Hypoglycemia Activates Orexin Neurons and Selectively Increases Hypothalamic Orexin-B Levels

Responses Inhibited by Feeding and Possibly Mediated by the Nucleus of the Solitary Tract

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Orexins are novel appetite-stimulating peptides expressed in the lateral hypothalamic area (LHA), and their expression is stimulated by hypoglycemia in fasted rats. We investigated activation of orexin and other neurons during insulin-induced hypoglycemia using the immediate early gene product Fos. Insulin (50 U/kg) lowered plasma glucose by >50% after 5 h and stimulated feeding sixfold compared with saline-injected controls. Hypoglycemic rats allowed to feed and normoglycemic controls both showed sparse Fos-positive (Fos$^+$) neurons in the LHA and the paraventricular nucleus (PVN) and arcuate nucleus (ARC) and showed none in the nucleus of the solitary tract (NTS), which relays visceral feeding signals to the LHA. In the LHA, total numbers of Fos$^+$ neurons were comparable in fed hypoglycemic and control groups (60 ± 6 vs. 52 ± 4 cells/mm^2, *P* > 0.05), as were Fos$^+$ neurons immunoreactive for orexin (1.4 ± 0.4 vs. 0.6 ± 0.4 cells/mm^2, *P* > 0.05). By contrast, hypoglycemic rats that were fasted showed significantly more Fos$^+$ nuclei in the LHA (96 ± 10 cells/mm^2, *P* < 0.05, vs. both other groups) and Fos$^+$ orexin neurons (8.4 ± 3.3 cells/mm^2, *P* < 0.001, vs. both other groups). They also showed two- to threefold more Fos$^+$ nuclei (*P* < 0.001) in the PVN and ARC than both fed hypoglycemic rats and controls and showed strikingly abundant Fos$^+$ neurons in the NTS and dorsal motor nucleus of the vagus. In parallel studies, whole hypothalamic orexin-A levels were not changed in hypoglycemic rats, whether fasted or freely fed, whereas orexin-B levels were 10-fold higher in hypoglycemic fasted rats than in control and hypoglycemic fed groups. These data support our hypothesis that orexin neurons are stimulated by falling glucose levels but are readily inhibited by signals related to nutrient ingestion and suggest that they may functionally link with neuronal activity in the NTS. Orexin-A and -B may play specific roles in behavioral or neuroendocrine responses to hypoglycemia. *Diabetes* 50:105–112, 2001

The lateral hypothalamic area (LHA) is important in the overall regulation of feeding behavior and body weight (1). Specifically, it is crucial for the intense hunger that is caused by hypoglycemia. Eating is one component of the concerted metabolic response that is mounted to restore normal blood glucose levels. Lesions of the LHA abolish the hyperphagic response to hypoglycemia induced by insulin (2) and also to neuroglycopenia caused by 2-deoxyglucose (3).

The neurochemical identities of the LHA neurons and pathways that mediate hypoglycemia-induced hyperphagia remain uncertain. The LHA contains glucose-sensitive neurons that are stimulated by hypoglycemia, and these account for ~25% of the LHA neurons (4,5). Hypoglycemia mainly activates LHA glucose-sensitive neurons indirectly (6), and pathways ascending from the brainstem are thought to be particularly important. These include a projection from the nucleus of the solitary tract (NTS) (7,8), which relays information from vagal afferents including glucoreceptors in the gut and liver (9). About 75% of recorded NTS neurons respond with altered electrical activity to blood glucose fluctuations within the physiological range (10).

Recent evidence suggests that some of the LHA neurons that respond to hypoglycemia express the peptides known as orexins (11) or hypocretins (12). The hypocretins have sequences similar to but longer than orexins. Hypocretin-1 includes the orexin-A sequence with five extra amino acids at the NH_2-terminus and one at the COOH-terminus, whereas hypocretin-2 has one extra residue at the COOH-terminus of the orexin-B sequence (12). Orexin-A and -B are 33- and 28-residue peptides, respectively, derived from prepro-orexin (13), and were named for their ability to stimulate feeding when injected intracerebroventricularly or into the LHA (13–19). Orexins are expressed solely in a neuronal population that is restricted to the perifornical LHA and zona incerta (20–24). This region has reciprocal connections with numerous hypothalamic and

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3-D, three-dimensional; ARC, arcuate nucleus; DMN, dorsomedial hypothalamic nucleus; DMNX, dorsal motor nucleus of the vagus; LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PBS, phosphate-buffered saline; PVN, paraventricular nucleus.

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extrahypothalamic structures involved in feeding behavior, including the hypothalamic paraventricular nucleus (PVN) and arcuate nucleus (ARC) and the NTS (20,24).

We and others previously showed that hypothalamic prepro-orexin mRNA levels were increased by prolonged fasting and by insulin-induced hypoglycemia when food was not available (13,25). We suggested that orexin neurons are stimulated by falling blood glucose levels but inhibited by food ingestion and that they might participate in the on-off regulation of short-term feeding behavior (25). It is now clear that orexin neurons are those previously identified as containing "prolactin-like immunoreactivity," which are known to be activated by hypoglycemia (26).

Here, we explored further the relationship between hypoglycemia, food intake, and orexin neuronal activation and the possible mediatory role of the NTS. We again induced acute (5-h) hypoglycemia with insulin in groups of rats that were either allowed to eat freely or were fasted throughout. We first used the early response gene marker Fos to map neuronal activation in the LHA, PVN, ARC, and NTS together with immunohistochemical identification of orexin neurons. In a parallel experiment, we used specific radioimmunoassays to measure changes in whole hypothalamic orexin-A and -B concentrations under the same experimental conditions.

RESEARCH DESIGN AND METHODS

Experiment 1.

Animals. Eighteen adult male Wistar rats were housed singly in environmentally controlled conditions (22 ± 2°C, humidity 40–60%) under a 12-h light/dark cycle (lights on at 0800) and habituated over 1 week to frequent handling, daily weighing, and food intake measurements. They were allowed free access to standard laboratory pelleted rodent diet (CRM; Biosure, Cambridge, U.K.), except during the 5-h experimental period described below, and tap water was freely available throughout. Each rat received a saline injection (0.2 ml intraperitoneally) at 0900 daily for 3 days before the study. Two groups ($n = 6$ for each) were given an intermediate-duration insulin (50 U/kg Insulatard) intraperitoneally at 0900 to induce hypoglycemia, as in our previous study (25). One hypoglycemic group was allowed to feed freely, whereas the other was food deprived. The third group (euglycemic controls) received the same volume of saline intraperitoneally and was allowed to eat freely ($n = 6$). The study was terminated 5 h after injection, when food intake was measured in the rats allowed to eat. The rats were killed, and tissue was prepared for immunohistochemistry as below.

Tissue and sample preparation. The rats were deeply anesthetized with a lethal dose of Hypnorm/Hypnovel (3 mg/kg), and blood was collected and centrifuged. Plasma was stored until assayed for plasma glucose using an autoanalyzer (glucose oxidase method; Yellow Springs Instruments, Yellow Springs, OH). The rats were perfused transcardially with 0.9% saline containing heparin (20 U/ml), followed by ice-cold fixative solution containing 4% paraformaldehyde in 0.01 mol/l phosphate-buffered saline (PBS). Brains were removed, post-fixed overnight by immersion in the same fixative, and cryoprotected with 0.5 mol/l sucrose for 24 h at 4°C. Brains were rapidly frozen in dry ice–cooled isopentane and sliced into 50-μm coronal sections on a cryostat (–23°C). Free-floating sections were rinsed several times and stored in immunobuffer (0.01 mol/l PBS containing 0.3% Triton X-100 and 0.12% thimerosal) at 4°C.

Immunohistochemistry. A preliminary study using chromogenic double-labeling detection of Fos and orexin immunoreactivities (i.e., avidin-biotin complex plus diaminobenzidine for Fos, and Vector blue alkaline phosphatase detection for orexin) did not in all cases allow us to determine whether Fos-positive (Fos$^+$) nuclear profiles lay within orexin-containing cells, especially where cell bodies were strongly orexin-positive. To overcome this difficulty, we developed a fluorescent double-labeling protocol similar to that used by others to study colocalization of receptors and Fos$^+$ neurons (27). This technique demonstrated patterns of Fos and orexin immunoreactivities that were identical to those we obtained using chromogenic detection and are similar to previous reports (28,29). The fluorescence method in combination with confocal analysis proved to be more powerful than histochemical detection methods because it revealed greater subcellular and three-dimensional (3-D) detail. In particular, we could interrogate the confocal 3-D volume in cases where the association of Fos and orexin was ambiguous (see "Image analysis.").

Fluorescence double-labeling was performed on every fifth frontal section through the hypothalamic region that contained orexin neurons and on every tenth frontal section through the medulla at the level of the NTS. Sections were rinsed three times in immunobuffer before incubation with avidin D solution followed by biotin solution (Vector Laboratories, Burlingame, CA) to block endogenous biotin-binding sites.

The sections were incubated overnight with a polyclonal sheep anti-Fos antibody (1:3,000; Genosys, Cambridge U.K.), then for 2 h with biotin-SP–conjugated donkey anti-sheep IgG (1:500; Jackson ImmunoResearch, West Grove, PA), followed by Alexa 488–conjugated NeutrAvidin (1:1,000; Molecular Probes, Eugene, OR) for 2 h. Sections were next incubated overnight in a rabbit polyclonal antibody against a partial sequence (amino acids 14–33) of orexin-A (1:1,000; raised in-house at SmithKline Beecham, as reported before [29]), followed by Cy5-conjugated donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch) for 2 h. Before each incubation, the sections were washed thoroughly three times for 10 min in immunobuffer. All incubations were at room temperature. Finally, the sections were mounted on cover slips in Citifluor (Citifluor, London, U.K.), sealed, and stored at 4°C. The specificity of the anti-orexin serum was tested by preincubating the primary antiserum with excess synthetic orexin-A (50 μg/ml) for 2 h before use or by omitting the primary antiserum from the incubation steps. In both cases, selective staining of cell profiles was abolished (29). To test for specificity of the Fos antiserum, the primary antiserum was omitted from the incubation procedure, and this resulted in no selective immunostaining.

Fluorescence double-labeling was visualized using a laser scanning confocal microscope (Leica TCS-SP; Leica, Heidelberg, Germany). Alexa 488 was excited by the 488-nm laser line and emitted light collected at 540 ± 35 nm to reveal Fos immunoreactivity. Cy5 was excited using the 633-nm laser line and emitted light collected at 675 ± 35 nm to reveal orexin immunoreactivity. With this arrangement, no cross talk was observed between the detection channels, so scanning and detection of Alexa 488 and Cy5 were performed simultaneously.

Image analysis. For each region to be analyzed, a series of 8–12 optical sections at intervals of 1.0–1.2 μm was examined to generate a brightest-point projection single image containing both Fos and orexin immunoreactivity. For the LHA, Fos$^+$ nuclei were counted and categorized as whether or not they lay within an orexin-containing neuronal cell body; in cases where coincidence was ambiguous, a 3-D volume image was constructed and rotated to clarify the relationship. Counts were expressed as the number of nuclei per 500 × 500-μm square field, bounded medially by the fornix and extending laterally toward the internal capsule and dorsally toward the zona incerta. For the PVN, dorsomedial hypothalamic nucleus (DMN), and ARC, sections were similarly imaged and Fos$^+$ nuclei counted in identified anatomical areas contained within a 1,000 × 1,000-μm square field. Results were expressed as means ± SE nuclei/mm^2 (bilaterally, 8–10 sections each animal).

Brainstem sections were examined in a dark field using a dissecting microscope (Leica FL3) to determine the rostrocaudal level relative to Bregma according to the atlas of Paxinos and Watson (30). Sections were first viewed using differential interference contrast (Leica DMR; Leica, Heidelberg, Germany) to identify neuroanatomical boundaries and then viewed in fluorescence mode to count Fos$^+$ nuclei. Using DIC, the boundary between the NTS and the dorsal motor nucleus of the vagus (DMNX) was identified by the larger cell bodies of the DMNX. Separate counts were made for the DMNX and NTS and expressed as total numbers in these smaller areas.

Experiment 2.

Animals. Three groups of Sprague-Dawley rats ($n = 8$) were given the same treatment as in experiment 1 and were killed 6 h after injection. The hypothalamus was immediately dissected en bloc under a binocular microscope from a frontal slice of fresh unfixed brain tissue between the optic chiasm and the mammillary bodies, cutting horizontally below the anterior commissure and vertically through the perihypothalamic sulci (31). The hypothalamus was boiled in 0.1 mol/l hydrochloric acid for 10 min to extract peptides, and the supernatant was kept at –80°C until assayed for protein concentrations (BCA protein assay kit; Pierce, Rockford, IL) and orexins.

Radioimmunoassays. Hypothalamic orexin-A and -B levels were measured using commercially available radioimmunoassay kits (Peninsula Laboratories, San Carlos, CA). Neither the orexin-A nor -B assay has significant cross-reactivity with neuropeptide Y (NPY), α-melanocyte–stimulating hormone, or other major neuropeptides, and the orexin-A and -B assays do not have significant cross-reactivity with each other. The orexin-A antiserum does not cross-react with mouse hypocretin-1, whereas the cross-reactivity of orexin-B antiserum with mouse hypocretin-2 is 100%. The within-assay coefficient of variation for orexin-A was 5.0% and for orexin-B 6.3%.

Statistical analyses. Data were expressed as means ± SE and analyzed by two-way analysis of variance followed by Student's t test.

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TABLE 1
Body weight, fat mass, food intake, and terminal plasma glucose in control and hypoglycemic rats with and without access to food

	Control	Hypoglycemic, fed	Hypoglycemic, fasted
n	6	6	6
Body weight (g)	308 ± 8	292 ± 5	300 ± 4
Fat mass (g)	2.77 ± 0.14	2.79 ± 0.32	2.79 ± 0.14
Food intake (g)	0.8 ± 0.2	5.4 ± 0.3*	0
Plasma glucose (mmol/l)	9.6 ± 0.5	4.7 ± 0.4*	3.0 ± 0.2*†

Data are means ± SE. *$P < 0.05$ vs control; †$P < 0.05$ vs. hypoglycemic freely fed group.

RESULTS

Hypoglycemia and hyperphagia. Saline-treated controls had terminal blood glucose concentrations of 9.5 ± 0.5 mmol/l, whereas insulin injection induced profound hypoglycemia, which was more severe in insulin-treated rats that were not allowed to feed (Table 1). Hypoglycemia markedly stimulated feeding by sixfold during the 5-h period ($P < 0.001$).

Orexin and Fos immunoreactivities in LHA neurons. Orexin-immunoreactive cell bodies were seen in the LHA, confined to the peri- and subfornical distribution previously reported (20–24). The cell bodies were spheroidal or elongated, and orexin-immunoreactive fibers were found in the PVN, LHA, ARC, and median eminence. Numbers of cell bodies immunoreactive for orexin were not different among euglycemic rats and hypoglycemic rats, either fasted or freely fed (Fig. 1).

Fos distribution induced by hypoglycemia was the same as in a previous report (28). In euglycemic rats, Fos immunoreactivity (confined to the nucleus) was seen in occasional cells of the thalamus and hypothalamus—notably in the PVN, LHA, DMN, and ARC (Fig. 2). In hypoglycemic fed rats, numbers of Fos-immunoreactive neurons in the LHA were slightly but not significantly higher than in vehicle-treated controls, whereas the hypoglycemic fasted rats had significantly more Fos^+ nuclei than both other groups, with nearly twice as many as in controls ($P < 0.001$) (Table 2, Figs. 1 and 2).

Double-labeling showed that only sparse Fos^+ nuclei belonged to orexin neurons in both controls and hypoglycemic fed rats (1.2 and 2.1% of Fos^+ nuclei, respectively) and that only 0.7 and 1.7% of orexin cells in control and hypoglycemic fed rats included a Fos^+ nucleus. However, coincidence of Fos with orexin was significantly increased in hypoglycemic rats that were not allowed to eat compared with both control and freely fed hypoglycemic rats (Table 2 and Fig. 1). In this group, 8.8% of Fos^+ nuclei belonged to orexin neurons, whereas 13.9% of orexin neurons were Fos^+.

Fos in the PVN and ARC. Controls and fed hypoglycemic rats had comparable low numbers of Fos^+ cells in the ARC (Table 2). In hypoglycemic rats that were fasted, numbers of Fos^+ nuclei in the ARC were significantly increased by 2-fold and 1.5-fold compared with those in controls and hypoglycemic fed rats, respectively (both $P < 0.05$). Similarly, Fos^+ cells in the PVN were increased by 1.5-fold and 2-fold, respectively (both $P < 0.0001$), in hypoglycemic fasted rats compared with the control and hypoglycemic fed groups (Table 2).

Fos in the NTS and DMNX. Neither euglycemic controls nor freely fed hypoglycemic rats showed any Fos^+ neurons in the NTS, whereas hypoglycemic fasted rats had abundant Fos^+ cells

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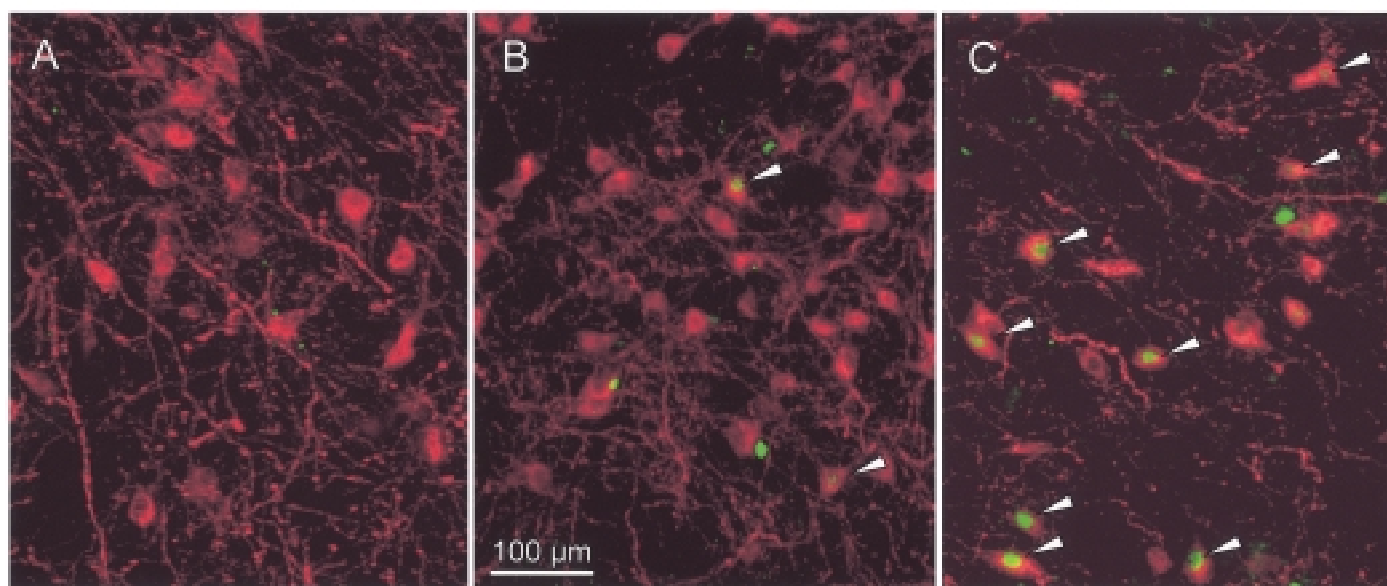

FIG. 1. Photomicrographs showing Fos and orexin in neurons of the LHA in saline control (*A*) and in insulin-treated rats that were either hypoglycemic and freely fed (*B*) or hypoglycemic and fasted (*C*). Fos appears as green fluorescence in the nucleus, whereas orexin-A appears as red fluorescence in the cytoplasm. Arrows indicate representative neurons with coincident Fos and orexin labeling.

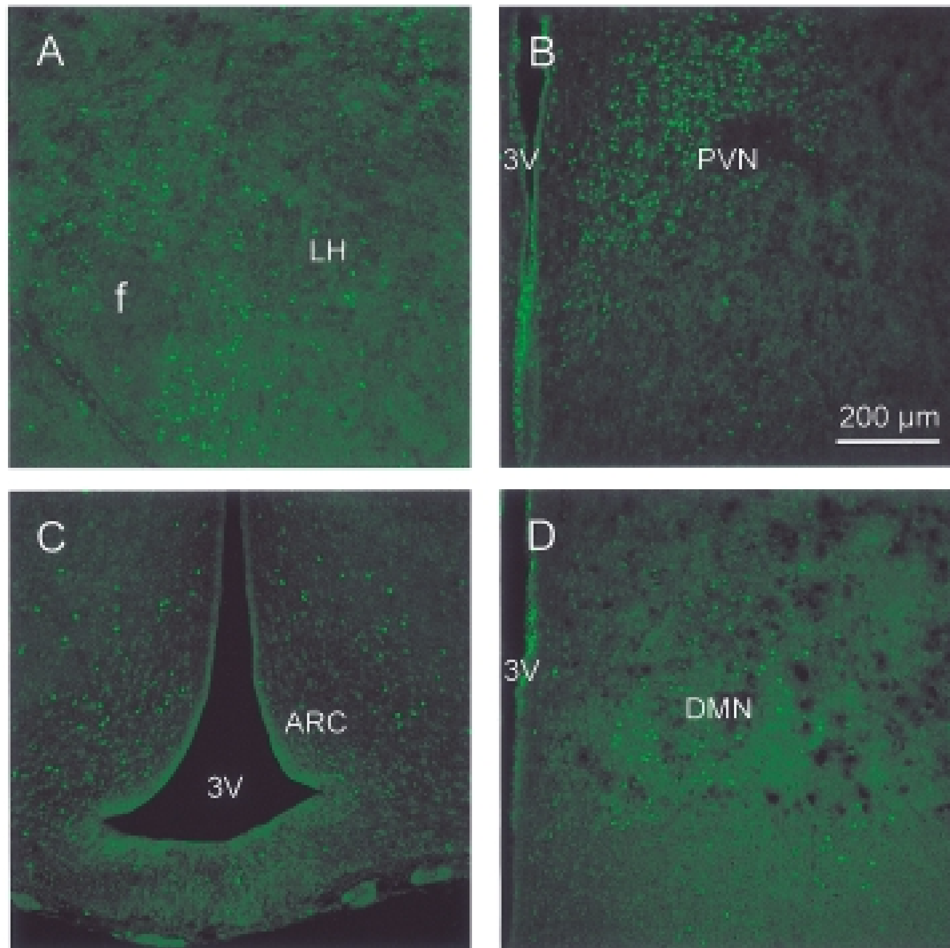

FIG. 2. Photomicrographs of Fos staining in the hypothalamic areas in hypoglycemic rats that were fasted for 5 h. *A*: Lateral hypothalamus area (LH); *B*: paraventricular hypothalamic nucleus (PVN); *C*: ARC; *D*: dorsal medial hypothalamic nucleus (DMN); 3V, third ventricle; f, fornix.

in the NTS. Fos^+ nuclei were observed in several subregions of the NTS, including the region of the C2 adrenergic cell complex and the ventral portion of the commissural NTS, with a sparser distribution near the border with the area postrema. Fos nuclei were also strikingly abundant in the dorsal motor nucleus of the vagus (DMNX), an area adjacent to the NTS, at a rostro-caudal level extending between 12.8 and 14.1 mm caudal to Bregma (Fig. 3 and Table 3). Labeled cell counts in the brainstem are shown in Table 3 and Fig. 3.

Orexin-A and -B concentrations in the hypothalamus. Orexin-A and -B were both detected in all hypothalamic extracts from all rats. In controls, orexin-A levels were approximately twice as high as orexin-B levels ($P < 0.0001$). Compared with euglycemic rats, orexin-A levels were not changed in hypoglycemic rats, whether freely fed or fasted throughout (Fig. 4). Orexin-B concentrations in hypoglycemic freely fed rats were comparable to those in controls. Notably, however, hypothalamic orexin-B concentrations were raised in the fasted

TABLE 2
Number of Fos- and orexin-immunoreactive cell bodies in hypothalamic nuclei and in the NTS in euglycemic and fasted or freely fed hypoglycemic rats

	Control	Hypoglycemic, fed	Hypoglycemic, fasted
n	6	6	6
LHA			
Fos^+	52 ± 4	60 ± 6	96 ± 10*†
Orexin with Fos^+	0.6 ± 0.4	1.4 ± 0.4	8.4 ± 3.3*†
PVN: Fos^+	42 ± 5	32 ± 4	105 ± 10*†
ARC: Fos^+	30 ± 4	38 ± 3	98 ± 12*†

Cell numbers are per millimeter squared, expressed as means ± SE. *$P < 0.001$ vs. controls; †$P < 0.001$ vs. hypoglycemic fed group.

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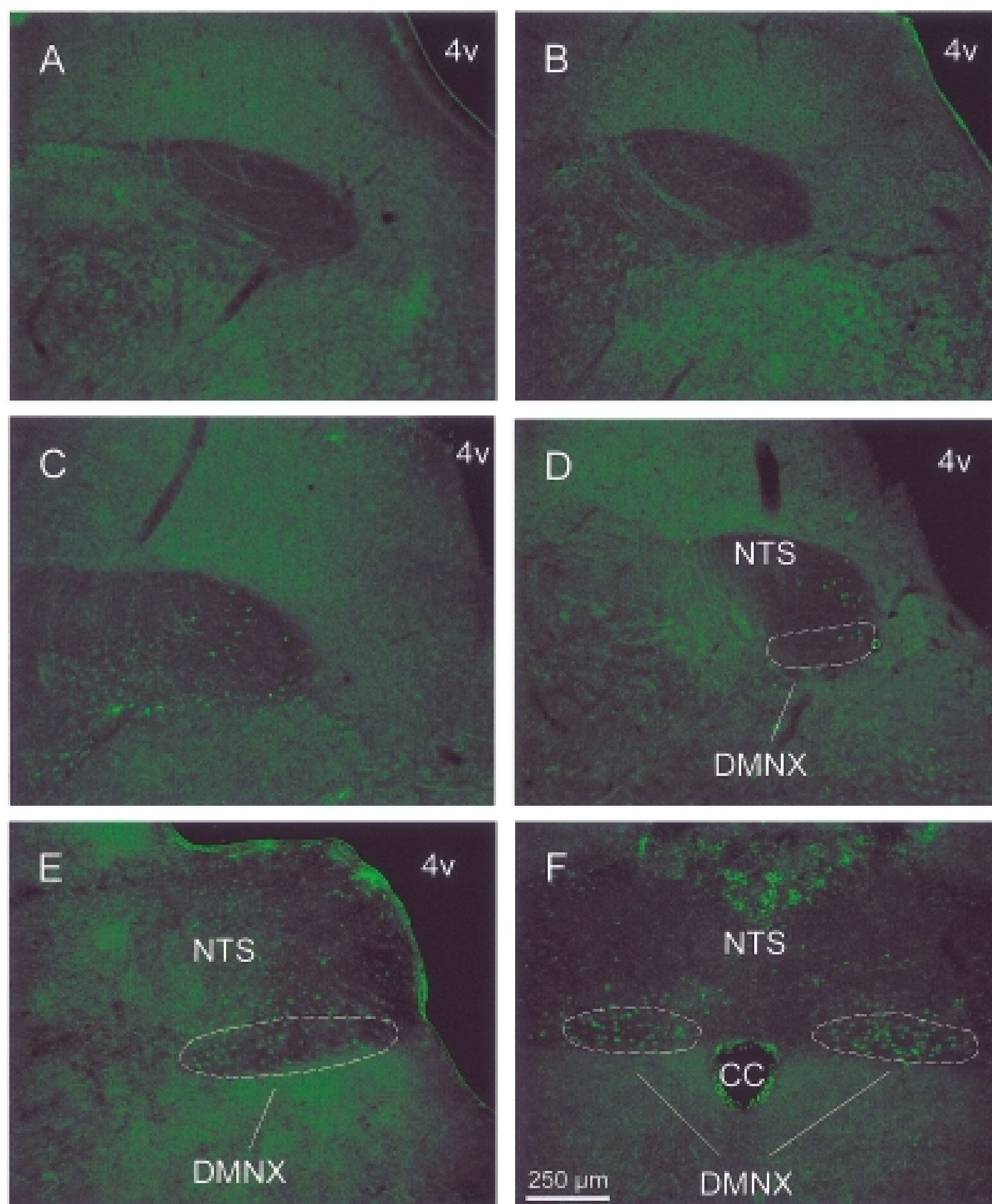

FIG. 3. Photomicrographs showing Fos expression in the NTS and DMNX in saline control (*A*) and in hypoglycemic rats that were freely fed (*B*) or food-deprived (*C–F*). Fos appears as green dots. Images in *A*, *B*, and *C* were taken at levels approximating to Bregma –12.72 mm. Images in *D*, *E*, and *F* were taken at levels approximating to Bregma –12.80, –13.30, and –14.08 mm, respectively. 4V, fourth ventricle; CC, central canal.

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hypoglycemic rats, being over 10-fold higher than those in both other groups (both $P < 0.0001$) (Fig. 4).

DISCUSSION

The LHA is one of several brain regions that cooperates in the control of energy homeostasis; it was previously regarded as a feeding center because lesions here can cause a sustained decrease in food intake and profound weight loss (1). The LHA contains distinct neuronal populations that express specific appetite-stimulating neurotransmitters, including the orexins and melanin-concentrating hormone (MCH) (32), and is also a site of projection of other neurons that release orexigenic neurotransmitters—notably the NPY neurons of the ARC (33). The LHA appears to be crucial in mediating the intense hyperphagia induced when blood glucose levels fall into the hypoglycemic range, because LHA lesions abolish this selectively without impairing the hyperphagic response to other conditions such as fasting (2).

Previous studies by our group and others (11,25,34) indicate that prepro-orexin expression in LHA neurons is stimulated by profound hypoglycemia, suggesting that increased release of the appetite-stimulating orexin-A could contribute to hypoglycemia-induced hyperphagia. Interestingly, prepro-orexin expression does not increase if hypoglycemic rats either have free access to food (25) or are given a glucose infusion to maintain euglycemia (34). Prepro-orexin expression

TABLE 3
Number of Fos+ nuclei in the brainstem in hypoglycemic rats that were not allowed to eat

Levels to Bregma	NTS	DMNX
–11.6	6.0 ± 0	—
–11.8	4.0 ± 1.15	2.0 ± 0
–11.96	21.0 ± 0.58	—
–12.3	12.3 ± 2.8	4.5 ± 0.3
–12.72	4.3 ± 1.3	6.0 ± 1.0
–12.8	19.3 ± 5.1	4.0 ± 0.7
–13.24	24.2 ± 9.9	13.4 ± 2.3
–13.68	18.8 ± 8.7	19.8 ± 5.1
–14.08	9.5 ± 3.7	47.0 ± 2.5
–14.30	9.0 ± 1.7	12.0 ± 0.5

Cell numbers are per nucleus, expressed as means ± SE. Fos+ nuclei were totally absent from control and hypoglycemic fed groups.

also increased in rats fasted for 48 h, but not in others that were food-restricted for several days to produce weight loss comparable to 48 h of starvation or in several other conditions of increased hunger, including insulin-deficient diabetes and provision of a highly palatable diet (25). Here, we demonstrated that activation of orexin neurons was significantly increased 5 h after insulin injection in profoundly hypoglycemic rats that were not allowed to eat, whereas no such increases were found in hypoglycemic rats that were allowed free access to food. These findings parallel our previous studies of prepro-orexin expression and support our suggestion that orexin neurons are stimulated by falling glucose and inhibited by feeding-related signals (25). Prandial signals in the hypoglycemic rats that ate could include the presence of food in the gut (e.g., gastric stretch receptors) or increases in glucose or other nutrients in the hepatic portal circulation.

However, it is not clear whether orexin neurons respond directly to changes in local glucose availability, as is the case for certain neurons in the ventromedial hypothalamus and NTS (10,35), or whether modulating signals are relayed indirectly to the LHA (6). One well-established indirect pathway is the projection ascending from the NTS, which receives input from visceral glucoreceptors and other vagal afferents (36). The NTS contains neurons that are stimulated by falling glucose in the systemic or portal circulations and inhibited by rising systemic or portal glucose and by gastric distension (10,37); these neurons therefore have the potential to integrate various feeding-related signals.

We found significantly increased numbers of activated orexin and nonorexin LHA neurons only in fasted hypoglycemic rats. Neuronal activation in the LHA was accompanied by the appearance of Fos+ neurons in the NTS and adjacent DMNX; these responses were entirely absent in both the euglycemic controls and in the fed hypoglycemic rats. The DMNX, intimately linked with the NTS, controls gut motility and the secretory responses that are particularly important in feeding behaviors (38). The strikingly similar pattern in the NTS and LHA leads us to believe that the signals that triggered orexin neurons may be relayed via the NTS, which has a major projection to the LHA (8).

Interestingly, orexin neurons accounted numerically for only about one-quarter of the additional number of LHA neurons activated in fasted hypoglycemic rats (Table 2). The neurochemical identities of the other Fos+ neurons are not clear. The activation of neurons in the ARC and PVN, both sites involved in regulation of feeding, suggests that some neurons from these areas may contribute to hypoglycemia-induced hyperphagia. Prolonged (24-h) fasting is known to activate neurons in these areas (39,40); however, the brief 5-h fast during the light phase (when the normoglycemic rats ate <1 g) was unlikely to have affected Fos expression per se in the hypoglycemic food-deprived group. An obvious candidate is NPY, the most potent appetite-stimulating peptide known, which is produced in the ARC and released in sites including the PVN and LHA (32). However, hypothalamic NPY gene expression and NPY levels were not affected by hypoglycemia in rats allowed to eat (41,42), although NPY has not been reported in fasted hypoglycemic rats. MCH, produced by a separate population of LHA neurons (32), stimulates feeding experimentally and may regulate energy homeostasis because MCH-deficient mice are hypophagic and lean (43). MCH expression is increased by insulin-induced hypoglycemia, but MCH neurons do not show Fos immunoreactivity during hypoglycemia (44). There are extensive synaptic connections between NPY, proopiomelanocortin, MCH, and orexin neurons (22,45), and functional interactions among these may drive hyperphagia during hypoglycemia.

We found ~14% of orexin neurons to be Fos+, a lower proportion than that reported by others (11). This finding may reflect a difference in the time course of hypoglycemia or the methodology used to demonstrate and localize Fos immunoreactivity. Using this dose of intermediate-acting insulin (which was selected for its consistent ability to stimulate feeding), plasma glucose dropped rapidly and remained low from 2 h after insulin administration; hypoglycemia was more profound in rats that were fasted throughout than in rats that were freely fed (25). As with other immunohistochemical methods, our double-fluorescence technique may not be sensitive enough to detect all activated orexin neurons, although false-positive results are less likely using this approach. The Fos method is widely used to identify and map neuronal activation across relatively large brain areas, but we acknowledge that it may miss neuronal activation via pathways other than Fos, as may be the case with MCH (11).

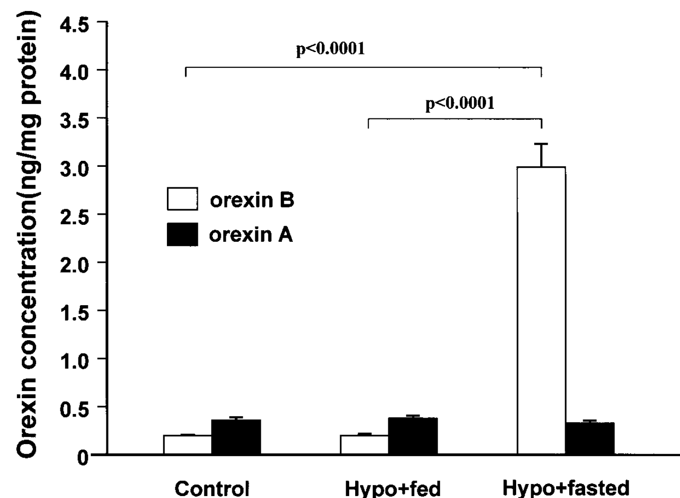

FIG. 4. Hypothalamic orexin-A and -B in saline-treated controls and insulin-treated rats. Data are means ± SE. *n* = 8. Hypo, hypoglycemic.

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We previously reported that prepro-orexin mRNA levels were increased twofold during 6 h of hypoglycemia with fasting—the same conditions that activated orexin and other neurons in the LHA and neurons in the NTS/DMNX. In this study, we found that whole hypothalamic concentrations of orexin-B were markedly raised by 10-fold above those for controls in the hypoglycemic fasted group; levels were unchanged in fed hypoglycemic rats, and orexin-A showed no changes in either hypoglycemic group. The increase in orexin-B levels specifically accompanied the other indexes of orexin neuronal activation, but the magnitude of the rise and its dissociation from the unchanged orexin-A levels is difficult to explain or to relate to the physiological and behavioral effects of hypoglycemia. Increased prepro-orexin expression should equally increase the production of orexin-A and –B because both are derived from the common precursor. Because orexin-A levels were not increased, it is possible that orexin-A release is enhanced in parallel with gene expression; increased synaptic availability of orexin-A would be predicted to stimulate feeding (13) and could contribute to the hyperphagia of hypoglycemia. The increase in orexin-B concentrations could be explained by a selective decrease in release, causing this peptide to accumulate within orexin neurons and terminals. Orexin-B has little, if any, effect on feeding (16) and instead may act preferentially on the OX-2 receptor, which is concerned with arousal: a mutation in OX-2 causes narcolepsy in dogs (46), whereas *orexin* knockout in mice causes disturbed arousal-sleep patterns (47). We therefore speculate that orexin-B release may be blocked in severe hypoglycemia, leading to its accumulation in the hypothalamus, and that this may contribute to the somnolence and ultimately coma induced by hypoglycemia. This discrepancy between orexin-A and -B may indicate that orexin neurons are functionally heterogeneous and that one subpopulation may be involved in mediating feeding (via orexin-A release), whereas another (releasing orexin-B) may regulate sleep and arousal. This explanation must remain speculative until orexin-A and -B release are measured separately in vivo in key hypothalamus regions and also in the locus coeruleus, which receives a dense orexinergic projection and controls the sleep-wake cycle (48).

In conclusion, we found striking activation of orexin and other LHA neurons in hypoglycemic rats that were not allowed to eat, but not if food was freely available. Increased activation of orexin neurons was accompanied by the emergence of Fos^+ neurons in the NTS and DMNX, indicating that the projection from the NTS to the LHA may be an important regulator of orexin neurons and their responses to changes in glucose availability and prandial signals. These findings support our suggestions that orexin neurons are some of the glucose-sensitive neurons in the LHA and that they are regulated by visceral signals relayed via the NTS. As such, they may be involved in triggering hunger and eating in response to hypoglycemia and perhaps in terminating feeding episodes. Orexins may be involved in various aspects of the hypothalamic responses to hypoglycemia, and their precise roles deserve further clarification.

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