**FULL PAPER** *Physiology*

# Relationship between Growth Hormone (GH) Pulses in the Peripheral Circulation and GH-Releasing Hormone and Somatostatin Profiles in the Cerebrospinal Fluid of Goats

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ABSTRACT. Growth hormone (GH) is secreted in a pulsatile manner, but the underlying mechanisms of GH pulse generation remain to be resolved. In the present study, we investigated the relationship between GH pulses in the peripheral circulation and GH-releasing hormone (GHRH) and somatostatin (SRIF) profiles in the cerebrospinal fluid (CSF) of male goats. The effects of an intracerebrovent ricular (icv) injection of neuropeptide Y (NPY), galanin and ghrelin were also analyzed. Blood and CSF samples were collected every 15 min for 8 hr from the jugular vein and third ventricle, respectively. GH pulsatility in the goat was found to consist of distinct large pulses of 5 hr periodicity and small pulses of 1 hr periodicity. GHRH and SRIF in the CSF fluctuated in a pulsatile manner with 1 hr periodicity, and most of the descending phase of SRIF pulses were associated with the initiation of GH pulses. Icv injections of NPY, galanin and ghrelin stimulated GHRH release without affecting SRIF release. In addition, NPY suppressed, and galanin and ghrelin induced large GH pulses, although ghrelin was much more effective than galanin. These results suggest that an hourly fall in SRIF is involved in generating intrinsic circhoral rhythm of GH pulsatility. The mechanisms underlying the generation of large GH pulses of 5 hr periodicity remain unknown, while direct action of NPY and/or ghrelin on the pituitary might be involved.

KEY WORDS: ghrelin, growth hormone, growth hormone-releasing hormone, NPY, somatostatin.

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Growth hormone (GH) is secreted in a pulsatile manner in all mammalian species studied to date, and this pulsatility plays an important role in the regulation of somatic growth and metabolism [7, 10, 15, 20]. Knowledge of the exact mechanisms underlying the generation of GH pulses is, therefore, of physiological and pathophysiological importance. It is generally accepted that GH pulses are governed by two hypothalamic peptides, GH-releasing hormone (GHRH) and somatostatin (SRIF), which are stimulatory and inhibitory for GH secretion, respectively [15, 29, 34, 41]. Most studies on the neuroendocrine regulation of GH pulsatility have been performed in male rats, in which GH secretion is characterized by a strikingly regular ultradian rhythm with large secretory bursts of every 3–4 hr [15, 41]. In the rat, indirect studies such as passive immunizations against GHRH and SRIF suggested that GH pulses depend on GHRH discharge in combination with a decrease in SRIF secretion [29, 41]. However, the secretory profiles of GHRH and SRIF under physiological conditions in this species remain to be elucidated because of the technical difficulties involved in collecting pituitary portal blood from unanesthetized animals. On the other hand, direct measurement of GHRH and SRIF in the portal blood from conscious animals has been reported in the sheep [4, 12, 44]. According to these studies, GH pulsatility in the sheep seemed less regular and the secretory patterns of GHRH and SRIF seemed more complex than those assumed in the rat, and thus a consistent relationship between GHRH, SRIF and GH has yet to be obtained.

In addition to GHRH and SRIF, many neuropeptides including neuropeptide Y (NPY), galanin and ghrelin are now considered to be involved in the control of GH secretion. NPY is one of the most abundant peptides within the hypothalamus [2] and well-known to be involved in appetite and energy metabolism [6, 21, 33, 47, 49]. In rats, intracerebroventricular (icv) injection of NPY inhibited GH secretion [30], while an antibody to NPY led to elevated GH levels [36]. NPY may mediate the negative-feedback action of GH upon its own secretion [5]. Galanin is also highly concentrated in the hypothalamus [3], and known to participate in the regulation of metabolism, especially fat metabolism [27, 43], as well as GH secretion [10, 20]. Ghrelin, which was found as an endogenous ligand of GH-secretagogue receptor, is distributed in the stomach and hypothalamus, and facilitates both food intake and GH secretion [25]. Involvement of these peptides in GH secretion suggests a close relationship between regulatory systems for GH secretion and metabolism, and to clarify the mechanisms of actions of these peptides will be of great importance in understanding the generation of GH pulsatility.

We recently found that the GH secretory pattern in male goats was distinctly pulsatile with regular 5 hr intervals throughout a day [32], and assumed them to be good experimental models for analyzing the neuropeptidergic control of GH pulsatility. To estimate GHRH and SRIF secretory

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profiles, we collected the cerebrospinal fluid (CSF) from the third ventricle in this study because CSF sampling via an indwelling cannula was expected to be less stressful to animals than portal blood sampling. A reliable correlation between gonadotropin-releasing hormone (GnRH) pulses in the CSF and luteinizing hormone pulses in the peripheral circulation has been well documented in the sheep [37, 38], cow [14] and rhesus monkey [45]. Here, we analyzed the correlation between GH pulses in the serum and GHRH and SRIF profiles in the CSF of the goat. The effects of icv injection of NPY, galanin and ghrelin on GH, GHRH and SRIF secretion were also investigated.

## MATERIALS AND METHODS

*Animals*: Five adult male Shiba goats (*Capra hircus*) of 2–3 years old maintained for experimental purposes as a closed colony at the experimental farm of the University of Tokyo were used in this study. They were used repeatedly with at least 3 weeks intermission period. All animals were castrated at least 6 months before the experiments and were loosely restrained by being tied to stanchions indoors, where they were allowed free access to food and water. The temperature was kept at 23 ± 2°C and lighting was controlled according to a 12L:12D cycle (lights on at 0900 hr).

*Surgical procedure*: At least 2 weeks before a goat was used in an experiment, a stainless steel cannula was stereotaxically introduced into the third ventricle as previously described [33]. The cannula was approximately 50 mm in length and was made from an 18-gauge spinal needle (Terumo, Tokyo, Japan) blunted at both ends. A 21-gauge stylet was extended 2 mm beyond the end of the cannula to penetrate the tissue and prevent CSF backflow as a stopper. When placing the cannula, the head of the goat anesthetized with isoflurane was positioned in a stereotaxic frame (Narishige, Tokyo, Japan), and then 500 $\mu l$ radioopaque liquid (Omnipaque, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) was injected into the lateral ventricle. A lateral x-ray was then taken, which gave specific landmarks of the ventricular system including the interthalamic adhesion and the interventricular foramen for the antero-posterior and vertical orientations. The cannula and stylet were stereotaxically lowered through the center of the sagittal sinus and aimed slightly before the vertical tangent of the interthalamic adhesion and about 10 mm below a horizontal line drawn through the middle of the interventricular foramen. If the tip of the cannula was placed correctly in the third ventricle, CSF came out when the stylet was pulled out. The position of the cannula was finally confirmed by an additional lateral x-ray. When the cannula was confirmed to be in position, it was immobilized by filling the burr hole with acrylic dental cement. A plastic cap with a screw-off top was centered over the cannula and anchored to the skull with six stainless steel screws and acrylic dental cement. The cannula and protective cap were further secured by filling the inside of the cap with acrylic dental cement.

*CSF and blood collection*: The CSF collection was performed at least 2 weeks after cannulation into the third ventricle. When a goat was used in an experiment, the top of the protective cap was removed and the stylet was pulled out. A silastic collection tube (id, 0.5 mm; od, 0.8 mm; 30 cm in length) filled with sterile saline was attached to the cannula with a 1 cm silastic collar, in which the proximal end of the collection tube was glued with an adhesive. This silastic collar was closely fitted onto the cannula to prevent leakage of CSF between the collecting tubing and the cannula. The distal end of the collection tube was sealed with a plug. To collect a sample of CSF, the plug was removed and CSF was siphoned out from the distal end of the collection tube. At each sampling, 120–180 $\mu l$ of CSF was collected after dead space CSF (60 $\mu l$) was discarded. CSF sampling was done at 15 min intervals for 8 hr (starting at 1,000 hr). Each animal was used twice, and a total of 10 experiments were conducted. Samples were stored at –80°C until GHRH and SRIF assays. To collect intravenous blood samples, a catheter (Argyle Medical Catheter, 18G and 70 cm in length, Nihon Sherweed, Tokyo, Japan) was inserted into the jugular vein 1 day before the experiment. Samples were taken immediately after the end of CSF-sampling. After centrifugation, the plasma was stored at –20°C until GH assay.

*Icv injection of NPY, galanin and ghrelin*: In the case of icv experiments, after the collection of CSF and blood samples for 4 hr (starting at 1000 hr), 10 $\mu$g of porcine NPY (Sigma, St. Louis, MO), 5 $\mu$g of porcine galanin (Sigma) or 13 $\mu$g of rat ghrelin, which was generously supplied by Dr. N. Murakami, Miyazaki University, was injected through the CSF collection tube with a 1 m*l* syringe with a 26-gauge blunted needle connected to the proximal end of the tube. The peptides were dissolved in 200 $\mu l$ saline and injected for 1 min. To prevent the solutions from remaining in the dead space, 100 $\mu l$ saline was additionally injected immediately after injection of the peptide solutions. In the control group, only saline (300 $\mu l$) was injected. Collection of CSF and blood samples was further continued for an additional 4 hr.

*Assays*: Plasma GH concentrations were measured by double-antibody radioimmunoassay (RIA) using monkey anti-bovine GH antiserum as previously described [32]. The parallelism between goat plasma and bovine GH was reported previously [18]. The minimum concentration of GH that could be detected was 0.8 *n*g/m*l* (the 95% binding point). Intra- and interassay coefficients of variation (CVs), calculated from pooled serum containing 4.2 *n*g/m*l* (the mean for 5 assays), were 6% and 8%, respectively. GHRH was assayed with commercial human GHRH RIA kits (RIK 8061, Peninsula Laboratory, Belmont, CA) according to the manufacturer's protocols with slight modifications. The parallelism between CSF and a standard of GHRH assays is shown in Fig. 1. The minimum assay sensitivity of GHRH was 10.4 *p*g/m*l*. Intra- and interassay CVs for GHRH, calculated from pooled CSF containing 22 *p*g/m*l* (the mean for 6 assays), were 7% and 10%, respectively. SRIF was assayed by RIA using commercial antibody (RAS 8001, Peninsula Laboratory) as previously described [33]. The minimum assay sensitivity was 3.2 *p*g/m*l*. Intra- and inter-

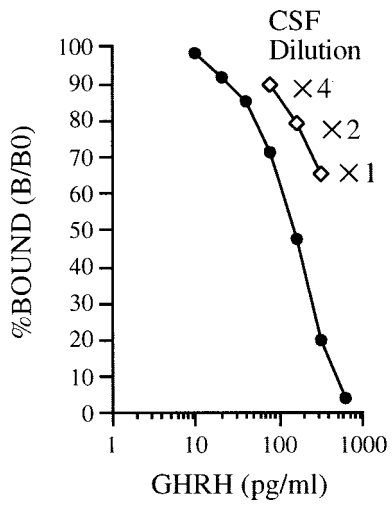


Fig. 1. Parallelism of serially diluted CSF samples of goats with GHRH standards in the RIA.

assay CVs, calculated from pooled CSF containing 35 *p*g/m*l* (the mean for 6 assays), were 5% and 10%, respectively.

*Data analysis*: Since secretory profiles of GH, GHRH and SRIF were found to be episodic, and pulse detection was done based on the methods reported previously [11, 13, 23]. A pulse was defined when CVs of assay values composing both ascending and descending phases of each peak exceeded 2 times the corresponding intraassay CV calculated for the pulse detection in each assay. The pulse amplitude denoted the difference between the peak and the preceding nadir, and the interpulse interval denoted the time between two peaks. In this study, a GH pulse having an amplitude of more than 20 *n*g/m*l* was tentatively defined as a 'large pulse' based on our previous study, in which the smallest pulse amplitude of GH pulse with 5 hr periodicity was about 20 *n*g/m*l* [32], otherwise as a 'small pulse'. The effects of icv injection of NPY, galanin and ghrelin on GH, GHRH and SRIF levels were analyzed by one-way ANOVA followed by the Fisher's PLSD test. Differences were considered significant at $p<0.05$.

## RESULTS

*Correlation between peripheral GH pulses and third ventricular GHRH and SRIF profiles*: Third ventricular CSF and blood samples were collected simultaneously at 15 min intervals for 8 hr (starting at 1000 hr) using 5 male goats. Each animal was used twice, and a total of 10 experiments were conducted. As shown in Fig. 2A-C (top panels), distinct large GH pulses having amplitude of more than 20 *n*g/m*l* were observed in 8 cases, in which small pulses were occasionally observed in addition to the large pulses. In the remaining 2 cases in two different animals, only small pulses were observed at relatively regular intervals (Fig. 2D, top panel). Mean pulse amplitudes of large and small GH pulses were 40.9 ± 2.8 *n*g/m*l* (mean ± SE, n=12) and 7.0 ± 0.7 *n*g/m*l* (n=33), respectively. Large pulses occurred with an interval of 5 hr (300.0± 6.1 min, n=4), which was almost exactly the same as that observed in our previous study [32]. As shown in Fig. 3A, frequency histograms of the overall interpulse intervals, which include all intervals between GH pulses irrespective of large or small, revealed that there were peaks at around 60 and 120 min.

Both GHRH and SRIF levels in the third ventricular CSF fluctuated in a pulsatile manner in all the animals examined (Fig. 2, middle and bottom panels). Mean pulse amplitudes of GHRH and SRIF pulses were 38.1 ± 4.0 *p*g/m*l* (n=68) and 50.2 ± 3.2 *p*g/m*l* (n=76), respectively. As shown in Fig. 3B and C, frequency histograms of the interpulse intervals revealed that both peptides had peaks at around 60 min in common; the mean interval of GHRH pulses was 60.3 ± 3.1 min (n=58) and that of SRIF pulses 59.6 ± 3.5 min (n=66). It was found that 91.1% of the initiation of the ascending phase of GH pulses, including both large and small ones, occurred during the descending phase (between the peak and following nadir) of SRIF pulses.

*Effect of icv injection of NPY, galanin and ghrelin on peripheral GH and third ventricular GHRH and SRIF levels*: In this study, only the data from animals that showed one large pulse during the 4 hr period before the icv injection were included for analysis to minimize the differences in GH secretory pattern before the injection among experimental groups. Representative profiles of peripheral GH and third ventricular GHRH and SRIF following icv injection of NPY, galanin and ghrelin are shown in Fig. 4. Changes in mean concentrations of GH, GHRH and SRIF during each 1 hr period between 2 hr before and 4 hr after the injection are summarized in Fig. 5. All the peptides at doses used in this study immediately induced a robust increase in GHRH concentrations in the CSF, while they did not affect the SRIF levels. The effects of ghrelin and galanin on GHRH were transient, but NPY increased the GHRH levels during the entire experimental period of 4 hr. Despite the increases in GHRH levels in the CSF, mean GH concentrations in the peripheral circulation were increased by only ghrelin, which induced several large pulses immediately after injection. No large pulse was observed during the experimental period of 4 hr following icv injection of NPY. Icv injection of galanin immediately induced a large GH pulse, but the effect on mean GH concentration was not significant.

## DISCUSSION

In the present study, distinct large GH pulses with regular 5 hr periodicity were observed in most cases (8 of 10 experiments) as in our previous study [32], indicating that the procedure for CSF sampling did not interfere with the hypothalamic generating system of GH pulsatility. In addition to large pulses, we detected small pulses with an interpulse interval of around 1 hr, which were more prominent in the remaining 2 cases in which large pulses were absent.

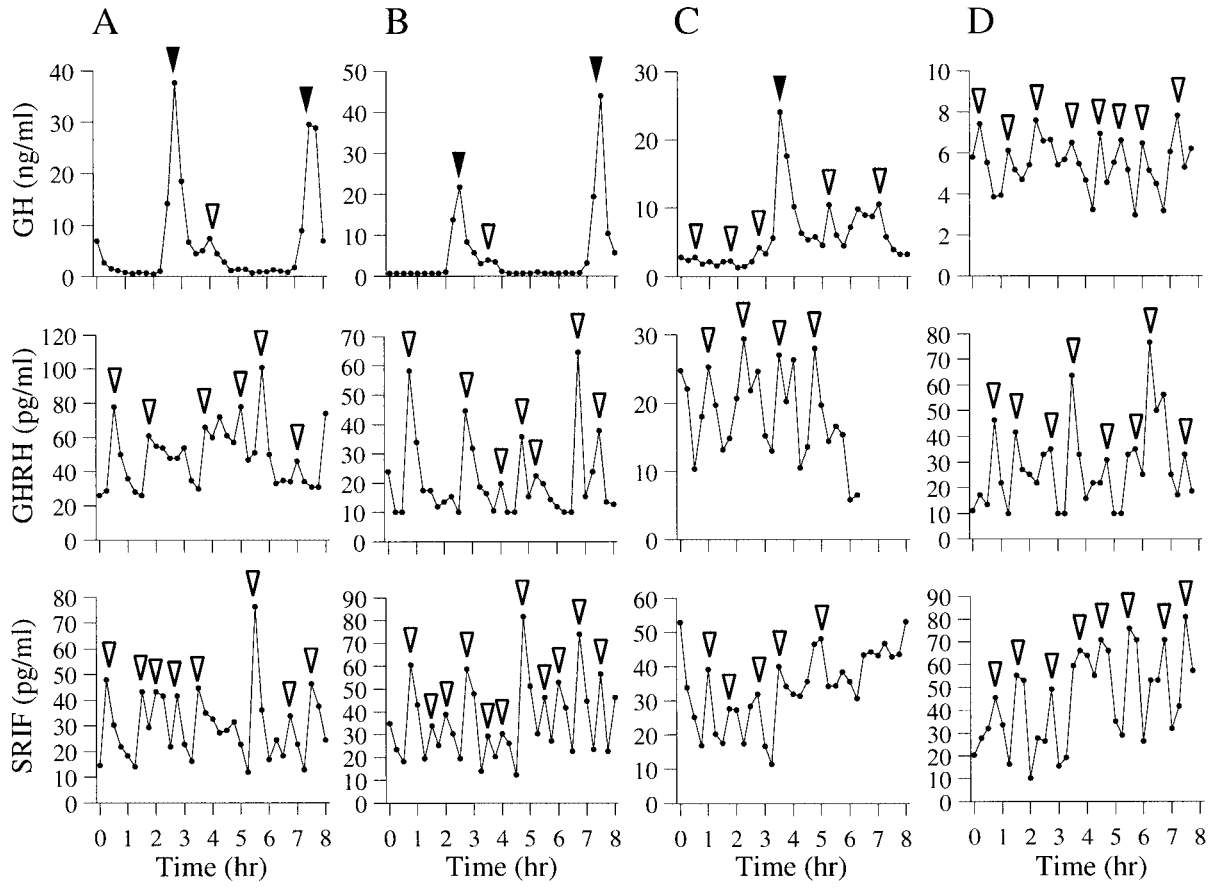


Fig. 2. Profiles of plasma GH (top panels), and those of GHRH (middle panels) and SRIF (bottom panels) in the CSF of four goats. Triangles indicate defined pulses. As for GH, open and filled triangles indicate small and large pulses, respectively. Note that vertical scale of GH in D is magnified.

Interestingly, two frequencies in GH secretion, namely the 3.3 hr periodicity of the GH secretory episode and 1 hr rhythm between the episodes, were also proposed in rats [46]. In human, 2 hr periodicity in basal secretion of GH was proposed in addition to large GH bursts occurring randomly [16, 48]. Taken together, it is suggested that there are two components in GH secretion in common among species; one is basal short interval oscillation, and the other is large secretory bursts with longer intervals.

GHRH and SRIF could be reliably measured in the CSF obtained from the third ventricle at 15 min intervals for 8 hr in this study. Both peptides fluctuated in the CSF in a pulsatile manner with an interval of almost exactly 1 hr. An intrinsic 1 hr rhythm of GHRH secretion was postulated mathematically in a rat model as well [46]. The pulsatile release of SRIF with an interval of about 1 hr was also reported by perfusion of the median eminence of unanesthetized rats [22]. The secretory patterns of GHRH and SRIF observed in the present study were consistent with those reported by Frohman *et al.* [12], who demonstrated that GHRH and SRIF were secreted into the pituitary portal blood of sheep in a pulsatile manner with an interval of 71 and 54 min, respectively. This suggests that GHRH and SRIF in the third ventricular CSF correlate well with those secreted into the portal blood. In support of this, it is suggested that neuropeptides once released into the pituitary portal circulation are inversely transported to the CSF [24]. The observation that more than 90% of GH pulses were initiated in the descending phase of SRIF pulses suggests that an hourly fall in SRIF is involved in the formation of 1 hr rhythm of small GH pulses in goats. This is supported by previous findings that an abrupt withdrawal of SRIF leads to a rebound of GH secretion both *in vitro* [26, 39] and *in vivo* [8, 31].

Then, how are the large GH pulses induced with 5 hr periodicity in goats? Our results suggest that neither GHRH nor SRIF is involved in determining 5 hr periodicity. Since GH secretion is influenced by multiple neurotransmitter pathways [15, 34], other hypothalamic factors such as NPY and galanin, which are released into hypophyseal portal circulation [28, 40], may be involved. Ghrelin, either of stomach or hypothalamus origin, may also take part in this process. We then investigated the effects of these three peptides on GHRH and SRIF release into the third ventricle as well as

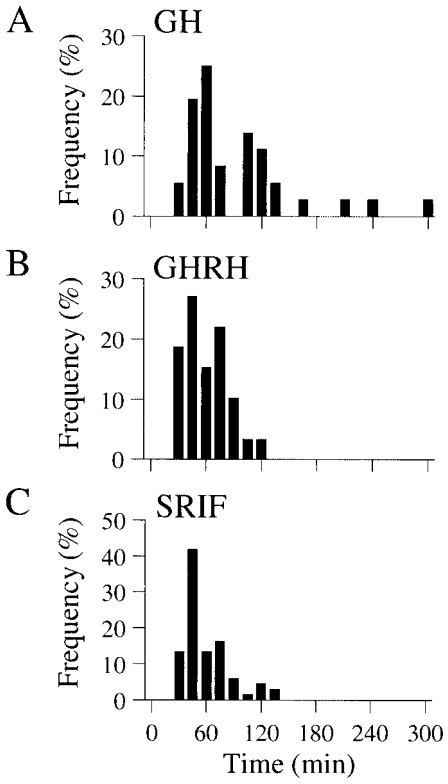


Fig. 3. Frequency histograms of overall interpulse intervals of GH (n=35, top panel), GHRH (n=58, middle panel) and SRIF (n=66, bottom panel) pulses.

peripheral GH profiles.

NPY is regarded as one of the inhibitory peptides in GH secretion [30, 36]. After the injection of NPY, mean GH concentrations were not changed and a large pulse was not observed during the 4 hr experimental period in all animals used, suggesting that the large GH pulse was suppressed by NPY. Icv injection of NPY induced a prolonged increase in GHRH secretion without significantly affecting SRIF levels, though NPY was demonstrated to stimulate SRIF release from rat median eminence fragments *in vitro* [36]. The discrepancy between these studies might be due to the differences in the experimental conditions, i.e. *in vivo* versus *in vitro*, and/or the differences in species used. Since direct inhibition of GH secretion by NPY was demonstrated in vitro using human pituitary somatotropic tumors [1], NPY might act directly on the pituitary to suppress large GH pulses.

Icv injection of galanin induced a large GH pulse consistent with the GH-releasing action of galanin reported in other species [3, 15], although mean GH concentrations were not significantly changed. Ghrelin also led to the induction of sequential large GH pulses with a resultant increase in mean GH concentration similarly to our previous study in goats [19]. Both peptides transiently stimulated GHRH secretion, while no change was observed in SRIF levels in the CSF. These results suggest that GH-releasing activities of both peptides were at least partially related to GHRH, but not to SRIF. It is also suggested that, in the absence of the direct inhibitory action of NPY on the pituitary, a large increase in GHRH could stimulate GH secretion. In support of this, the involvement of GHRH in both galanin and ghrelin actions has been shown by previous studies using passive immunization [9, 35]. An increase in GHRH levels by GHS in the hypophyseal portal blood was also demonstrated in the sheep [17]. In addition, it has been suggested that SRIF is not involved in ghrelin action [9, 15, 34], consistent with the present results. Although the inhibition of SRIF release by galanin was reported [42], we could not confirm it in this study.

At the doses used in the present study, galanin and ghrelin equally increased GHRH levels in the CSF. However, ghrelin induced much larger GH pulses than galanin. Since ghrelin could stimulate GH release by acting directly on pituitary cells [25], ghrelin administered into the third ventricle might induce GH pulses by acting directly on the pituitary as well as through an increase in GHRH release. Although further study is needed to elucidate the precise mechanisms underlying the generation of large GH pulses, a withdrawal of NPY and/or a rise in ghrelin might be involved.

In summary, the present study demonstrated that: 1) the GH secretory pattern in the goat consisted of large pulses of 5 hr periodicity and small pulses of 1 hr periodicity; 2) GHRH and SRIF in the third ventricular CSF fluctuated in a pulsatile manner with 1 hr periodicity; 3) all of NPY, galanin and ghrelin stimulated GHRH release without affecting SRIF release, while NPY suppressed and galanin and ghrelin induced large GH pulses. These observations suggest that an hourly fall in SRIF is involved in generating the intrinsic 1 hr rhythm of small GH pulses. The mechanisms underlying the generation of large GH pulses remain unknown, but the direct action of NPY and/or ghrelin on the pituitary might be involved.

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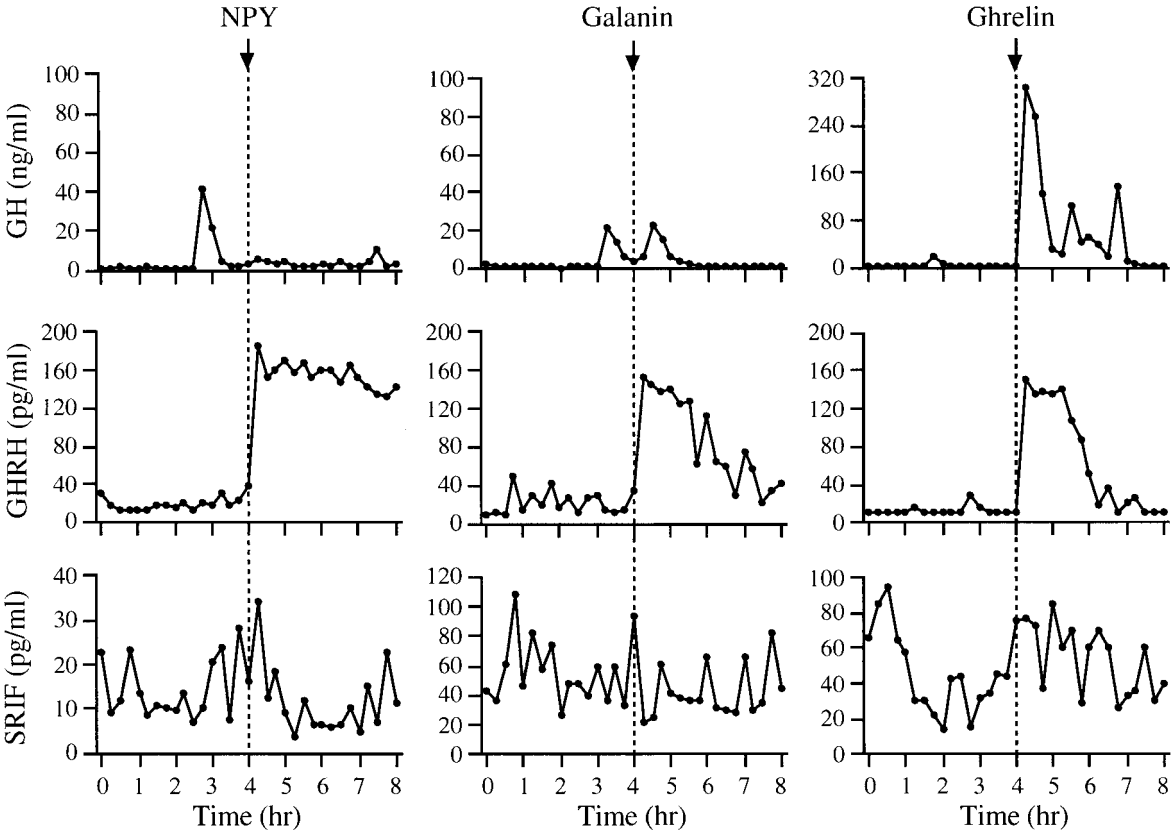


Fig. 4. Effect of icv injections of NPY (left panels), galanin (middle panels) and ghrelin (right panels) on the profiles of plasma GH (top panels), and those of GHRH (middle panels) and SRIF (bottom panels) in the CSF.

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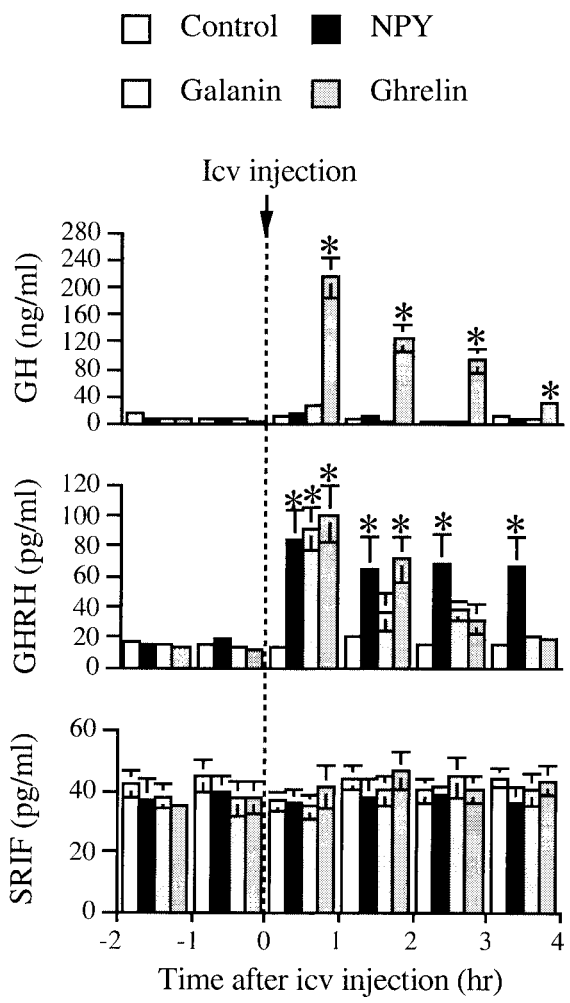


Fig. 5. Effects of icv injections of NPY, galanin and ghrelin on mean concentrations of plasma GH (top panels), and those of GHRH (middle panels) and SRIF (bottom panels) in the CSF during each 1 hr period between 2 hr before and 4 hr after the injections. Values are mean ± SE (n=3). *, $p<0.05$ vs. Saline control.

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