Feeding Reduces Activity of Growth Hormone-Releasing Hormone and Somatostatin Neurons (44482)

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Abstract. **Secretion of growth hormone (GH) is synchronized among castrate male cattle (steers) around feeding when access to feed is restricted to a 2-hr period each day. Typically, concentrations of GH increase before and decrease after feeding. Our objectives were to determine whether i) concentrations of GH decrease in blood after start of feeding; ii) activity of immunoreactive growth hormone-releasing hormone (GHRH-ir) neurons decreases in the arcuate nucleus (ARC) after feeding; iii) activity of immunoreactive somatostatin (SS-ir) neurons in the periventricular nucleus (PeVN) and ARC increase after feeding; and iv) GHRH stimulates release of GH to a similar magnitude at 0900 and at 1300 hr, in steers fed between 1000 and 1200 hr. Blood samples were collected at 20-min intervals from 0700 to 1300 hr. Groups of steers were euthanized at 0700, 0900, 1100, and 1300 hr ($n = 5$ per group). Dual-label immunohistochemistry was performed on free-floating sections of hypothalami using antibodies directed against Fos and Fos-related antigens (Fos/FRA) as a marker of neuronal activity in immunoreactive GHRH and SS neurons. Concentrations of GH were high before and decreased after feeding. The percentage of SS-ir neurons containing Fos/FRA-ir in the PeVN was 50% lower ($P < 0.01$) at 1100 hr and 36% lower ($P < 0.05$) at 1300 hr than at 0900 hr. There was no change in percentage of SS-ir neurons containing Fos/FRA-ir in the ARC. The percentage of GHRH-ir neurons containing Fos/FRA-ir in the ARC was 66% lower ($P < 0.05$) at 1100 hr and 65% lower ($P < 0.05$) at 1300 hr than at 0700 hr. In contrast, the number of GHRH-ir neurons increased from 0700 to 1300 hr. GHRH-induced release of GH was suppressed at 1300 hr compared with 0900 hr. In conclusion, reduced basal and GHRH-induced secretion of GH after feeding was associated with decreased activity of GHRH neurons in the ARC and decreased activity of SS neurons in the PeVN.** [P.S.E.B.M. 2000, Vol 223]

Growth hormone (GH) is secreted from somatotropes in the anterior pituitary gland in episodic bursts, or pulses, that are not synchronized among animals. However, secretion of GH can be synchronized around feeding when castrate male cattle (steers) are offered *ad libitum* access to feed for a 2-hr period each day (meal-feeding). Typically, concentrations of GH increase for 1–3 hr before feeding, decrease during feeding, and remain low for several hours after feeding (1–3). Therefore, meal-feeding provides a unique opportunity to assess the activity of neurons regulating release of GH when hormone secretion among animals is synchronized.

Two hypothalamic peptide hormones principally regulate release of GH. Growth hormone-releasing hormone (GHRH) stimulates release, whereas somatostatin (SS) inhibits release of GH (4, 5). GHRH neurons are located in the arcuate nucleus (ARC), whereas most SS neurons in the hypothalamus are located in the periventricular nucleus (PeVN) and ARC (6). Somatostatin that inhibits release of GH, *via* an action on somatotropes, originates from neurons in the PeVN, whereas SS neurons in the ARC are thought to communicate between GHRH neurons in the ARC and SS

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neurons in the PeVN (7). Axons from GHRH and SS neurons in the PeVN terminate in the external layer of the median eminence where GHRH and SS are released into hypophysial-portal vessels and transported to the anterior pituitary gland (4, 5).

Activity of neurons can be measured with dual-label immunohistochemistry, which uses the presence of immediate-early gene proteins as markers of neuronal activity (8, 9). Immediate-early genes such as *c-fos* regulate long-term gene responses. After cell membrane-receptor mediated signal transduction, the translated protein, Fos, is transported into the nucleus, where it forms dimers with other immediate-early gene proteins, and these bind to activator protein 1 sites (10). Therefore, Fos increases in nuclei of recently activated neurons. Conversely, Fos-related antigens (FRA) are expressed in tonically active neurons, and their presence decreases on removal of a stimulus to those neurons. Collectively, Fos/FRA reflect trans-synaptic changes in neuronal activity.

We hypothesized that activity of immunoreactive (ir) GHRH neurons would be high before feeding and decrease after feeding, whereas activity of SS-ir neurons would be low before feeding and increase after feeding. Our objectives were to determine whether i) concentrations of GH decrease in blood after the start of feeding; ii) activity of GHRH-ir neurons decreases in the ARC after feeding; iii) activity of SS-ir neurons in the PeVN and ARC increase after feeding; and iv) GHRH stimulates release of GH to a similar magnitude at 0900 and at 1300 hr, in steers fed between 1000 and 1200 hr.

Materials and Methods

Animals and Maintenance. Twenty male Holstein calves born at Michigan State University's Dairy Cattle Teaching and Research Center were castrated at 1 week and fed whole milk until weaning at 8 weeks of age. Steers were moved into individual stalls in rooms (four steers per room) where they had free access from 1000 to 1200 hr to a diet containing 18% crude protein and 19.6% acid detergent fiber (Land O'Lakes, Indianapolis, IN) and free access to water. Lights were on for 16 hr each day, and the temperature was maintained at 20 ± 1°C in the animal rooms. At the start of experiments steers were 17 ± 1 weeks old and weighed 130 ± 6 kg (mean ± SEM). The Institutional Animal Care and Use Committee approved this experiment at Michigan State University.

Experiment 1: Percentage of GHRH-ir and SS-ir Neurons Containing Fos Protein and Fos-Related Antigens (Fos/FRA). *Blood Sampling and Tissue Collection.* Twenty steers were randomly allocated into four groups (n = 5 per group) that were euthanized at 0700, 0900, 1100, and 1300 hr. A jugular vein of each steer was cannulated 24 hr before the experiment. Patency of each cannula was maintained with sterile 3.5% sodium citrate. Blood samples (6 ml) were collected every 20 min from 0700 until euthanasia. Blood was allowed to clot at 20°C for 2 hr, stored at 4°C for 22 hr, and then centrifuged. Harvested serum was stored at –20°C until assayed for GH.

Each steer was injected intravenously with 33,000 I.U. sodium heparin 20 min and 1 min before sacrifice to prevent coagulation of blood. Steers were euthanized with an intravenous injection of sodium pentobarbital (85 mg/kg body weight) and then decapitated. Within ≈ 5 min of death, brains were perfused *in situ via* the carotid arteries for 20 min with 8 l of fresh 4% paraformaldehyde, containing 0.5% sodium nitrite (to dilate blood vessels). After perfusion, hypothalami were removed and post-fixed in fresh 4% paraformaldehyde, containing 0.5% sodium nitrite and 2.5% acrolein (Polysciences Incorporated, Warrington, PA) (11) for 24 hr at 4°C. Hypothalami were then infiltrated with 20% sucrose in 0.1 *M* phosphate buffered saline at 4°C for 1 week, then transferred to 30% sucrose at 4°C for 2 weeks.

Dual-Label Immunohistochemistry of Fos/FRA-ir in GHRH-ir and SS-ir Neurons. Hypothalami were sectioned on a freezing microtome at 40-μm intervals and stored frozen in a cryoprotectant solution (12) containing ethylene glycol until dual-label immunohistochemistry could be carried out. Sections were rinsed six times over 60 min in 0.05 *M* tris buffered saline (TBS, pH 7.6) to remove cryoprotectant and between each of the following steps. Sections were immersed sequentially in 3% hydrogen peroxide for 30 min to reduce endogenous peroxidase activity (13), 1% sodium borohydride (Sigma Chemical Company, St. Louis, MO) to partially neutralize aldehydes (14), and then avidin (Vector Laboratories, Burlingame, CA; 100 μl per 10 ml TBS) followed by biotin (Vector Laboratories; 100 μl in 10 ml TBS) to block endogenous biotin.

Optimal concentrations of antibodies to SS, GHRH, and Fos/FRA for immunohistochemistry were determined on adjacent sections at dilutions ranging from 1:1000 to 1:300,000. Specificity of each antibody was performed by absorbing primary antibodies for 24 hr with 10 μ*M* SS (SS-14; Peninsula Laboratories, Belmont, CA), 10 μ*M* bovine $GHRH^{1-44}$ (Bachem California, Inc., Torrance, CA), or 10 μ*M* Fos protein (c-Fos [PP10]; Oncogene Research Products, Cambridge, MA), respectively, before exposure to sections. Immunostaining was absent in sections in which primary antibody was absorbed with its corresponding peptide before exposure to sections. Dilution studies demonstrated decreasing intensity of immunostaining with sequential dilution of anti-SS, anti-GHRH, and anti-Fos/FRA antibodies, whereas labeling was not evident in the absence of these antibodies.

Immunohistochemistry was performed on free-floating sections. Presence of Fos/FRA-ir was detected in cell nuclei first using a polyclonal rabbit anti-Fos/FRA antiserum (Ab-5; Oncogene Research Products) at a dilution of 1:80,000. This antibody is directed against amino acids 4–17 of human c-Fos, but this amino acid sequence is also common to Fos-B, FRA1 and FRA2 (15). After a 72-hr incubation at 4°C, tissues were transferred to biotinylated goat anti-rabbit

second antibody at a dilution of 1:500 (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubated for 2 hr at room temperature. Tissues were then transferred to an avidin-biotin-peroxidase-complex (ABC, Vector Laboratories) and incubated for 2 hr at room temperature. The antibody complex was developed by transferring tissue to a 0.05% solution of tetrahydrochloride 3,3'diaminobenzedine (Sigma Chemical Company) and 0.25 g nickel sulfate (Sigma Chemical Company), which, in the presence of 0.01% hydrogen peroxide, produces a blue/black reaction product in Fos/FRA-ir nuclei (16, 17).

Somatostatin in the cytoplasm was detected with a polyclonal rabbit anti-rat SS antiserum (Incstar Corporation, Stillwater, MN) at a dilution of 1:10,000 for the PeVN and 1:7000 for the ARC. After a 48-hr incubation at 4°C, tissues were transferred to biotinylated goat anti-rabbit second antibody as described above for detection of Fos/FRA-ir, before being developed in 3,3'diaminobenzedine without the addition of nickel sulfate, which, in the presence of peroxide, produces a reddish-brown reaction product in the cytoplasm of SS-ir cells. GHRH-ir in the cytoplasm was detected with a polyclonal rabbit anti-human GHRH (Peninsula Laboratories, Inc., Belmont, CA) antiserum at a dilution of 1:40,000 using the same protocol for SS described above. Bovine GHRH differs from human GHRH by five amino acids (18). However, the anti-human GHRH antibody used in this experiment cross-reacts 100% with bovine GHRH (Peninsula Laboratories).

Experiment 2: Effect of GHRH on Secretion of GH Before and After Feeding. Eight steers were randomly assigned to be injected intravenously with bGHRH ([Leu^{27}, Hse^{45}] bGHRH$^{1-45}$ lactone Pharmacia-Upjohn, Kalamazoo, MI; 0.2 μg/kg body weight) at either 0900 hr (1 hr before feeding) or 1300 hr (1 hr after feeding) in a simple crossover design (n = 8 per group). Two days separated each replicate in the design. A jugular vein of each steer was cannulated 24 hr before the experiment. Blood samples (6 ml) were collected at –20, –10, 0, 5, 10, 15, 20, 30, 40, 60 min relative to injection of bGHRH. Serum was harvested as described above and stored at –20°C until assayed for GH.

Growth Hormone Assay. Growth hormone was measured using a radioimmunoassay (3) in which the intra-assay coefficient of variation was 6.8%.

Statistical Analyses. In Experiment 1, mean concentrations of GH were presented for the group euthanized at 1300 hr only because all blood samples were collected from 0700 to 1300 hr for this group. To determine whether concentrations of GH were lower after than before feeding, mean concentrations from 1200 to 1300 hr were compared with mean concentrations from 0900 to 1000 hr.

The number of SS-ir and GHRH-ir neurons and the number of Fos/FRA-ir nuclei in SS-ir and GHRH-ir neurons were counted bilaterally in PeVN and ARC nuclei on four sections of hypothalami from each steer. These four sections were selected from 10 collected serially from a total of 20 comprising the PeVN and from 10 collected serially between sections 20–30 from a total of 50 comprising the ARC in each steer. Thus, without stereotaxic coordinates, sections were a reasonable representation of the PeVN, and mid-ARC. Sections were selected on the basis of the distribution map of Leshin *et al.* (6). One observer, who was blind to the identity of animals and their treatment groups, performed all cell counts. Presence of Fos/FRA-ir detected in SS-ir or GHRH-ir neurons is expressed as a percentage of the number of SS-ir or GHRH-ir per section.

In Experiment 2, net areas under the GH curve were calculated as follows. Total areas under the GH curve were calculated for each animal from 0 to 60 min after injection of GHRH using the trapezoid method. The area of the rectangle calculated from the concentration of GH at Time 0 and projected to 60 min was used to estimate baseline, which was subtracted from the total area under the GH curve to yield net areas (net GH AUC) for each animal.

Data for GH, percentage of GHRH-ir and SS-ir neurons containing Fos/FRA-ir, number of GHRH-ir and SS-ir neurons, and net GH AUC were subjected to ANOVA using the generalized linear models (GLM) procedure in SAS (19) with either time (before or after feeding) or group (0700, 0900, 1100, or 1300 hr) as the main effect. Differences between means, when the F-test was significant ($P < 0.05$), were evaluated using the PDIFF option of the GLM procedure of SAS and were adjusted for multiple comparisons using the method of Tukey where appropriate. Data are reported as least squares means ± the pooled standard error of the mean (SEM).

Results

Feeding-Induced Changes in Concentrations of GH. Concentrations of GH from 0700 to 1300 hr are shown in Figure 1. Concentrations of GH decreased after the start of feeding and were lower ($P < 0.05$) from 1200 to 1300 hr (4.4 ng/ml) than from 0900 to 1000 hr (13.4 ng/ml) (pooled SEM = 3.1).

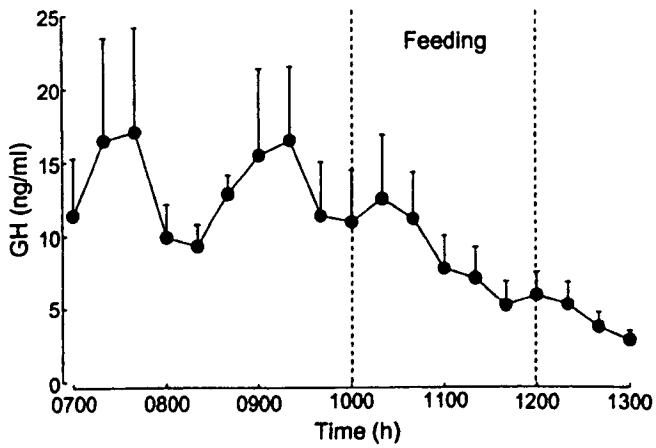


Figure 1. Concentrations of GH in serum from 3 hr before to 1 hr after feeding in Holstein steers. Pooled SEM = 3.7 ng/ml.

Immunostaining of GHRH, SS, and Fos/FRA. Immunostaining of SS neurons in the PeVN was intense with neurons located within 500 μm of the third ventricle (Figs. 2A and 2C). Fos/FRA-ir nuclei were more numerous at 0900 hr (Figs. 2A and 2B) than at 1100 hr (Figs. 2C and 2D). In contrast, intensity of immunostaining of SS neurons in the ARC was weak, and a lower dilution of primary antibody (1:7,000) was required to detect these neurons than SS neurons in the PeVN (1:10,000; photomicrograph not shown). Intensity of immunostaining of GHRH neurons in the ARC was stronger at 1300 hr than at 0700 hr, though not as intensely immunostained as SS neurons, resulting in more clearly defined neurons and processes at 1300 hr (Figs. 3A and 3B). Immunostaining for Fos/FRA did not differ regionally within PeVN or ARC from 0700 to 1300 hr.

Percentage of GHRH-ir Neurons in the ARC Containing Fos/FRA-ir Nuclei. The percentage of GHRH-ir neurons containing Fos/FRA-ir in the ARC was 66% lower ($P < 0.05$) at 1100 hr and 65% lower ($P < 0.05$) at 1300 hr than at 0700 hr (Fig. 4). In contrast, the number of GHRH-ir neurons detected increased ($P < 0.05$) from 0700 to 1300 hr. Concentrations of GH did not correlate well with the percentage of GHRH-ir neurons containing Fos/FRA ($P = 0.66$).

Percentage of SS-ir Neurons in the PeVN and ARC Containing Fos/FRA-ir Nuclei. The percentage of SS-ir neurons in the PeVN containing Fos/FRA-ir nuclei was 50% lower ($P < 0.01$) at 1100 hr and 36% lower ($P < 0.05$) at 1300 hr than at 0900 hr (Fig. 5). There was no difference ($P = 0.78$) in the number of SS-ir neurons detected in the PeVN from 0700 to 1300 hr. In contrast, the percentage of SS-ir neurons in the ARC containing Fos/FRA-ir nuclei did not change from 0700 to 1300 hr ($P = 0.15$) and, similarly, there was no difference ($P = 0.21$) in the number of SS-ir neurons detected in the ARC from 0700 to 1300 hr (Fig. 6). Concentrations of GH did not correlate well with the percentage of SS-ir neurons in the PeVN and ARC containing Fos/FRA ($P = 0.11$ and $P = 0.67$, respectively).

Effect of GHRH on Secretion of GH Before and After Feeding. Intravenous injection of bGHRH stimulated release of GH at 0900 and at 1300 hr (Fig. 7), but net GH AUC was greatly suppressed at 1300 hr compared with 0900 hr ($P < 0.001$).

Discussion

Secretion of GH into blood is episodic in steers (20), other animals (4, 21), and humans (22). Currently, it is not clear how each pulse of GH released from somatotropes in the anterior pituitary gland is generated. A long-held postulate is that SS and GHRH are secreted into hypophysial-portal blood alternately to inhibit or stimulate, respectively, release of GH from somatotropes (4, 5). However, a number of studies do not support this hypothesis, and instead, show poor relationships among pulses of SS, GHRH, and GH (23–25).

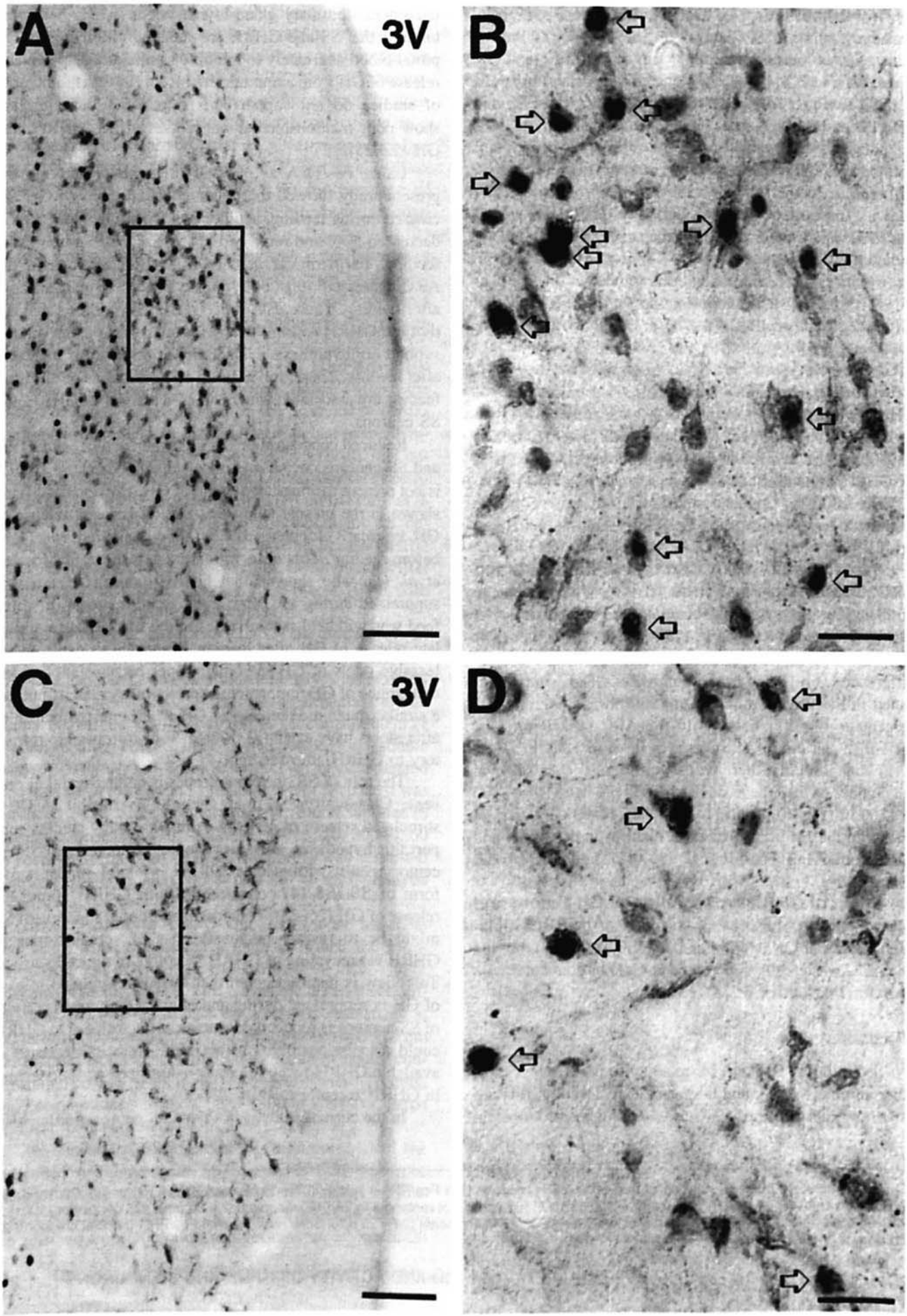
Using Fos/FRA as markers of neuronal activity, the present study showed that when concentrations of GH decreased during feeding, activity of SS neurons in the PeVN decreased 50% and remained low for at least an hour after feeding. Furthermore, activity of GHRH neurons in the ARC decreased up to 66% during and for at least an hour after feeding. These data supported our hypothesis that activity of GHRH neurons decreases after feeding, but did not support our hypothesis that activity of SS neurons increases after feeding. Therefore, reduced concentrations of GH after feeding are associated with decreased activity of GHRH and SS neurons.

Reduced concentrations of GH when activity of GHRH and SS neurons decrease is understandable because GHRH is not present to stimulate release of GH. However, we have shown in the present study that GHRH-induced release of GH is greatly suppressed 1 hr after compared with 1 hr before feeding. This observation extends those of Moseley *et al.* (1), who showed that GHRH-induced release was suppressed during and after feeding and in steers in which feed was withheld (sham-fed). Failure of GHRH to stimulate release of GH after feeding is not due to depleted releasable pools of GH in somatotropes because another secretagogue of GH, quipazine, a serotonin agonist, stimulates a similar magnitude release of GH when injected before or after steers have eaten (3). Rather, somatotropes are refractory to GHRH after feeding.

The role of SS on somatotropes is controversial at present. Classically, SS was thought to inhibit GHRH-stimulated release of GH (4). Although many studies support this hypothesis, administration of octreotide, an SS receptor agonist with a long half-life in blood, or the active form of SS (SS-14) concurrently with GHRH stimulates release of GH (26–28). Perhaps an additional function of SS might be to restore responsiveness of somatotropes to GHRH *via* recycling of GHRH receptors to the cell surface. This view is justified given that GHRH-stimulated release of GH is suppressed during and after feeding when activity of SS neurons is reduced. However, refractoriness to GHRH could also be attributed to a GHRH-induced decrease in available GHRH receptors on somatotropes and a decrease in GHRH receptor mRNA (29, 30).

In the current study, activity of SS neurons in the ARC

Figure 2. Brightfield photomicrograph of dual-label SS-ir neurons with Fos/FRA-ir nuclei in the PeVN at 0900 (A, B) and 1300 hr (C, D). (A) Low power (100x) at 0900 hr (scale bar = 100 μm); (B) high power of rectangle in 2A (400x; scale bar = 25 μm); (C) low power (100x) at 0900 hr (scale bar = 100 μm); (D) high power of rectangle in 2C (400x; scale bar = 25 μm). SS-ir neurons with Fos/FRA-ir nuclei are identified with arrows (3V = third ventricle).


A
3V
B
C
3V
D

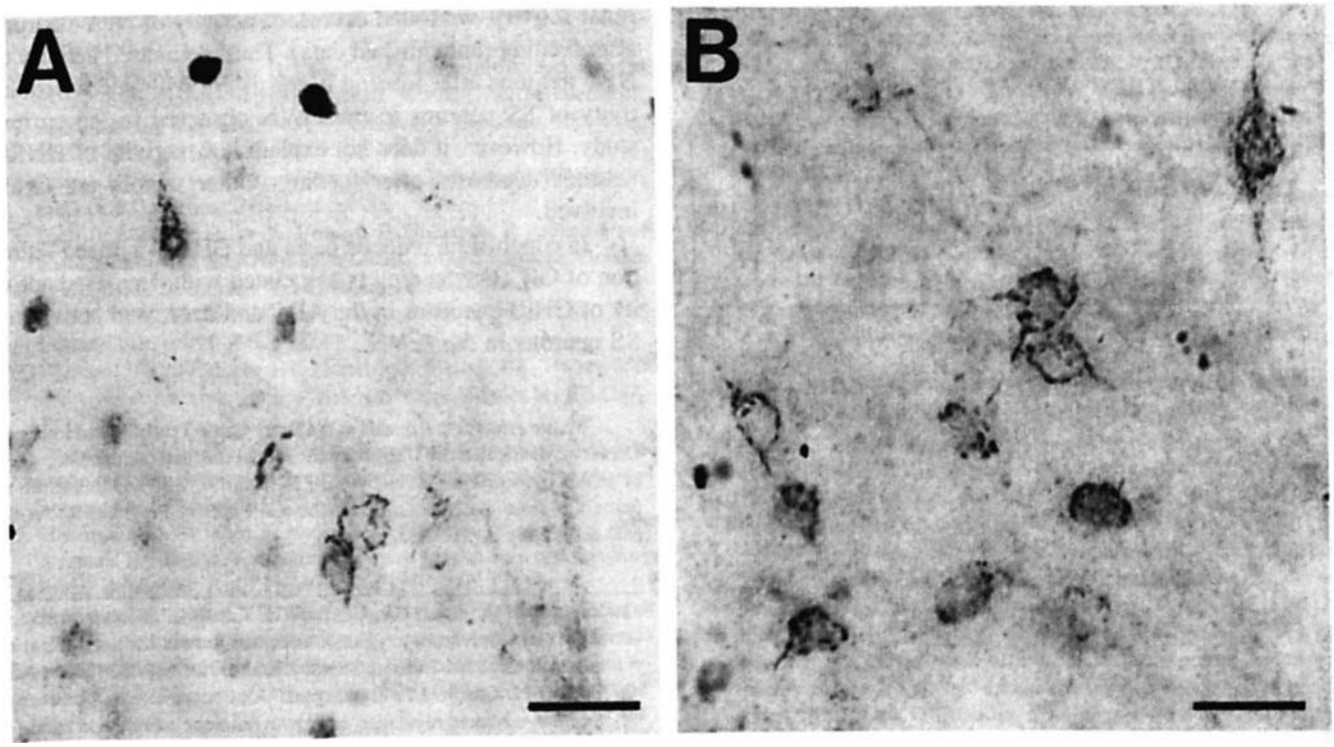


Figure 3. Brightfield photomicrograph (400x) of GHRH-ir neurons in the ARC at (A) 0700 hr and (B) 1300 hr (scale bar = 25 μm). Immunostaining was more intense at 1300 hr than at 0700 hr, resulting in more clearly defined neurons and processes.

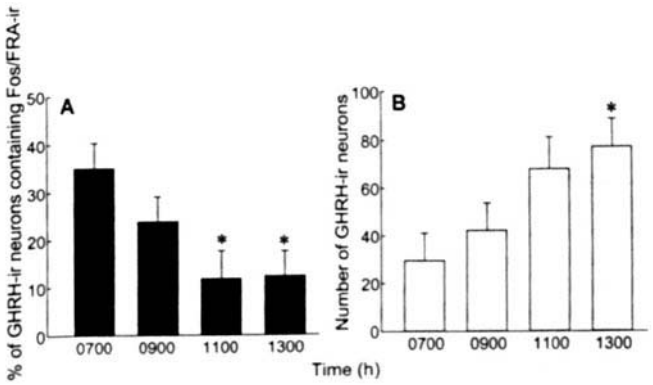


Figure 4. (A) Percentage of GHRH-ir neurons in the ARC containing Fos/FRA-ir nuclei; and (B) number of GHRH-ir neurons detected in the ARC. Symbols indicate differences from 0700 hr (*, $P < 0.05$).

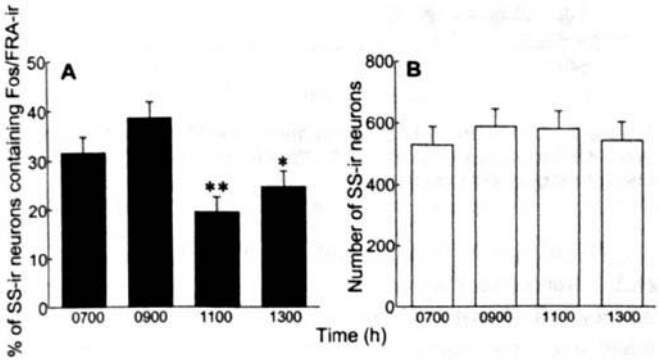


Figure 5. (A) Percentage of SS-ir neurons in the PeVN containing Fos/FRA-ir nuclei; and (B) number of SS-ir neurons detected in the PeVN. Symbols indicate differences from 0900 hr (**, $P < 0.01$; *, $P < 0.05$).

did not change from 0700 to 1300 hr. In addition, intensity of immunostaining differed between somatostatin neurons in the PeVN and those in the ARC. Most somatostatin (80%) released into hypophysial-portal vessels originates from SS neurons in the PeVN, which are also 8–9 times more active than SS neurons elsewhere in the hypothalamus (7). Therefore, weaker immunostaining of SS neurons in the ARC, than the PeVN, in our study may reflect lower activity and synthesis of SS in SS neurons of the ARC.

Activity of GHRH neurons decreased with time in conjunction with increased numbers of GHRH-ir neurons. The total number of GHRH-ir neurons in the ARC is difficult to obtain without treating animals with colchicine to block axonal transport and release from terminals, resulting in increased concentrations of GHRH in these neurons (31). Therefore, immunostaining of GHRH neurons in the present study represents a sample of the total population of GHRH neurons in the ARC. We suggest that increased numbers and intensity of immunostained GHRH-ir neurons during and after feeding reflect either an increased rate of synthesis of GHRH, or a decreased rate of transport out of neurons, or both. Increased intensity of immunostaining from 0700 to 1300 hr can be explained by removal of negative feedback of GH on GHRH neurons *via* SS and other neurotransmitters, resulting in increased synthesis of GHRH (32, 33). In support of this argument, Tannenbaum *et al.* (34) observed increased intensity of immunostaining and increased numbers of GHRH-ir neurons after depleting concentrations of SS in hypothalami.

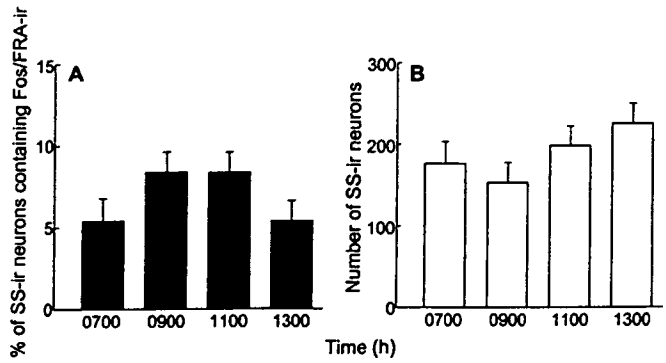


Figure 6. (A) Percentage of SS-ir neurons in the ARC containing Fos/FRA-ir nuclei; and (B) number of SS-ir neurons detected in the ARC.

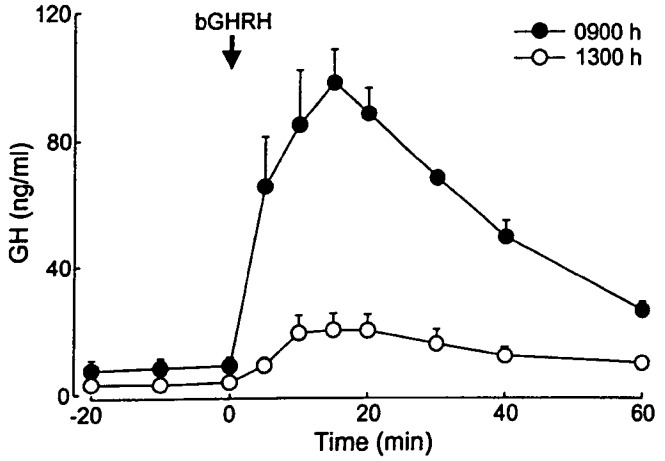


Figure 7. Concentrations of GH in serum for 20 min before and 60 min after intravenous injection of bGHRH (0.2 μg/kg body weight). Pooled SEM = 6.3 ng/ml.

Regulation of feeding-induced downregulation of GH and refractoriness of somatotropes to GHRH is not well understood. Downregulation of the GH axis is not prevented when steers are sham-fed (1). Therefore, feed alone does not contribute to this phenomenon, and it may be a rhythm entrained to time of feeding. In addition, it is not known whether peripheral or central factors regulate feeding-induced downregulation of the GH axis. However, we have recently demonstrated that blocking the postprandial increase in insulin does not prevent concentrations of GH from decreasing during feeding and does not restore responsiveness of somatotropes to GHRH (35). Therefore, we suggest that downregulation of the GH axis is mediated in the hypothalamus.

Neuropeptide Y (NPY) is a likely candidate regulating the GH-axis at feeding. Neuropeptide Y is thought to mediate negative feedback of GH on SS neurons in the PeVN (36). Expression of NPY mRNA increases in neurons with duration of fasting and returns to normal after feeding (37, 38). Furthermore, GH receptors are located on NPY neurons (39), and GH stimulates expression of *c-fos* mRNA in NPY neurons (40). In addition, NPY axons synapse on SS neurons in the PeVN (41). Using Fos/FRA as markers of neuronal activity, we found decreased activity of NPY neurons after feeding (unpublished data). Thus, decreased activity of NPY neurons after feeding is consistent with decreased activity of SS neurons in the PeVN observed in the current study. However, it does not explain how activity of GHRH neurons decreases after feeding. Other factors are likely involved.

In conclusion, reduced basal and GHRH-induced secretion of GH after feeding is associated with decreased activity of GHRH neurons in the ARC and decreased activity of SS neurons in the PeVN.

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