FULL PAPER *Physiology*

Possible Involvement of Neuropeptide Y in Photo-Induced Suppression of Growth Hormone Pulses

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(Received 4 September 2012/Accepted 6 October 2012/Published online in J-STAGE 23 October 2012)

ABSTRACT. It is well established that growth hormone (GH) is secreted in a pulsatile manner. Although the GH pulse-generating mechanism is not fully understood, we have previously reported that neuropeptide Y (NPY) profiles in the cerebrospinal fluid were negatively correlated with serum GH pulses. In addition, it is known that photic stimulation suppresses GH pulses for a certain period of time. In the present study, to investigate the involvement of NPY in regulating GH pulse generation, NPY gene expression in the arcuate nucleus (ARC) of the hypothalamus in rats was analyzed at around the lights on. First, we confirmed that GH pulses did not occur for around 1.5 hr after the start of the light phase. Then, we analyzed the activity of neurons and expression of NPY mRNA 1 hr before and 0.5 and 2 hr after lights on. Both the activity of neurons, which was evaluated by immunohistochemical detection for phosphorylated-cAMP response element binding protein (pCREB), and NPY mRNA levels in the caudal ARC were higher at 0.5 hr after lights on than the other two time points, while pCREB-positive cell numbers in the rostral ARC remained unchanged throughout the experimental period. In addition, NPY immunoreactivity in the periventricular nucleus (PeVN) was also higher at 0.5 hr after lights on than the other time points. These results suggest that NPY neurons in the caudal ARC projecting to the PeVN play a role in inhibiting GH pulses at the commencement of the light phase.

KEY WORDS: arcuate nucleus, growth hormone, neuropeptide Y, photic stimulation, pulsatile secretion.

doi: 10.1292/jvms.12-0390; *J. Vet. Med. Sci.* 75(3): 275–281, 2013

It is well known that the secretion pattern of growth hormone (GH) is described as a pulsatile manner in various mammalian species [11, 17]. As well as other pituitary-derived hormones, GH secretion is thought to be under the control of several hypothalamic neuropeptides. From the results of passive immunization, antagonization and *in vivo* administration experiments, GH-releasing hormone (GHRH) and somatostatin (SRIF) have been recognized as major neuropeptides regulating GH pulses [4, 19, 22, 34, 36]. The general hypothesis is that GHRH, the stimulator of GH secretion, triggers the pulsatile secretion of GH, and SRIF, the inhibitor, keeps the baseline at low levels [35]. Furthermore, recent studies suggest that some other peptides, such as neuropeptide Y (NPY) and ghrelin, are also involved in the GH pulse-generating system [11, 37].

There are several factors known to modify the pulsatile secretion of GH. For example, stress is one of the GH-pulse inhibiting stimuli, and glucocorticoids, a major stress-responsive hormone, are thought to suppress GH secretion [31]. Other hormones such as thyroxin and insulin-like growth factor-I also have modifying effects on GH pulses [11]. Photic stimulation is known to be another GH pulse suppressive environmental factor [38]. Davies *et al.* [7] reported that nocturnal photic stimulation inhibited both spontaneous and induced GH secretion in rats and that a similar trough in GH was also observed during the first hour of the normal light phase. They also suggested that SRIF neurons located in the periventricular nucleus (PeVN) may play a role in this transient entrainment by photic stimulation. We also observed that GH pulses did not occur for a certain period of time after lights on in goats (our unpublished observation). Inhibition of GH pulses by photic stimulation may be common among mammalian species and, therefore, could be a good experimental model for analyzing mechanisms underlying GH pulse generation.

NPY, a potent orexigenic neuropeptide, also has a suppressive effect on GH secretion [29]. There are some reports that NPY in the arcuate nucleus (ARC) plays a role in mediating the negative feedback effect of GH, *i.e.* peripheral GH increases NPY release to inhibit its own secretion [2, 9, 18]. In addition, we have previously found a negative correlation between NPY levels in the cerebrospinal fluid and peripheral GH profiles in goats [40]. Moreover, NPY neurons in the ARC are supposed to have anatomical connections with SRIF neurons in the PeVN to suppress GH secretion [8, 15, 20]. These findings led us to hypothesize that NPY in the ARC may be involved in photo-induced suppression of GH pulses. In the present study, to verify this hypothesis, excitability of neurons and NPY gene expression in the ARC were analyzed at around the lights on.

MATERIALS AND METHODS

Animals: Adult male rats of the Wistar-Imamichi strain were obtained from the Institute for Animal Reproduction (Tsuchiura, Japan). The animals were maintained under controlled light (lights on: 1200–2400 hr) and given free access to food and water. The rats were subjected to the experiments described below after at least 2 weeks of habituation to the

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light condition. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Blood sampling: Silastic cannula (Kaneka medics, Tokyo, Japan) was inserted into the right jugular vein of 12 rats under ether anesthesia. The distal end of the cannula was tunneled subcutaneously to the back of the neck. After 2 days of recovery, the cannula was connected to the auto-blood sampling system DR-2 (Eicom, Kyoto, Japan), and kept to pass by intermittent flush for at least 11 hr. A blood sample (150 μl) was withdrawn through the indwelling jugular cannula from freely moving animals at 15-min intervals for 4 hr starting at 1100 hr, *i.e.* 1 hr before lights on. The collected blood samples were allowed to clot overnight at 4°C, and were centrifuged at 9,000 × g for 15 min. The separated serum was stored at −80°C until assayed for GH.

Hormone assay: The serum concentrations of GH were measured with a rat GH RIA kit (NIDDK, Bethesda, MD, U.S.A.). All samples were measured in a single assay, and intraassay coefficient of variation, which was calculated from 18 replicated determinations for the pool of male rat serum containing 1.83 *n*g/m*l* of GH, was 11.13%. The minimum and maximum assay ranges of GH were 0.4 *n*g/m*l* and 400 *n*g/m*l*, respectively.

Brain sample preparation for histological analysis: Brain samples were obtained at 1100, 1230 and 1400 hr. Animals were anesthetized with overdose pentobarbital sodium (50 mg/kg, *i.p.*) and perfused with 4% paraformaldehyde/PBS (pH 7.2), via the left ventricle of the heart. Brains were dissected out and sliced coronally in 4 mm section containing the ARC, and further fixed in the paraformaldehyde solution overnight and then in a solution of 30% sucrose/PBS for 2 days. Frozen sections of 10-μm thickness were made on a cryostat, and one out of every 6 slices was used for immunostaining for NPY, and another one for *in situ* hybridization for NPY. Thirty-μm thickness frozen sections were also made, and one out of every 6 slices was used for floating method immunostaining for phosphorylated-cAMP response element binding protein (pCREB).

Immunohistochemistry: Sections were rinsed with PBS for 10 min 3 times. Sections were then incubated in blocking solution (Block Ace, Snow Brand Milk Products Co., Sapporo, Japan; 1:10 with PBS) for 1 hr. Tissue sections were incubated in primary antibody; anti-NPY (sc-14728; Santacruz, Santa Cruz, CA, U.S.A.; 1:100 with 0.3% Triton X-100 PBS (PBST)), and anti-pCREB (06–519; Millipore, Billerica, MA, U.S.A.; 1:5,000), at 4°C for 72 hr, and then washed 3 times with PBS. Characteristics of these antibodies were described in manufacturer's datasheets. Thereafter, sections were incubated in secondary antibody; Alexa Fluor 488-conjugated anti-goat IgG and 594-conjugated anti-rabbit IgG (Life Technologies, Grand Island, NY, U.S.A.; 1:500 with PBST) for NPY and pCREB, respectively, at room temperature for 2 hr, and washed three times with PBS. Specimens were observed under a fluorescent microscope, and pCREB-immunoreactive (pCREB-ir) cell number in the ARC was counted in the ARC. Since NPY was stained as granular- or fibriform-manner, NPY-positive area in the PeVN was calculated with the image analyzing application Image J (NIH). NPY-positive area was presented as percent of the whole area of the PeVN in each section. The border of each nucleus was defined using the brain map [28].

Generation of cRNA probes and in situ *hybridization*: DNA fragments were isolated from rat hypothalamic cDNAs by PCR. Primers' sequences were quoted from NCBI accession no. NM_012614.1 (forward primer, caagctcattcctcgcagag; reverse primer, gaatgcatgatactttattt). The DNA fragments were subcloned into the pGEM-T vector system (Promega, Madison, WI, U.S.A.). The clone was linearized with the appropriate restriction enzyme (Takara, Otsu, Japan), and antisense RNA probes were generated by *in vitro* transcription using DIG-labeled UTP with T7 RNA polymerase (Roche Diagnostics Inc., Penzberg, Germany). The frozen sections were rinsed in PBS for 5 min twice, then treated with 0.75% Glycine/PBS for 5 min twice, and with 0.3% Tween/PBS for 15 min. After washing twice, sections were digested with 5 mg/m*l* proteinase K in Tris-HCl buffer (pH 7.5) for 30 min at 37°C. Thereafter, sections were postfixed with 4% paraformaldehyde. Then, sections were performed 10 min acetylation and washed with PBS for 10 min 3 times. During preincubation for at least 15 min at 37°C with hybridization buffer (50% formamide, 5 x SSC, 5 x Denharts' solution, 0.1% SDS, 0.1% NLS and 0.5 mg/m*l* ssDNA), hybridization mix was prepared with denatured DIG-labeled RNA probes, 0.5 mg/m*l*. After 12–16 hr of hybridization at 45°C, sections were washed at 52°C and treated with 18 μg/m*l* RNase A (Roche Diagnostics Inc.) for 30 min at 37°C. Following incubation in blocking solution (Block Ace) for 1 hr, the DIG signals were detected by double-antibody immunohistological method using anti DIG-IgG (1:1,000, Roche Diagnostics Inc.) for primary antibody, and Alexa Fluor 488-conjugated anti-sheep IgG (1:500, Invitrogen) for secondary antibody. Specimens were observed under a fluorescent microscope, and positive area in the caudal ARC was calculated with the image analyzing application Image J (NIH). The border of the ARC was defined using the brain map [28], and the ARC was divided into 2 parts (rostral and caudal ARC) at approximately −3.5 mm from the bregma.

Semi-quantitative RT-PCR: Animals were sacrificed under deep ether anesthesia, then brains were dissected out and the caudal ARC was punched out and frozen immediately. Tissues were put into the TRIzol reagent (Invitrogen) to isolate total RNA according to the manufacturer's protocol. Total RNA was converted to cDNA using 2.5 mM Oligo dT primer (Invitrogen) in 20 μl reverse transcription reactions (Superscript 2 RT kit, Invitrogen) and then used for PCR. Oligonucleotide PCR primers' sequences were quoted from NCBI accession no. NM_012614.1 for NPY (forward primer, caagctcattcctcgcagag; reverse primer, gaatgcatgatactttattt), and no. NM_012583.2 for HPRT (forward primer, gaccggttctgtcatgctg; reverse primer, acctggttcatcatcactaatcac) as an internal standard. Using Thunderbird SYBR qPCR kit (Roche Diagnostics Inc.), quantity of DNA was measured according to manufacturer's protocol. Annealing temperatures were 55°C for NPY and 58°C for HPRT, respectively.

Amplification protocol consisted of 35 cycles of denaturing (95°C), annealing and extention (72°C). Diluted series of cDNA samples was used for drawing the standard curve.

Statistical analysis: The data were analyzed by one-way ANOVA followed by Tukey HSD test. Differences were considered significant at $P<0.05$.

RESULTS

Serum GH profiles: Serum GH profiles of all rats examined (n=12) are shown in Fig. 1. Although small fluctuations in GH levels were observed for around 90 min after lights on, GH pulses that had a peak more than 10 *n*g/m*l* were observed only before 1200 hr and after 1330 hr. Based on this observation, we determined the sampling point at 1100, 1230 and 1400 hr, *i.e.* 1 hr before and 0.5 and 2 hr after lights on.

pCREB immunoreactivity in the ARC: To detect the neurons activated after lights on, expression of pCREB in the ARC at each time point was evaluated by immunohistochemistry. Fig. 2A-C shows representative examples of pCREB-positive cells in the caudal ARC at 1100, 1230 and 1400 hr, respectively. Immnoreactivity for pCREB was observed in the nucleus and mainly distributed in the lateral side of the ARC. Although the number of pCREB-ir cells in the rostral ARC remained unchanged throughout the experimental period (Fig. 2D), those in the caudal part of the ARC were significantly increased at 1230 hr as compared with 1100 hr and 1400 hr (Fig. 2E). These results suggest that the neurons in the caudal part of the ARC are transiently activated in response to photic stimulation.

NPY mRNA expression in the ARC: *In situ* hybridization was performed in order to determine the neurons expressing NPY mRNA in the caudal ARC. As shown in Fig. 3A-C, the ARC contained dense NPY mRNA throughout the experiment period. As presented in Fig. 3D, the positive area tended to increase at 1230 hr. NPY mRNA expression in the caudal ARC was also evaluated by semi-quantitative RT-PCR. As shown in Fig. 3E, the level of NPY mRNA was significantly increased at 1230 hr as compared with at 1100 hr, and thereafter returned to the level of the dark phase. Thus, both *in situ* hybridization and semi-quantitative RT-PCR analyses suggest that NPY mRNA synthesis in the caudal part of the ARC was increased 0.5 hr after lights on.

NPY immunoreactivity in the PeVN: To examine the accumulation of NPY, immunostaining for NPY was performed in the PeVN, where NPY nerve terminals are known to connect with SRIF neurons. As shown in Fig. 4, valicocitic NPY-ir granules were observed in the PeVN, and the positive area at 1230 hr was significantly higher than that at 1100 and 1400 hr, suggesting that NPY accumulates at the nerve terminal in the PeVN at 1230 hr.

DISCUSSION

In the present study, GH pulses did not appear for around 1.5 hr at the commencement of the light phase, which is consistent with the report by Davies *et al.* [7] in rats and our previous observations in goats (unpublished). Davies *et al.* [7] suggested that photo-induced suppression of GH secretion is related to the entrainment of the ultradian pattern of GH secretion to the photic environment. They also suggested that this entrainment is transient, since individual profiles of GH secretion are no longer synchronized by the end of the light phase, probably due to individual variation in hypothalamic periodicity. Although there are large species differences in intrinsic inter-pulse intervals in GH secretion, there seems to be common mechanisms among species to suppress GH pulses in response to photic stimulation. Entrainment of ultradian GH secretion to circadian photic environment may be important for animals to accommodate their internal physiological functions such as endocrine function and metabolism to external environment. Animals, regardless of nocturnal (rats) or diurnal (goats) ones, may reset GH periodicity once a day utilizing photic cue as a zeitgeber. Then, we planned to elucidate the involvement of NPY in this mechanism and targeted the ARC, where dense collection of NPY neurons exists [1, 3, 10].

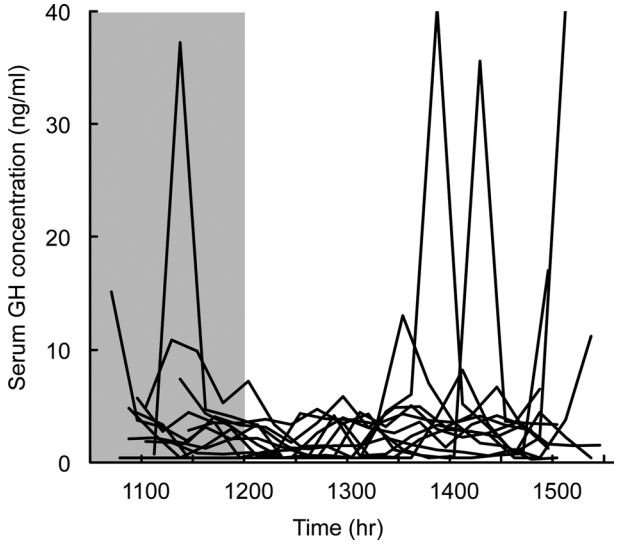

Fig. 1. Profiles of GH secretion in male rats between 1100-1500 hr (lights on: 1200 hr). The data are presented as superimposed profiles of 12 rats used. The shaded area represents the dark phase.

First, neural activity in the ARC was visualized by detecting pCREB in place of generally used c-Fos as a marker of neural excitation. c-Fos is reported as a transcriptional factor, and its expression is upregulated by cAMP- and calcium-dependent cascade [13]. On the other hand, CREB protein gains its binding ability to cAMP response element by phosphorylation of 133th serine, and activation of CREB is triggered by an increase in concentrations of cAMP or calcium ion [6, 25]. In contrast to CREB, the activity of which is regulated by momentary occurring phosphorylation and dephosphorylation, c-Fos takes 30–60 min until its expression and lasts 2–3 hr after neural activation [13, 14, 33]. Using pCREB as a marker, therefore, permits to detect faster processes of neural excitation than using c-Fos [24]. In the present study, since changes of neural activity were expected

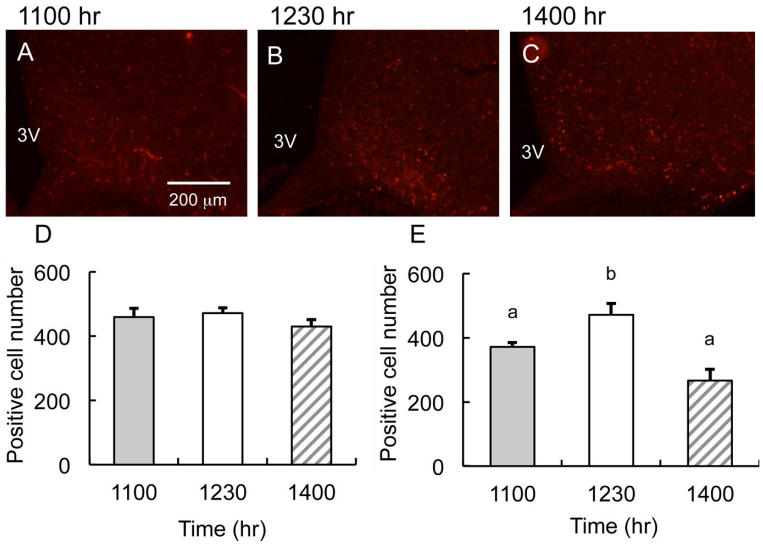

Fig. 2. Immunohistochemistry for pCREB in the caudal ARC (A-C) and accumulated pCREB-ir cells number in the rostral (D) and caudal ARC (E) at 1100, 1230 and 1400 hr. 3V=third ventricle. Each column and vertical bar of accumulated graphs (D and E) represents mean ± SEM (n=6). Values with different letters are significantly different (P<0.05, one-way ANOVA followed by the Tukey HSD test).

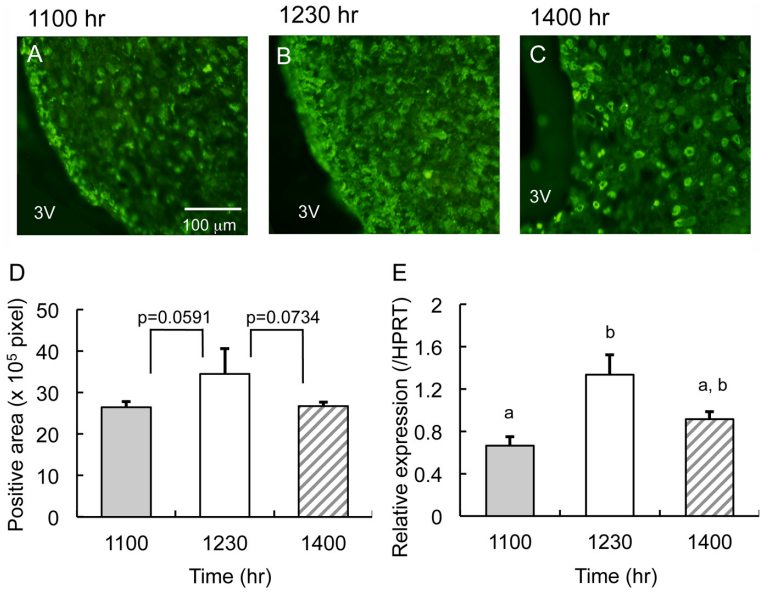

Fig. 3. *In situ* hybridization for NPY mRNA in the caudal ARC (A-C) and summed up NPY mRNA positive area (D) at 1100, 1230 and 1400 hr. 3V=third ventricle. Each column and vertical bar of accumulated graph (D) represents mean ± SEM (n=6). Semi-quantitative RT-PCR analysis for NPY mRNA in the caudal ARC at each time point is also shown (E). Each column and vertical bar represents mean ± SEM (n=5). Values with different letters are significantly different (P<0.05, one-way ANOVA followed by the Tukey HSD test).

to occur immediately after lights on, c-Fos could not be used as a neural activation marker. Although pCREB-ir cells were observed throughout the ARC, changes in the number of them during the experimental period were observed only in the caudal ARC, suggesting that information of the light is transmitted to the caudal part in the ARC. Since NPY neu-

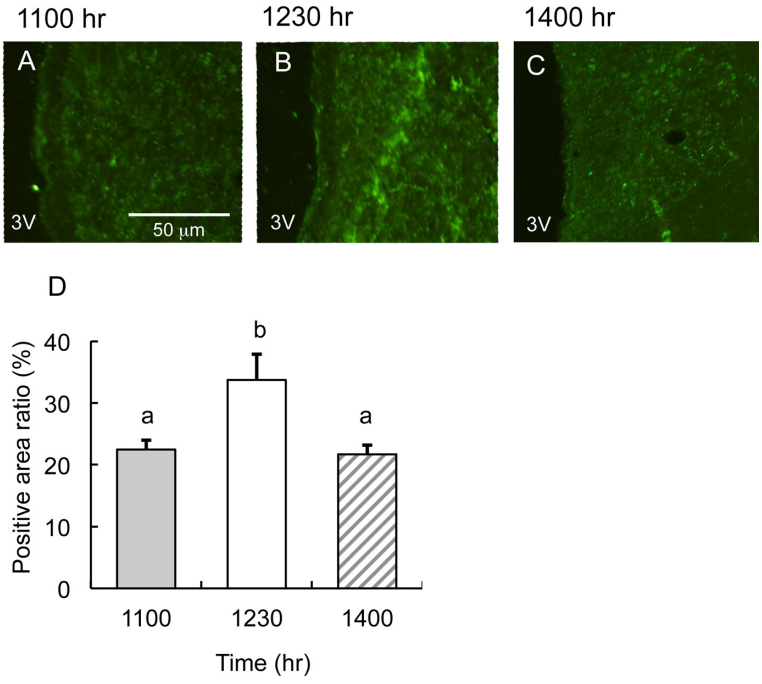

Fig. 4. Immunohistochemistry against NPY in the PeVN (A-C), and summed up NPY mRNA positive area (D) at 1100, 1230 and 1400 hr. 3V=third ventricle. Each column and vertical bar represents mean ± SEM (n=4). Values with different letters are significantly different (P<0.05, one-way ANOVA followed by the Tukey HSD test).

rons were reported to exist from middle to caudal direction of the ARC [1, 3, 10], we postulated that activated neurons in response to photic stimulation in the caudal ARC may contain NPY neurons, which are potentially involved in the suppression of GH pulses.

We then examined gene expression of NPY in the caudal part of the ARC by *in situ* hybridization and semi-quantitative RT-PCR. The analysis by *in situ* hybridization revealed that NPY mRNA-positive area tended to increase at 1230 hr, as compared with 1100 and 1400 hr. The amount of NPY mRNA in punched out tissues evaluated by RT-PCR showed similar but more significant changes. These results suggest that transcription of NPY gene is upregulated 0.5 hr after lights on. It is generally recognized that excitation of neurons synthesizing neuropeptides is associated with transcriptional activation of corresponding neuropeptide genes. Phosphorylation of CREB could be associated with c-Fos expression, and both cAMP response element and AP1 site (the binding site for Fos-Jun complex) are indeed located in the promoter region of the NPY gene [23, 30, 32], suggesting that excitation of NPY neurons leads transcriptional activation of NPY gene in themselves. Thus, the observations in the present study support the notion that photic stimulation induces transient excitation of NPY neurons in the caudal part of the ARC, which is followed by transcriptional activation of NPY gene.

Finally, we analyzed NPY immunoreactivity in the PeVN. In many neurosecretory neurons, synthesized neuropeptides are transported to the axon terminals and accumulated in synaptic vesicles. It is often difficult to stain neuropeptides immunohistochemically in the neural soma without colchicine, which blocks axonal transport [20]. In the present study, we tried to stain NPY immunohistochemically in both the ARC and PeVN, where the neural soma and axon terminals exist, respectively [15], but failed to obtain distinctive NPY-ir cell bodies in the ARC without colchicine. This is the reason why we used *in situ* hybridization to localize NPY neurons in the ARC. In the PeVN, we could detect valicocitic NPY-ir granules, the positive area of which was significantly larger at 1230 hr than the other time points, suggesting that accumulation and release of NPY may transiently increase after lights on. It has been reported that SRIF neurons in the PeVN have Y2 and Y5 receptors for NPY [7, 21], and that NPY works as a GH inhibitor via SRIF [29]. In addition, Davies *et al.* [7] suggested that photo-induced suppression of GH secretion may be mediated by excitation of SRIF neurons in the PeVN. Taken together, it is suggested that photo stimulation is conveyed to NPY neurons in the caudal ARC, which in turn activates SRIF neurons in the PeVN to suppress GH pulses.

In conclusion, we found in the present study that neural exitation and NPY gene expression in the caudal part of the ARC as well as accumulation of NPY in the PeVN take place synchronously at the commencement of the light phase. All these observations are consistent with the hypothesis that photic stimulation activates SRIF neurons in the PeVN via

NPY neurons in the ARC with resultant suppression of GH pulses. Although how photic stimulation is transmitted to the ARC remains to be elucidated, there are several possible neural pathways from the suprachiasmatic nucleus, which receives photic information to integrate circadian rhythm [5], to the ARC, *e.g.* corticotropin-releasing hormone neurons via the periventricular nucleus [12, 16], serotonergic and histaminergic neurons via the raphe and tuberomammillary nucleus [26, 27, 39]. To clarify the communication pathways for photic stimulation to the ARC could consolidate the present hypothesis.

ACKNOWLEDGMENT. This work was supported in part by JSPS KAKENHI Grant Number 23228004.

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