metabolism (20–22), and LHA lesions abolish the hyperphagia usually caused by both agents (10). However, different CNS pathways must mediate the hyperphagia that they induce, because lesions of the zona incerta or amygdala abolish the feeding caused by 2-DOG but not by insulin (36,37). Modulatory inputs from these other pathways may explain the divergent effects of hypoglycemia and 2-DOG on orexin neurons.

In conclusion, we suggest that the orexin neurons are stimulated by falls in plasma glucose and inhibited by signals related to feeding. As such, they may be important in the short-term "glucostatic" feeding mechanisms postulated by Mayer (38) to maintain glucose availability. Further work will clarify whether they respond to the transient decreases in plasma glucose that are suggested to trigger spontaneous feeding episodes (39), and whether they correspond to the glucose-sensitive neurons of the LHA that respond to visceral signals transmitted via the NTS.

ACKNOWLEDGMENTS

X.J.C. was supported by a CASE award from SmithKline Beecham and the Medical Research Council, and by an Overseas Research Scholarship from the Committee of Vice-Chancellors and Principals of the U.K. J.H. is supported by Astra Hässle, Gothenberg, Sweden.

We are grateful to Leigh Thompson for conscientious care of the animals.

REFERENCES

1. Sakurai T, Amemiya A, Tshii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M: Orexins and orexin receptors: a family of hypothalamic neuropeptides and G-protein–coupled receptors that regulate feeding behavior. *Cell* 92:573–585, 1998
2. Peyron C, Tighe DK, van den Pol AN, De Lecea L, Heller HC, Sutcliffe JG, Kilduff TS: Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996–10015, 1998
3. Horvath TL, Diano S, van den Pol AN: Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci* 19:1072–1087, 1999
4. Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kaugawa K, Sakurai T, Yanagisawa M, Nakazato M: Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine, and neuroregulatory systems. *Proc Natl Acad Sci U S A* 96:748–753, 1999
5. Trevidi P, Yu H, MacNeil D, Van der Ploeg LHT, Guan X-M: Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett* 438:71–75, 1998
6. De Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuliara C, Battenberg EL, Gautvik VT, Bartlett FS II, Frankel WN, van den Pol AN, Bloom FF, Fautvik KM, Sutcliffe JG: The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 95:322–327, 1998
7. Lubkin M, Stricker-Krongrad A: Independent feeding and metabolic action of orexins in mice. *Biochem Biophys Res Commun* 253:241–245, 1998
8. Edwards CMB, Abusnana S, Sunter D, Murphy KG, Ghatei MA, Bloom SR: The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone, and galanin. *J Endocrinol* 160:R7–R12, 1999

9. Dube MG, Kalra SP, Kalra PS: Food intake elicited by central administration of orexins: identification of hypothalamic sites of action. *Soc Neurosci Abstracts* 24:448, 1998
10. Bernardis LL, Bellinger LL: The lateral hypothalamus revisited: ingestive behavior. *Neurosci Biobehav Rev* 20:189–287, 1996
11. Oomura Y, Ooyama H, Sugimori M, Nakemura T, Yamada Y: Glucose inhibition of the glucose-sensitive neurons in the rat lateral hypothalamus. *Nature* 247:284–286, 1974
12. Orsini JC, Armstrong DL, Wagner MJ: Responses of lateral hypothalamic neurons recorded in vitro to moderate changes in glucose concentration. *Brain Res Bull* 29:503–505, 1992
13. Shimizu N, Oomura Y, Novin D, Grijalva CV, Cooper PH: Functional correlations between lateral hypothalamic glucose-sensitive neurons and hepatic portal glucose-sensitive units in rat. *Brain Res* 265:49–54, 1983
14. Chambert G, Kobashi M, Adachi A: Convergence of gastric and hepatic information in brain stem neurons of the rat. *Brain Res Bull* 32:525–529, 1993
15. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763–770, 1998
16. Dryden S, Frankish HM, Wang Q, Williams G: Neuropeptide Y and the hypothalamus: one way forward for the treatment of obesity? *Eur J Clin Invest* 246:293–308, 1994
17. Strack AM, Sebastian RJ, Schwartz MW, Dallan MF: Glucocorticoids and insulin: reciprocal signals for energy balance. *Am J Physiol* 268:R142–R149, 1995
18. Widdowson PS, Upton R, Buckingham R, Arch J, Williams G: Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet-induced obesity. *Diabetes* 46:1782–1785, 1997
19. Le Magnen J: Body energy balance and food intake: a neuroendocrine regulatory mechanism. *Physiol Rev* 63:315–386, 1983
20. Smith GP, Epstein AN: Increased feeding in response to decreased glucose utilization in the rat and monkey. *Am J Physiol* 217:1083–1087, 1969
21. Stricker EM, Rowland N: Hepatic versus cerebral origin of stimulus for feeding induced by 2-deoxy-D-glucose in rats. *J Comp Physiol Psychol* 92:126–132, 1978
22. Granneman J, Friedman MI: Feeding after recovery from 2-deoxyglucose injection: cerebral and peripheral factors. *Am J Physiol* 244:R383–R388, 1983
23. Williams G, Steel JH, Cardoso H, Ghatei MA, Lee YC, Gill JS, Burrin JM, Polak JM, Bloom SR: Increased hypothalamic neuropeptide Y in the streptozotocin-diabetic rat. *Diabetes* 37:763–772, 1988
24. Griffond BG, Risold PY, Jacquemard C, Colard C, Fellmann D: Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. *Neurosci Lett* 262:77–80, 1999
25. Brady LS, Smith MA, Gold PW, Herkenham M: Altered expression of hypothalamic neuropeptide Y mRNA in food-restricted and food-deprived rats. *Neuroendocrinology* 52:441–447, 1999
26. Beck B, Jhanwar-Uniyal M, Burlet A, Chapleur-Chateau M, Leibowitz SF, Burtlet C: Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. *Brain Res* 529:245–249, 1990
27. White JD, Olchovsky D, Kershaw M, Berlowitz M: Increased hypothalamic content of preproneuropeptide Y messenger RNA in streptozotocin-diabetic rats. *Endocrinology* 126:765–772, 1990
28. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, Mackellar W, Rosteck PR, Schoner B, Smith D, Tinsley FC, Zhang XY, Heiman M: Fat regulation: the role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530–532, 1995
29. Wang Q, Bing C, Al-Barazanji K, Morralowaska DE, Wang XM, McBay DL, Neville WA, Tadayyon M, Pickavace L, Dryden S, Thomas ME, McHale MT, Gloyer IS, Wilson S, Buckingham R, Arch JR, Trayhurn P, Williams G: Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes* 46:335–341, 1997
30. Schwartz MW, Figlewicz DP, Baskin DG, Woods SC, Porte D: Insulin in the brain: a hormonal regulator of energy balance. *Endocr Rev* 13:387–414, 1992
31. Katafuchi T, Oomura Y, Yoshimatsu H: Single unit activity in the lateral hypothalamus during 2-deoxy-D-glucose induced and natural feeding behavior. *Brain Res* 359:1–9, 1985
32. Orsini JC, Himmi T, Wiser AK, Perrin J: Local versus indirect action of glucose on the lateral hypothalamic neurons sensitive to glycemic levels. *Brain Res Bull* 25:49–53, 1980
33. Yettefti K, Orsini JC, El Ouazzani T, Himmi T, Boyer A, Perrin J: Sensitivity of nucleus tractus solitarius neurons to induced moderate hyperglycemia, with special reference to catecholaminergic regions. *J Auton Nerv Syst* 51: 191–197, 1995
34. Gjedde A, Grone C: Induction processes in blood-brain transfer of ketone bodies during starvation. *Am J Physiol* 229:1165–1169, 1975
35. Kumagai AK, Kang YS, Boado RJ, Pardridge WM: Upregulation of blood-brain barrier GLUT1 glucose transporter protein and mRNA in experimental chronic hypoglycemia. *Diabetes* 44:1399–1404, 1995
36. Tordoff MG, Geiselman PJ, Grijalva CV, Kiefer SW, Novin D: Amygdaloid lesions impair ingestive responses to 2-deoxy-D-glucose but not insulin. *Am J Physiol* 242:R129–R135, 1982
37. Walsh LL, Grossman SP: Loss of feeding in response to 2-deoxy-D-glucose but not insulin after zona incerta lesions in the rat. *Physiol Behav* 15:481–485, 1975
38. Mayer J: Regulation of energy intake and body weight: the glucostatic theory and lipostatic hypothesis. *Ann N Y Acad Sci* 63:15–42, 1955
39. Campfield LA, Smith FJ: Systemic factors in the control of food intake: evidence for patterns as signals. In *Neurobiology of Food and Fluid Intake: Handbook of Neurobiology*. Vol. 10. Stricker EM, Ed. New York, Plenum, 1990, p. 183–206