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Central Administration of Glucagon-Like Peptide-1 Activates Hypothalamic Neuroendocrine Neurons in the Rat*

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ABSTRACT

Within the central nervous system, glucagon-like peptide-1-(7–36) amide (GLP-1) acts as a transmitter, inhibiting feeding and drinking behavior. Hypothalamic neuroendocrine neurons are centrally involved in the regulatory mechanisms controlling these behaviors, and high densities of GLP-1 binding sites are present in the rat hypothalamus. In the present study we have, over a period of 4 h, followed the effect of centrally injected GLP-1 on plasma levels of the neurohypophysial hormones vasopressin and oxytocin. Plasma levels of corticosterone and glucose were also followed across time after central administration of GLP-1. In conscious, freely moving, and unstressed rats, central injection of GLP-1 significantly elevated plasma levels of vasopressin 15 and 30 min after administration (basal, 0.8 ± 0.2 pg/ml; 15 min, 7.5 ± 2.0 pg/ml; 30 min, 5.6 ± 1.1 pg/ml; mean ± SEM) and elevated corticosterone 15 min after administration (52 ± 13 *vs.* 447 ± 108 ng/ml, basal *vs.* 15 min; mean ± SEM). In contrast, plasma oxytocin levels were unaffected by intracerebroventricular (icv) injections of GLP-1 over a period of 4 h after the injection. The animals given a central injection of GLP-1 developed transient hypoglycemia 20 min after the injection, which was fully restored to normal levels at 30 min.

Furthermore, we used c-*fos* immunocytochemistry as an index of stimulated neuronal activity. The distribution and quantity of GLP-1-induced c-*fos* immunoreactivity were evaluated in a number of hypothalamic neuroendocrine areas, including the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei and the parvicellular neurons of the medial parvicellular subregion of the PVN. The number of c-*fos*-expressing nuclei in those areas was assessed 30, 60, and 90 min after icv administration of GLP-1.

Intracerebroventricular injection of GLP-1 induced c-*fos* expression in the medial parvicellular subregion of the PVN as well as in magnocellular neurons of the PVN and SON. A slight induction of c-*fos* expression was seen in the arcuate nucleus and the nucleus of the solitary tract, including the area postrema. In contrast, the subfornical organ, which is a rostrally situated circumventricular organ, was free of c-*fos*-positive cells after central administration of GLP-1. When the GLP-1 antagonist exendin-(9–39) was given before the GLP-1, c-*fos* expression in these neuroendocrine areas was almost completely abolished, suggesting that the effect of GLP-1 on c-*fos* expression is mediated via specific receptors. A dual labeling immunocytochemical technique was used to identify the phenotypes of some of the neurons containing c-*fos*-immunoreactive nuclei. Approximately 80% of the CRH-positive neurons in the hypophysiotropic medial parvicellular part of the PVN coexpressed c-*fos* 90 min after icv GLP-1 administration. In contrast, very few (~10%) of the vasopressinergic magnocellular neurons of the PVN/SON contained c-*fos*-positive nuclei, whereas approximately 38% of the magnocellular oxytocinergic neurons expressed c-*fos*-positive nuclei in response to GLP-1 administration. This study demonstrates that central administration of the anorectic neuropeptide GLP-1 activates the central CRH-containing neurons of the hypothalamo-pituitary-adrenocortical axis as well as oxytocinergic neurons of the hypothalamo-neurohypophysial tract. Therefore, we conclude that GLP-1 activates the hypothalamo-pituitary-adrenocortical axis primarily through stimulation of CRH neurons, and this activation may also be responsible for the inhibition of feeding behavior. (*Endocrinology* **138:** 4445–4455, 1997)

THE POSTTRANSLATIONAL processing of the mammalian preproglucagon precursor is tissue specific and exhibits clear differences between the pancreas and the brain (1). In the pancreas, glucagon is the major splicing product, whereas the major splicing products found in the small intestine and brain are glucagon-like peptide-1-(7–36) amide (GLP-1) and glicentin (1, 2). In the small intestine, carbohydrate ingestion stimulates the release of GLP-1 from the L cells, and its subsequent stimulatory action on insulin release from pancreatic β-cells suggests that GLP-1 is an incretin (3, 4). In the central nervous system, GLP-1 is a potent inhibitor of feeding and drinking behavior (5–7). Thus, central, but not peripheral, administration of GLP-1 specifically and dose dependently inhibits food intake in hungry as well as fed rats via a mechanism unlikely to involve taste aversion (8). *In vivo*, these effects are believed to be mediated by a neuronal GLP-1-synthesizing system projecting from the caudal part of the nucleus of the solitary tract (NTS) mainly to the hypothalamus (1, 9). In the hypothalamus, dense innervation by GLP-1-immunoreactive terminals is seen in the paraventricular nucleus (PVN), suggesting that GLP-1 exerts its influence on ingestive behavior and water homeostasis via circuits localized to this area (1). Also, the neurons projecting from the NTS to the PVN constitute a population clearly discernible from the ascending catecholaminergic bundle, indicating that they affect targets within the PVN differently from the catecholaminergic input, which has also been shown to have an effect on food intake (1). Furthermore, receptor autora-

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diographic studies have identified high densities of GLP-1-binding sites in both hypothalamic nuclei and rostral circumventricular specializations involved in the regulation of water homeostasis (10–12).

The PVN is centrally involved as an overall regulator of feeding behavior. Thus, feeding elicited by intracerebral administration of neuropeptide Y (NPY) and catecholamines has its most potent site of action within the PVN and its immediate surroundings (13). The inhibitory effect of CRH-41 on food intake is probably mediated by CRH-synthesizing neurons of the medial parvicellular PVN. The recent observation of GLP-1-elicited c-*fos* expression in the PVN suggests that the inhibitory effect of GLP-1 on food intake involves the CRH-containing neuroendocrine neurons of the PVN (7).

In contrast to effects of GLP-1 on feeding, both central and ip administration of GLP-1 dose dependently inhibited basal drinking and induced a paradoxical increase in urinary output (8). Moreover, angiotensin II-induced drinking was completely abolished by prior ip or central administration of GLP-1, suggesting that the inhibitory effect of GLP-1 is mediated via a central site also accessible from the periphery (8). Obvious candidates for such sites are the blood-brain barrier-free regions of the third ventricle [subfornical organ (SFO) and organum vasculosum laminae terminalis] and the area postrema. Although it has been shown that various hypothalamic nuclei are among the areas with the highest density of GLP-1 nerve fibers, it remains to be shown whether this is also the case with these sensory circumventricular organs (9).

In a previous study we observed that central administration of GLP-1 is associated with increased plasma argnine vasopressin (AVP) levels (8). Whether GLP-1 exerts its effect directly on magnocellular neurons of the hypothalamo-neurohypophysial tract or whether the secretory response is a secondary water-conserving mechanism occurring as a consequence of a pronounced natriuresis elicited by central GLP-1 is unknown. Also, it has been demonstrated that centrally administered GLP-1 inhibits food intake (7, 8), and it seems likely that endogenous GLP-1 during some types of gastric stimulation is released from terminals within various hypothalamic targets affecting both food intake and oxytocin (OT) secretion. In the rat, OT acts as a natriuretic agent (14, 15), and the concurrent increase in natriuresis seen after intracerebroventricular (icv) injection of GLP-1 may be caused by a direct activation of the oxytocinergic cells in the PVN and supraoptic nucleus (SON). The secretory response of the hypothalamo-pituitary-adrenocortical (HPA) axis is stimulated upon hypoglycemia, and consequently, fluctuations in plasma glucose could influence both neuroendocrine responses as well as appetite-controlling systems.

To investigate the effects *in vivo* of centrally administered GLP-1 on the secretory responses of two hypothalamic neuropeptides known to be involved in fluid homeostasis and food intake, we measured plasma levels of the neurohypophysial hormones vasopressin and OT. In addition, by the use of immunohistochemical staining for c-*fos*, vasopressin, OT, and CRH, we phenotypically characterized the hypothalamic neurons activated by central injection of GLP-1. This combined hormonal and immunohistochemical investigation has enabled us to elucidate the pathways of GLP-1 action within the hypothalamus.

Materials and Methods

Intracerebroventricular injections and hormone measurements

Adult male Wistar rats housed under standard conditions with free access to rat chow and water were used in all experiments. Seven days before experimentation, all animals were equipped with an indwelling guide cannula (26 gauge) in the lateral ventricle. The guide cannula was positioned 1.5 mm lateral to the bregma on the coronal suture with its tip protruding 4.5 mm below the surface of the skull. Implantation was performed under tribromethanol anesthesia (20 mg/100 g, ip).

All injections were given to conscious, freely moving rats between 0900–1200 h. On the day of experimentation, cannulas extended by SILASTIC brand tubing (Dow Corning, Midland, MI) were inserted into the guide cannula, and the animals were left undisturbed in individual cages for approximately 2 h. Before insertion, the tubing connecting the cannulas was filled with the test substances in question. Thus, GLP-1 (10 μg/animal) was infused icv in a total volume of 10 μl. The dose of GLP-1 was chosen on the basis of its ability to significantly inhibit feeding and drinking (8). At 0, 15, 30, 60, 120, and 240 min after the injection of GLP-1, animals were decapitated, and trunk blood was collected in heparinized plastic tubes. The blood samples were centrifuged (3000 rpm for 5 min), and the plasma was recovered and frozen until analysis of the vasopressin, OT, and corticosterone contents was performed using standard in-house RIAs (16, 17). Plasma glucose was monitored in peripheral blood samples obtained by consecutively bleeding the tail vein of a separate group of animals receiving icv injections of either GLP-1 (10 μg) or vehicle. Blood glucose was measured using a glucose oxidase-based method (One Touch or LifeScan, Johnson & Johnson, Milpitas, CA).

Single antigen immunohistochemistry of c-fos

Thirty, 60, or 90 min after icv injection of either 10 μg GLP-1 or vehicle (0.9% saline and 1.0% BSA), the animals were anesthetized with tribromethanol (20 mg/100 g, ip) and perfused transcardially with heparinized (15,000 IU/liter) PBS (0.05 M sodium phosphate, 0.15 M NaCl, and 2.7 mM KCl, pH 7.4), followed by 350 ml of either 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) or Stefaninis fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.2). To evaluate the pharmacological specificity of GLP-1, a group of animals (n = 4) received an icv injection of the GLP-1 antagonist truncated exendin-(9–39) (20 μg) 10 min before GLP-1 was injected. This dose of the antagonist was chosen on the basis of its ability to significantly inhibit the effect of 10 μg GLP-1 on feeding and drinking behavior (8). Brains were postfixed in the same fixative overnight and cryoprotected in a phosphate-buffered 30% sucrose solution for 2 days. Frozen one in six series of 40-μm thick frontal sections were cut and collected in PBS. Immunohistochemical visualization of Fos was carried out on free floating sections using the avidin-biotin bridge method, as described in detail previously (18). After blocking endogenous peroxidase and preincubation in 5% nonimmune swine serum, the sections were incubated for 14 h in primary rabbit anti-Fos antiserum (no. 94012) at 4 C diluted 1:8000 in PBS and 1% BSA and 0.3% Triton X-100 (PBS-TX). The anti-Fos antiserum was raised in our laboratory (in collaboration with Dr. J. D. Mikkelsen) against the N-terminal sequence of 16 amino acids conserved in the human, mouse, and rat c-*fos* protein. In all cases studied to date, this antiserum gives rise to a staining pattern identical to that described for a thoroughly characterized c-*fos* antiserum (19). Furthermore, immunostaining was lost after preincubation with the synthetic c-*fos* peptide epitope or omission of the primary antiserum. The primary antiserum was visualized with biotinylated swine antirabbit IgG diluted 1:600 (Dako, Glostrup, Denmark), followed by avidin-biotin-peroxidase-streptavidin-horseradish peroxidase complex diluted 1:125 (Elite kit, Vector, Burlingame, CA). Sections were reacted for peroxidase activity in a solution of 0.025% diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer (pH 7.6) and 0.01% H_2O_2 for 20 min. Reactions were terminated by washing the sections in excessive amounts of water. Finally, the sections were mounted on gelatinized slides, dried, and embedded in Depex (Bie & Berntsen, Rødovre, Denmark). To evaluate

the effect of cannulation *per se*, a number of noncannulated nonmanipulated animals served as basal controls, and the perfusion-fixed brains from these animals were processed similarly. None of these animals displayed significantly higher expression of c-*fos* that with the animals receiving the vehicle, which thereafter served as the proper control group. The number of Fos-immunoreactive nuclei was counted in selected hypothalamic nuclei and circumventricular organs. Counting was performed bilaterally on a single counterstained section from each animal, and the average of the two sides served as a representative figure of the number of c-*fos*-positive nuclei in the specific area counted. Sections from all investigated animals were chosen at identical rostrocaudal levels, making direct comparison between animals possible without necessitating counting of all cells within a given nucleus. In the PVN, counting was performed at the level of the posterior magnocellular subdivision, whereas counting of the SON was performed at the level of the central portion of the suprachiasmatic nucleus. Three to five animals were included in each group. Statistical analysis was performed using ANOVA followed by *post-hoc* Fisher analysis, and values were considered significantly different when $P < 0.05$.

Double antigen immunohistochemistry of Fos and neuropeptides

Concomitant visualization of Fos immunoreactivity and relevant neuropeptide antigen was carried out using a modified version of a dual label immunocytochemical method (20). Immunohistochemical detection of Fos immunoreactivity was carried out as described above, except that the DAB reaction product was blackened by reacting the sections in a solution consisting of 100 mM $NiSO_4$, 125 mM NaCOOH, 10 mM imidazole, 0.03% DAB, and 0.003% H_2O_2, pH 6.5. After visualization of Fos immunoreactivity, sections were washed thoroughly in PBS (six times, 10 min each time) and incubated overnight in PBS-TX containing a second rabbit antiserum recognizing OT (1:2000), AVP (1:2000), or CRH-41 (1:2000). The specificities of these antisera have previously been described (16, 17). These second antisera were visualized with a peroxidase-antiperoxidase method, employing a second incubation in swine antirabbit IgG (1:100; Dako) and a final incubation in the rabbit peroxidase-antiperoxidase complex (1:200), as described previously (21). After incubation in a solution of 0.025% DAB in 0.05 M Tris-HCl buffer (pH 7.6) and 0.01% H_2O_2 for 20 min, the reaction was terminated by washing the sections in excessive amounts of water. Finally, the sections were mounted on gelatinized slides, dried, and embedded in Depex.

Results

GLP-1-induced hormonal secretion

Central administration of 10 μg GLP-1 significantly increased plasma levels of vasopressin 15 and 30 min after the injection (15 min, 7.6 ± 2.0; 30 min, 5.6 ± 1.1; basal, 0.8 ± 0.2 pg/ml; mean ± SEM; Fig. 1A). In contrast, GLP-1 did not significantly affect plasma levels of OT across time (Fig. 1B). GLP-1 significantly elevated plasma corticosterone, which remained elevated throughout the 60 min after injection (15 min, 444 ± 106; 30 min, 367 ± 98; 60 min, 443 ± 60; basal, 52 ± 12 ng/ml; mean ± SEM; Fig. 2). When following the time course of plasma glucose levels at 10-min intervals after icv administration of GLP-1, a significantly lower level was observed only 20 min after the injection (GLP-1, 20 min, 3.99 ± 0.42 mmol/liter; vehicle, 20 min, 5.63 ± 0.24 mmol/liter; mean ± SEM; Fig. 3).

Distribution of c-fos-immunoreactive elements

Initially, the distribution c-*fos*-immunoreactive nuclei in the hypothalamus and circumventricular organs of 90 min after vehicle injection was compared with that seen at three different time points after icv administration of GLP-1 (Table 1). As early as 30 min after GLP-1 treatment, maximal levels of c-*fos*-expressing nuclei were observed in the periventricular parvicellular part of the PVN, the magnocellular subdivision of the PVN, as well as the SON. In contrast, GLP-1-induced expression of c-*fos* was submaximal in the medial parvicellular parts of the PVN, where it first reached maximal levels 60–90 min after the injection. Animals injected with the vehicle exhibited very sparse c-*fos* expression after 30, 60 (not shown), and 90 min in the SON and PVN. As observed in previous experiments using similar conditions, however, the expression of c-*fos*-positive nuclei was slightly elevated in ventromedial, dorsomedial, and arcuate nuclei 90 min after icv administration of GLP-1 compared with basal expression in nonmanipulated animals (22). These observations confirm that the expression of c-*fos* immunoreactivity was unaffected by icv injection of the vehicle. To assess the pharmacological specificity of GLP-1-induced c-*fos* expression, 20 μg of the GLP-1 receptor antagonist exendin-(9–39) were infused 10 min before the GLP-1 injection. In animals pretreated with exendin-(9–39), GLP-1-induced expression of c-*fos* immunoreactivity was essentially abolished in the nuclei of interest (Figs. 4, B and D, and 5B).

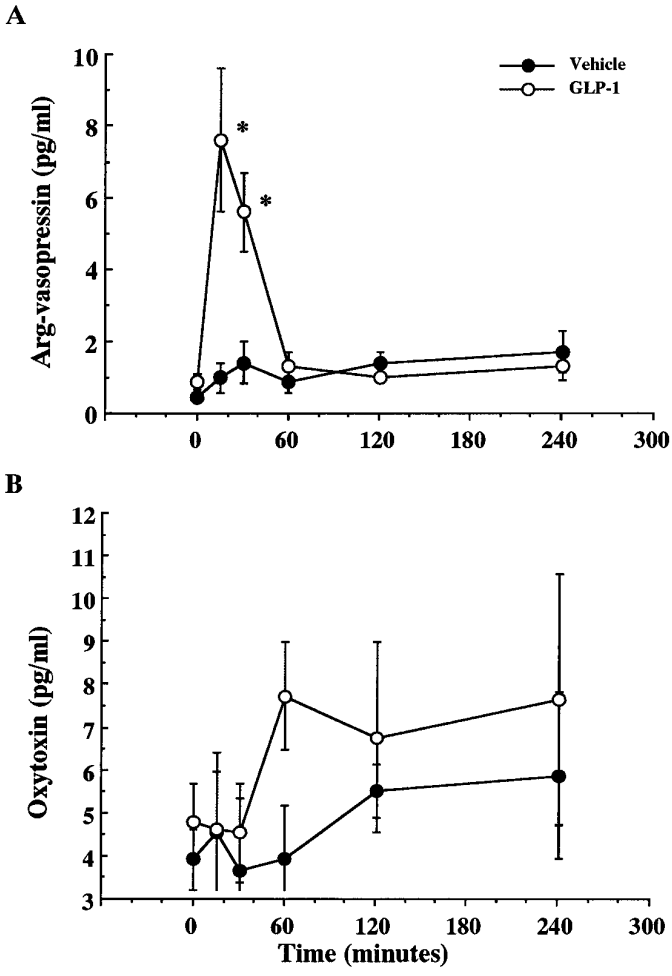

FIG. 1. Time course of the effect of icv administration of 10 μg GLP-1 on plasma vasopressin (A) and OT (B). Data are shown as the mean ± SEM (n = 6 in all points). *, $P < 0.05$ *vs.* vehicle, as determined by ANOVA followed by Fisher's *post-hoc* analysis.

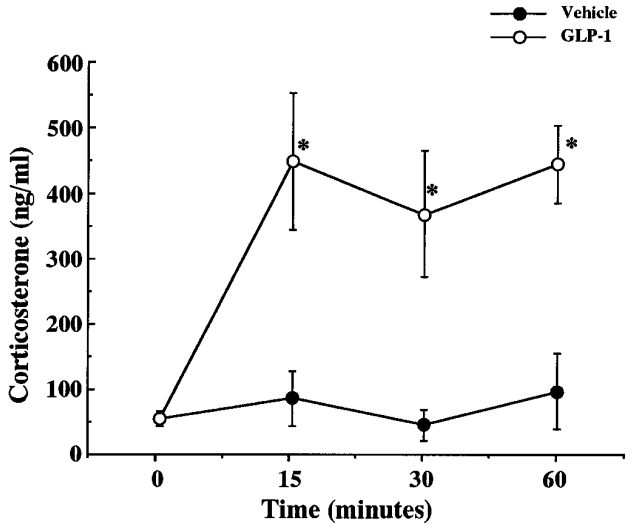

FIG. 2. Time course of the effect of icv administration of 10 μg GLP-1 on plasma corticosterone levels. Data are shown as the mean ± SEM (n = 6 in all points). *, $P < 0.05$ *vs.* vehicle, as determined by ANOVA followed by Fisher's *post-hoc* analysis.

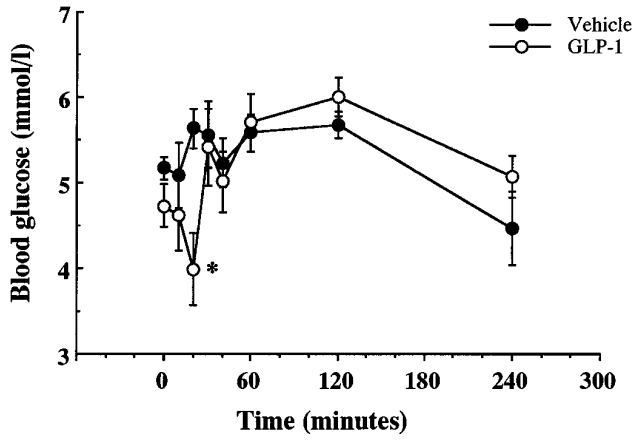

FIG. 3. Time course of the effect of icv administration of 10 μg GLP-1 on blood glucose. Data are shown as the mean ± SEM (n = 6 in all points). *, $P < 0.05$ *vs.* vehicle, as determined by ANOVA followed by Fisher's *post-hoc* analysis.

Intracerebroventricular administration of 10 μg GLP-1 induced expression of c-*fos* immunoreactivity in a number of forebrain areas, including many medial hypothalamic nuclei, the ventricular ependyma, and circumventricular organs. Thus, icv administration of GLP-1 induced c-*fos* expression in the PVN, SON, and Arc (Figs. 4 and 5). In addition, central administration of GLP-1 induced c-*fos* expression in the ependymal lining covering both the third and lateral ventricles. Central administration of GLP-1 induced c-*fos* expression within the perimeter of the magnocellular portion of the PVN, whereas the central core of the posterior magnocellular portion, where vasopressinergic neurons predominate, was virtually devoid of c-*fos*-immunoreactive nuclei (Fig. 4A). All parvicellular subdivisions responded with c-*fos* expression in response to icv GLP-1, but the number of positive nuclei differed considerably between individual subdivisions (Table 1). The most intense induction of c-*fos* immunoreactivity in the PVN was seen in the dorsal portion of the medial parvicellular subdivision (Fig. 4A). In contrast, modest GLP-1-induced c-*fos* expression was seen in the other parvicellular subnuclei of the PVN.

Intracerebroventricular injection of GLP-1 significantly increased the number of c-*fos*-immunoreactive nuclei in the dorsolateral region of the SON (Fig. 4C and Table 1). This part of the SON corresponds to the region where oxytocinergic neurons are massed. Prior administration of the GLP-1 antagonist exendin-(9–39) completely blocked GLP-1-induced c-*fos* expression in the SON (Fig. 4D), whereas c-*fos* expression in the adjacent regions of the lateral hypothalamic area appeared to be stimulated by icv administration of exendin-(9–39). In the Arc, GLP-1-induced c-*fos* expression was observed preferentially in the ventrolateral division of this nucleus, whereas the adjacent ventromedial region was left unaffected (Fig. 5A). As was the case with the SON, prior administration of exendin-(9–39) almost completely blocked GLP-1-induced c-*fos* expression in the Arc (Fig. 5B).

Central administration of GLP-1 had no effect on c-*fos* expression in the SFO (Fig. 5C) and its rostral continuum of the ependymal specialization covering the anteroventral tip of the third ventricle (Av3V) as well as in the organum vasculosum laminae terminalis. In contrast, pronounced GLP-1-induced expression of c-*fos* immunoreactivity was seen in the most caudal of the circumventricular organs, the area postrema (Fig. 5D). Other areas responding to icv injection of GLP-1 with increased numbers of c-*fos*-immunoreactive nuclei were the oval subnucleus of the bed nucleus of the stria terminalis (BST) and the lateral part of the central nucleus of the amygdala (CeA_l). Both of these areas are known to contain CRH-immunoreactive neurons.

Immunocytochemical identification of GLP-1-responsive neuroendocrine neurons

The topographical distribution of neurons displaying GLP-1 induced c-*fos* immunoreactivity in the PVN resembled the well recognized distribution of parvicellular CRH-synthesizing and magnocellular OT-synthesizing neuroendocrine neurons. To identify the transmitter phenotype of GLP-1-responsive neuron in the PVN, we carried out a dual immunocytochemical analysis for c-*fos*, on the one hand, and CRH, OT, or vasopressin, on the other hand (Fig. 6). At the central portion of the PVN, 80 ± 3.5 vasopressinergic magnocellular neurons and 75.5 ± 4.2 oxytocinergic cells were identified (mean ± SEM; n = 6). Very few vasopressin-immunoreactive neurons were present in the medial parvicellular portion of the PVN, and those encountered were always magnocellular. At the midlevel of the SON, 52 ± 2 magnocellular vasopressinergic cells and 42.9 oxytocinergic cells were identified (mean ± SEM; n = 6). Parvicellular vasopressinergic cells in the medial parvicellular part of the PVN were not observed with the current immunohistochemical procedure. The proportion of magnocellular oxytocinergic neurons coexpressing c-*fos* was 37.7 ± 9.3% in the SON and 38.3 ± 3.9% in the posterior magnocellular part of the PVN (mean ± SEM; n = 4; Fig. 6, C and D). In contrast, the proportions of magnocellular vasopressinergic cells expressing c-*fos* were much smaller (11.5 ± 1.8% in the SON and 13.6 ± 2.7% in the posterior magnocellular part of the PVN;

TABLE 1. Number of c-*fos*-immunoreactive nuclei per section in selected brain areas after icv administration of GLP-1 plus saline, GLP-1 with or without exendin-(9-39), or vehicle alone

Nucleus	GLP-1 (10 μg), icv, 30 min	GLP-1 (10 μg), icv, 60 min	GLP-1 (10 μg), icv, 90 min	GLP-1 (10 μg)+ exendin-(9-39), icv, 90 min	Vehicle icv, 90 min
mpdPVN	47.4 ± 6.2[a]	98 ± 5.2	103 ± 6.2[b]	10.5 ± 7.5	4.3 ± 1.9
mpvPVN	28.2 ± 3.1[a]	59 ± 2.2	66.2 ± 7.2[b]	13 ± 10	0.7 ± 0.3
pePVN	21 ± 1.2	17.2 ± 2.6	22.5 ± 3.6[b]	4 ± 2	0
pmPVN	23.2 ± 4.6	33.3 ± 2.3	29.1 ± 3.9[b]	7.5 ± 1	1.0 ± 0.6
SON	32.8 ± 4.2	39.7 ± 3.7	38.4 ± 2.7[b]	4 ± 1	5.3 ± 1.5

Three to five sections taken at comparable levels of the central portion of the PVN or SON were counted.
[a] $P < 0.05$ *vs.* 60 and 90 min (by ANOVA followed by Fisher *post-hoc* analysis).
[b] $P < 0.05$ *vs.* vehicle (by ANOVA followed by Fisher *post-hoc* analysis). $P < 0.05$ *vs.* GLP-1 plus exendin-(9-39) (ANOVA followed by Fisher *post-hoc* analysis).

mean ± SEM; n = 4; Fig. 6E). The relatively sparse content of visible CRH in perikarya of the medial parvicellular region of the PVN precluded the use of conventionally fixed tissue in the dual labeling protocol. Instead, sections used for dual immunocytochemical analysis of c-*fos* and CRH were obtained from animals perfused with Stefaninis fixative, allowing partial visualization of the CRH-immunoreactive cell bodies at the central level of the PVN. Despite the use of this fixation, the number of CRH-immunoreactive cell bodies varied considerably between animals, making proper assessment of the exact number of CRH neurons in each animal impossible (20.0 ± 2.9 cells/section; mean ± SEM; n = 5). However, it was still possible to determine that the proportion of c-*fos*-expressing CRH neurons in the medial parvicellular part of the PVN was 77.6 ± 3.7% (mean ± SEM; n = 5; Fig. 6, A and B). In contrast, none of the CRH-immunoreactive neurons of the BST and CeA_l colocalized c-*fos*-immunoreactive nuclei (not shown). In particular, c-*fos*-immunoreactive nuclei in the central amygdala were localized medial to the CRH-immunoreactive perikarya residing in this nucleus.

Discussion

Hormonal measurements and *in situ* morphological methods were used to assess to what extent centrally infused GLP-1 affects neuroendocrine neurons of the PVN synthesizing and secreting stress hormones. The conclusions drawn from the c-*fos* immunocytochemistry rely on the current literature assumption that c-*fos* is a marker of functionally activated neurons. The versatility of this immediate early gene as a marker of activation of neuroendocrine neurons by various types of chronic and/or acute stress has been well established (23–26). A fixed concentration of GLP-1 was employed in the present series of experiments, but it is by no means certain that all neurons in the intricate circuit believed to mediate the anorectic effects of the GLP-1 challenge express c-*fos* in response to this stimulus. The abolition of GLP-1-induced c-*fos* expression by prior administration of the antagonist exendin-(9–39) supports the idea that specific receptors mediates the effect of GLP-1. Exendin-(9–39) in the currently employed dose induced c-*fos* expression in a number of central sites, which could reflect the fact that these areas experience a constant GLP-1-mediated inhibitory tone that is released by the antagonist. The underlying rationale for using the relatively high dose of antagonist was the previous finding that abolition of the inhibitory effect of GLP-1 upon feeding and drinking behavior requires 2–5 times higher amounts (7, 8) (our unpublished observations). Also, a lack of c-*fos* expression should not necessarily lead to the conclusion that certain neurons are nonresponsive to icv injections of GLP-1 and, consequently, should not be considered part of a given functional circuit. This is because neither c-*fos* nor any other immediate early gene provides a useful marker for inhibitory input to the neurons. Given the fact that centrally applied GLP-1 actually induces net inhibition of both drinking and feeding, it is most likely that the neurons directly driving these behavioral outputs remain electrophysiologically silent and, consequently, do not express c-*fos*.

It was evident that icv injection of GLP-1 triggered c-*fos* expression in the majority of the CRH-containing perikarya within the medial parvicellular subset of the PVN. Messenger RNA encoding the GLP-1 receptor protein is expressed at high levels in neurons of the medial parvicellular PVN, and high densities of GLP-1-binding sites are also present within this portion of the PVN (11, 27), supporting a direct influence of GLP-1 on the CRH-containing motor neurons of the HPA axis. This is further corroborated by the relatively rapid c-*fos* expression in response to icv GLP-1. The transient decrease in plasma glucose was very moderate, and GLP-1-induced corticosterone secretion preceded the transient hypoglycemia, rendering the latter an unlikely cause of c-*fos* expression in CRH neurons. Because of technical limitations it was not possible in the present study to exactly quantify the total number of c-*fos*-expressing parvicellular CRH-positive neurons in this part of the PVN. It is well known that immunocytochemical visualization of CRH-containing cell bodies in the rat PVN is virtually impossible in conventionally paraformaldehyde-fixed brains (22). Often colchicine is used as a tool to enhance CRH immunocytochemical staining, but the potential triggering of c-*fos* expression in central neurons of the HPA axis by this compound makes it less desirable to use (23). Rather, we have chosen to use another fixative that makes possible partial visualization of the CRH-immunoreactive perikarya in the PVN; despite this limitation, our current dual labeling experiments suggest that the inhibitory effect of centrally applied GLP-1 on feeding involves approximately 78% of the CRH neurons in the medial parvicellular part of the PVN. The hypophysiotropic CRH neurons of the medial parvicellular PVN have the ability to coexpress and release a variety of other neuropeptide transmitters, of which vasopressin is by far the best described (28,

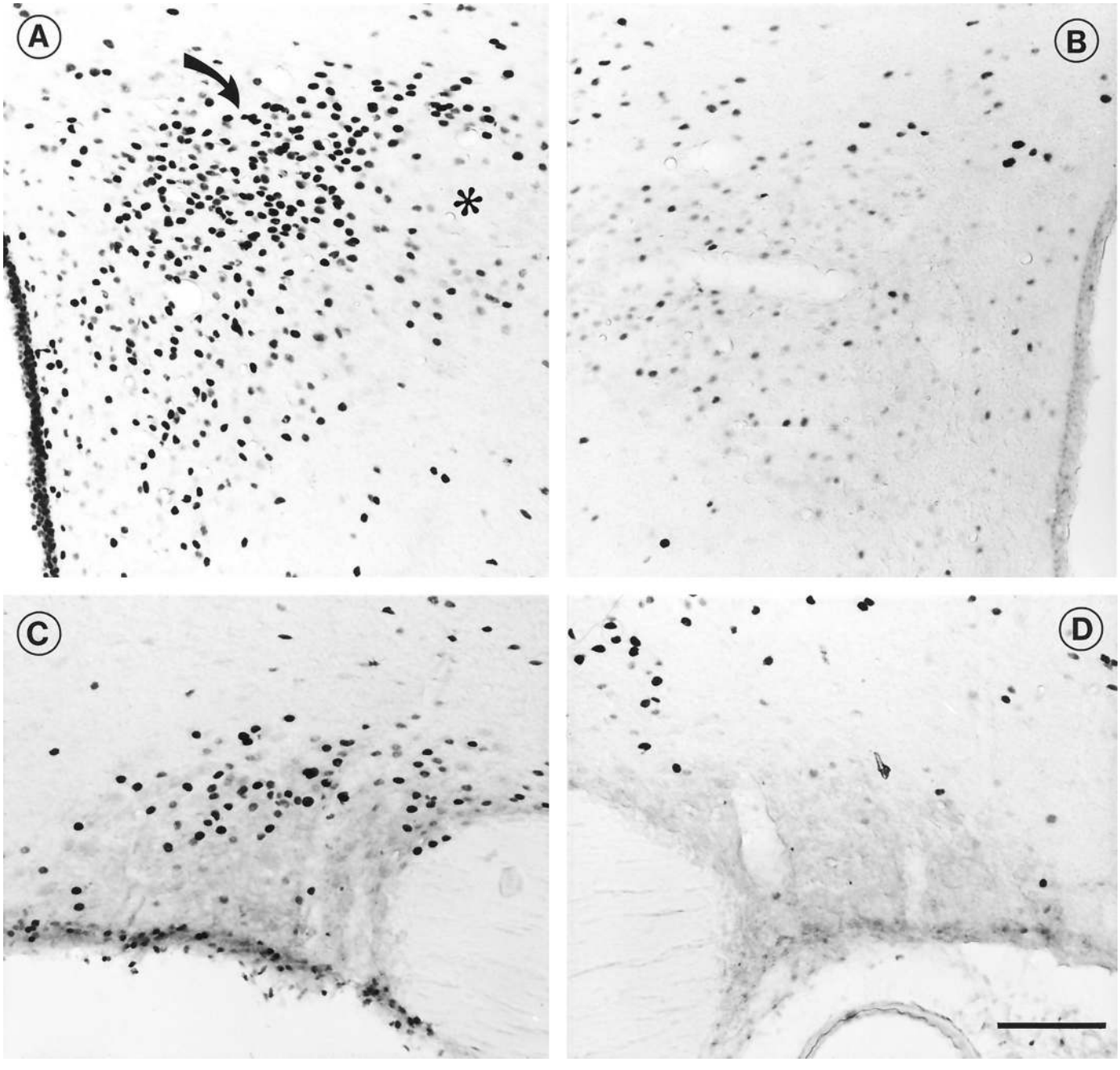

FIG. 4. Photomicrographs demonstrating c-*fos*-immunoreactive nuclei in the hypothalamic paraventricular (A and B) and supraoptic nuclei (C and D) 90 min after icv injection of GLP-1 alone (A and C) or GLP-1 preceded by a dose of exendin-(9–39) given 10 min earlier (B and D). *Scale bar* = 100 μm.

29). Under resting conditions, vasopressin is expressed at very low levels in the medial parvicellular PVN (30), and in agreement with this, we could not detect any vasopressin immunoreactive in this region. A plethora of data suggest that the expression, storage, and release of vasopressin in the parvicellular CRH/AVP neurons is activated by both chronic and acute stressors (31–33), and it is possible that GLP-1 induces the expression and release of vasopressin from parvicellular neurons to the portal circulation. The current combination of GLP-1-induced elevation of plasma vasopressin and the paradoxical absence of c-*fos* expression in magnocellular vasopressin neurons are discussed in detail below. Given that GLP-1 activates hypophysiotropic parvicellular CRH/AVP neurons, a remote possibility exists that the currently observed increase in plasma vasopressin is caused by a spillover from the portal circulation to the systemic circuit. However, the amounts of vasopressin released to the portal circulation during stress conditions are normally insufficient to cause a detectable elevation of systemic vasopressin concentrations (34, 35).

Until now, NPY is the most potent stimulator of feeding described (13, 36), and GLP-1 (7), like CRH (37), reduces

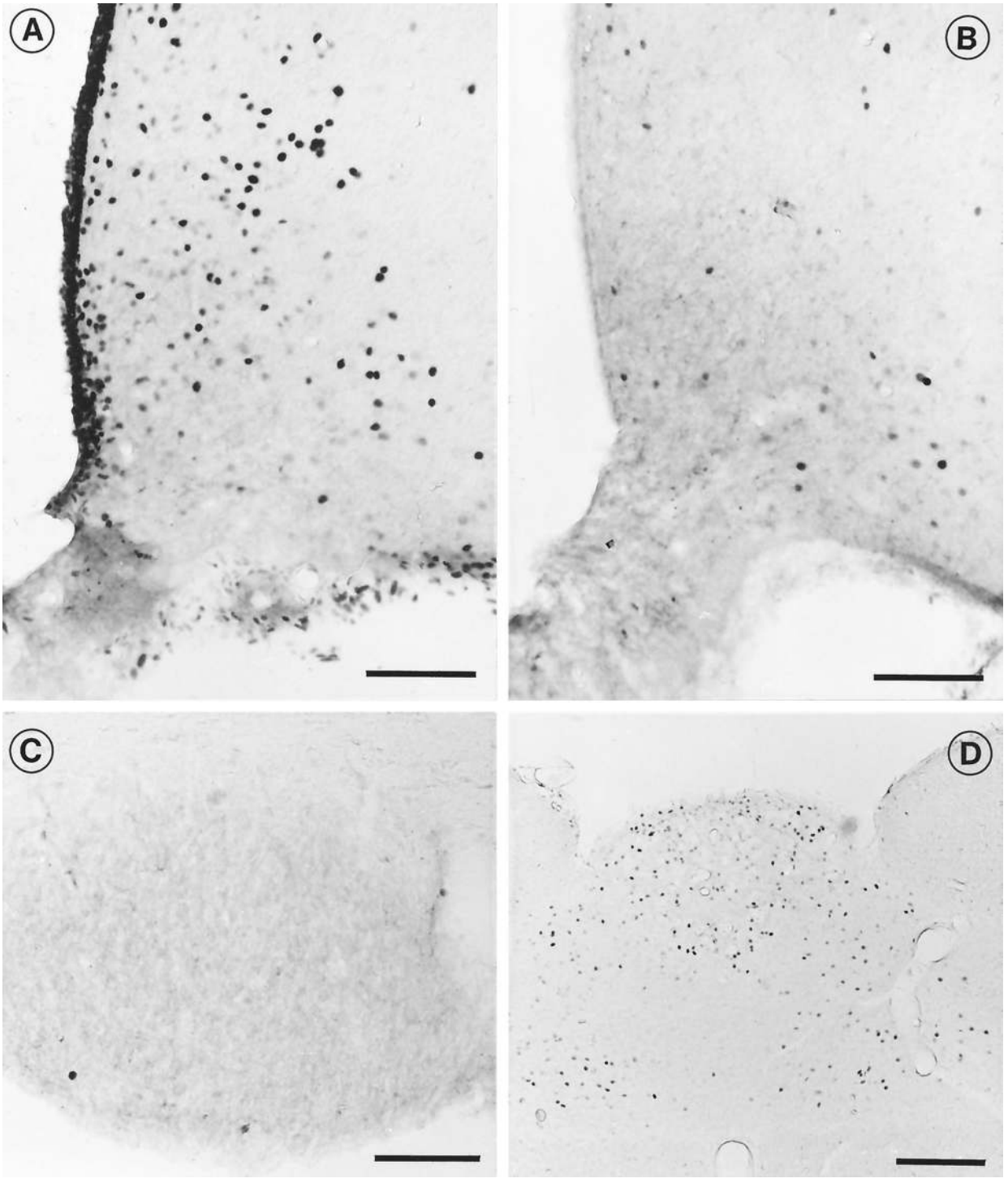

FIG. 5. GLP-1 induced c-*fos* expression in the arcuate nucleus and circumventricular areas. The photomicrographs demonstrate c-*fos*-immunoreactive nuclei in the arcuate nucleus from a rat given an icv injection of GLP-1 alone (A) or from a rat in which the icv injection of GLP-1 was preceded 10 min by an injection of 20 μg exendin-(9–39) (B). Intracerebroventricular injection of GLP-1 had no effect on c-*fos* expression in the subfornical organ (C), whereas numerous c-*fos*-immunoreactive cell bodies are seen in the area postrema and the surrounding subpostreme region of the nucleus of the solitary tract (D). *Scale bars* = 100 μm (A–C) and 200 μm (D).

feeding induced by central application of NPY. Obviously, the present study does not allow determination of whether GLP-1 inhibits feeding at the same site as NPY stimulates food intake or at a topographically distant site. However, a high density of both GLP-1-containing nerve terminals and binding sites is present in the PVN and the adjacent lateral extension of the lateral parvicellular part of the PVN (1, 11), where NPY exerts its most potent stimulatory action on feeding. Also, the recent demonstration of expression of messenger RNAs encoding Y1 and Y5 receptors in the medial

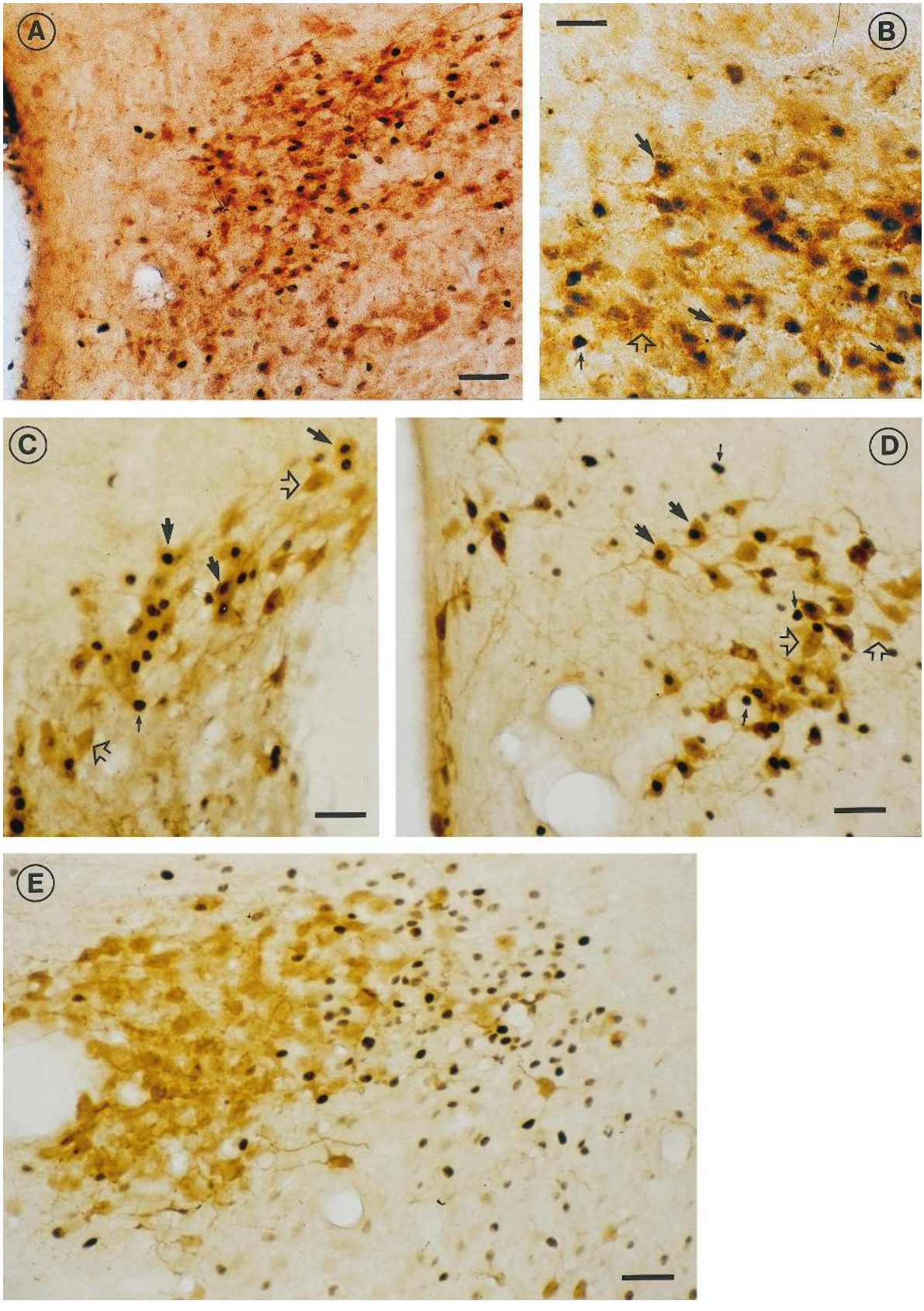

FIG. 6. GLP-1 induced c-*fos* expression in neurochemically identified neurons in the PVN (A, B, D, and E) and SON (C), as shown in photomicrographs of sections double labeled for c-*fos* and CRH (A and B), for c-*fos* and OT (C and D), or for c-*fos* and AVP (E). The medium power photomicrograph in A shows an overlapping distribution of CRH-positive neurons and c-*fos*-immunoreactive nuclei in the dorsal part

parvicellular part of the PVN (38, 39) suggest that GLP-1 and NPY may interact within the PVN, although it remains to established whether both GLP-1 and Y1/Y5 receptors are expressed in parvicellular CRH-containing neurons. The inhibitory action of CRH on feeding is primarily elicited within the PVN, further substantiating that this hypothalamic site acts as a central integrator of neuronal activity orchestrating feeding behavior. Possible involvement of CRH neurons in the BST and the CeA_l in ingestive behavior has not been properly addressed (40) but the absence of GLP-1-induced c-*fos* expression in neurons of the BST and CeA_l speaks against involvement of these nuclei in central circuits inhibiting food intake via a GLP-1-sensitive mechanism.

A number of anorectic agents can activate central OT neurons. Systemically administered cholecystokinin (CCK) is a powerful inhibitor of food intake and gastric motility (41), and electrophysiological recordings have demonstrated that peripheral administration of CCK activates magnocellular OT neurons, leading to increased levels of circulating OT while the magnocellular AVP neurons remain silent (42, 43). This peripheral increase in circulating OT has no effect on food intake and merely mirrors the central events occurring in response to CCK, which is believed to mediate its inhibitory action on feeding via brainstem-projecting OT-containing neurons (44, 45). Peripherally administered CCK induces c-*fos* expression in the PVN with a pattern almost identical to that currently observed for GLP-1, *i.e.* a profound activation of OT and CRH-containing neurons and little if any activation of AVP neurons (46). Thus, it is likely that the CCK-induced activation of PVN neurons is mediated via ascending GLP-1 neurons in the caudal nucleus of the solitary tract recently shown to project to the PVN (1). In the nucleus of the solitary tract, CCK has convincingly been shown to activate a large proportion of the ascending catecholaminergic neurons innervating the PVN (47). However, a minor proportion of CCK-activated NTS neurons are noncatecholaminergic, suggesting that other transmitters may convey information about CCK activation of the NTS to the PVN (47). The GLP-1-containing neurons of the NTS are noncatecholaminergic (1), and it is likely that they participate in the neuronal circuitry mediating systemic CCK-induced c-*fos* expression of PVN neurons, although it remains to be established whether CCK induces c-*fos* expression in medullary GLP-1-synthesizing neurons. Some controversy exists as to whether GLP-1 induces its anorectic effect via the production of learned taste aversion. As suggested by some investigators, the effect of GLP-1 could represent a nonspecific response to nausea rather than inducing specific activation of central pathways involved in ingestive behavior (48, 49). In line with this, it is well known that CCK can produce learned taste aversion (50) as well as specific satiety (43). The administration of agents causing gastrointestinal illness and conditioned taste aversion are often accompanied by a dose-dependent secretion of OT (51). Thus, neurohypophysial secretion of OT represents a marker of activity in central pathways processing sensory information, ultimately leading to nausea, but circulating OT is not the nauseating agent itself (52). In this study, we could not detect increased plasma levels of OT in response to injection of GLP-1 into the lateral ventricle, supporting our previous observation that GLP-1 does not cause taste aversion (8).

In addition to inhibitory actions on feeding, we previously observed that centrally applied GLP-1 acutely inhibits water intake concomitantly with increased urine output due to accompanying natriuresis (8). We have previously observed increased AVP secretion and suggested that this could be secondary to fluid depletion accompanying the GLP-1-induced natriuresis. This suggestion, however, seems unlikely in light of the current observation that centrally applied GLP-1 has little if any stimulatory action on c-*fos* expression in magnocellular AVP-containing neurons. The most potent stimulus for vasopressin secretion is an increase in plasma osmolality, and it is well established that dehydration and volume depletion mediated by such diverse stimuli as hypertonic saline injection, ip polyethylene glycol, or hemorrhage all induce pronounced c-*fos* expression within magnocellular vasopressinergic neurons of the hypothalamo-neurohypophysial system (25, 26, 53, 54). Our data show a paradoxical lack of c-*fos* expression in vasopressinergic hypothalamo-neurohypophysial neurons during a robust plasma vasopressin response that undoubtedly is of neurohypophysial origin. The secretory activity of the neurohypophysis is, however, not always coupled with c-*fos* expression, as demonstrated recently in a study employing orthodromic activation of the pituitary stalk (55). In that study it was shown that electrically stimulated activation of neurohypophysial secretion is not accompanied by c-*fos* expression in magnocellular neurons of the SON and PVN. In contrast, central pharmacological activation of magnocellular neurons leading to neurohypophysial secretion was followed by c-*fos* expression, further emphasizing that synaptic activation at the level of the perikarya is required to activate this immediate early gene. The fact that increased spike activity at the level of the pituitary terminals does not necessarily induce c-*fos* expression leaves open the possibility that the currently observed AVP secretory response is caused by a local stimulatory effect on vasopressinergic terminals within the neurohypophysis. A high density of GLP-1-binding sites has been demonstrated within the neurohypophysis (10), and it is possible that part of the centrally administered GLP-1 may have gained access to this distant site, thereby influencing vasopressinergic nerve terminals. However, another possibility that should be considered is that OT, either

of the mpPVN. At higher magnification (B), some CRH neurons are seen to costore c-*fos*-immunoreactive nuclei (*bold solid arrows*), whereas other CRH neurons are devoid of c-*fos*-immunoreactive nuclei (*open arrow*). A number of cells display only c-*fos* immunoreactivity (*thin arrows*). In the magnocellular SON (C) and PVN (D), OT-immunoreactive neurons colocalized c-*fos*-immunoreactive nuclei to a large degree (*solid bold arrows*). Few OT-immunoreactive cells were devoid of c-*fos*-immunoreactive nuclei (*open arrows*), whereas a number of cells displayed only c-*fos* immunoreactivity (*thin arrows*). None of the AVP-immunoreactive magnocellular neurons of the PVN (E) colocalized c-*fos*-immunoreactive nuclei, as shown by this photomicrograph of the central part of the posterior magnocellular part of the PVN. *Scale bars* = 80 μm (A, C, D, and E) and 40 μm (B).

alone or together with a costored transmitter, was released at the level of the neurohypophysis to have a local stimulatory role in vasopressin release. Such modulatory mechanisms are well known for a number of neuropeptides costored with either OT or vasopressin in neurohypophysial terminals (56). A number of neuropeptides have been shown to coexist with OT in magnocellular neurons, including CRH, CCK, NPY, and pituitary adenylate cyclase-activating polypeptide (PACAP) (57–60) (our unpublished observations). CCK, NPY, and CRH have all been shown to stimulate the release of vasopressin from isolated neurointermediate lobes (61–63). The *in vivo* effect of CCK remains questionable, because peripheral administration of this peptide selectively stimulates oxytocinergic cells and has no effect on magnocellular vasopressinergic neurons (64). The stimulatory effect of CRH on vasopressin secretion from the neurohypophysis requires the presence of the intermediate lobe, rendering this mechanism rather complex (62). However, NPY is present in both OT- and AVP-containing magnocellular neurons, and NPY potentiates the release of AVP from neurohypophysial terminals via a high density of NPY-binding sites pharmacologically characterized as Y2 receptors (60, 63). Also, PACAP has recently been shown to coexist with OT in magnocellular neurohypophysial neurons (65) (our unpublished observations), and the recent discovery that PACAP stimulates both vasopressin and OT release from isolated neurohypophysial neurosecretory terminals suggests that this peptide could mediate the GLP-1-induced effect on vasopressin secretion (66).

Both centrally and peripherally applied GLP-1 abolishes angiotensin II-induced drinking (8). Peripherally applied angiotensin II mediates its effect via rostral sensory circumventricular organs, whereas centrally applied angiotensin II exerts its stimulatory effect on drinking via receptors in the Av3V (67). Thus, it is likely that peripheral GLP-1 inhibits angiotensin II-induced drinking via the SFO, whereas central GLP-1 inhibits angiotensin II-sensitive neurons in the Av3V, both of which stimulate water intake via projections to the PVN and SON (68–70). The almost complete absence of c-*fos*-positive nuclei in the SFO and Av3V after central GLP-1 administration suggests that GLP-1 may inhibit neurons at these sites or may have no effect, once again emphasizing that events leading to electrophysiological quiescence may not be detected by c-*fos* immunocytochemistry.

In conclusion, we have shown that centrally applied GLP-1 significantly stimulates circulating levels of AVP and corticosterone, with no effect on circulating OT. Furthermore, GLP-1 induces c-*fos* expression in OT- and CRH-containing neurons in the PVN, suggesting that these are involved in the neuronal circuits governing the inhibitory effect of GLP-1 on food intake. The effect of GLP-1 on AVP secretion is most likely mediated at the level of neurohypophysial terminals, either via GLP-1 itself or via a transmitter coexisting with OT in magnocellular hypothalamo-neurohypophysial neurons.

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