表　題　　光刺激による室傍核 oxytocin ニューロンの活性化と摂食抑制
Light-induced activation of paraventricular nucleus oxytocin neurons and feeding suppression

論 文 の 区 分　　博士課程

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TABLE OF CONTENTS

ABBREVIATIONS

$[Ca^{2+}]_i$: Cytosolic calcium concentration

aCSF: artificial-cerebrospinal fluid

AgRP: agouti-related peptide

ARC: arcuate nucleus

AVP: arginine vasopressin

DM: dorsomedial

DMH: dorsomedial hypothalamus

eGFP: enhanced-green fluorescent protein

CTB: cholera toxin subunit B

HKRB: HEPES-buffered Krebs-Ringer bicarbonate buffer

ICV: intracerebroventricular

IR: immunoreactive

LH: lateral hypothalamus

mRFP: monometric-red fluorescent protein

NPY: neuropeptide Y

NTS: nucleus tractus solitarius

OVT: ornithine-vasotocin

Oxt: oxytocin

Oxt-R: oxytocin receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

PVN: paraventricular nucleus

SCN: suprachiasmatic nucleus

SON: supraoptic nucleus

SPVZ: subparaventricular zone

VIP: vasoactive intestinal polypeptide

VL: ventrolateral

VMH: ventromedial hypothalamus

SUMMARY OF DISSERTATION

Background and Aim

Feeding behavior is regulated by complex processes involving both central and peripheral mechanisms. In rodents, feeding behavior exhibits a diurnal pattern, being highly promoted during dark phase and diminished during light phase. The feeding rhythm could be disrupted by irregular light exposure and thereby promotes metabolic diseases including obesity, diabetes and hypertension. Importantly, these metabolic disorders are also observed in humans who experience extended and/or irregular light exposure, due to the modern lifestyle. These findings suggest that light could entrain feeding behavior and thereby influence metabolism. However, how light could entrain feeding behavior, including feeding inhibition in rodents, remains unclear.

The master clock suprachiasmatic nucleus (SCN) in the hypothalamus functions as the endogenous circadian pacemaker of the body. SCN activity is implicated in maintaining of behavioral and physiological rhythms including feeding rhythm. Irregular and/or extended light exposure impairs the rhythm of SCN activity, and SCN lesion and blockade of SCN neurotransmission promote arrhythmic feeding in rodents. These findings suggest a role of SCN in light-entrained feeding behavior.

It is likely that the light-controlled SCN neurons regulate feeding behavior through the feeding centers located primarily in the hypothalamus. Arginine vasopressin (AVP) neurons in SCN are reported to project to feeding center paraventricular nucleus (PVN). Moreover, the expression of anorexigenic neuropeptide oxytocin (Oxt) in PVN tends to synchrony with light-dark cycle, suggesting its possible link to the light signals. It also has been suggested that activation of oxytocinergic signaling in the PVN profoundly suppresses feeding. Hence, it is intriguing whether the SCN-PVN pathway could be involved in the

light-induced inhibition of feeding. The present study aimed to elucidate the neurocircuit in the hypothalamus that mediates light-induced feeding inhibition with a particular attention to the neurocircuit from SCN AVP to PVN Oxt.

Materials and Methods

Male Wistar rats, Oxt-mRFP1 rats, and AVP-eGFP rats were reared in normal light-dark cycle and fed *ad libitum*. For the purpose of central injection experiments (AVP or Oxt-receptor antagonist injection), animals were stereotaxically cannulated in the lateral ventricle or intra-PVN. In the light exposure experiments, animals were exposed to the white light (200 lux) for 2 h (21:30-23:30) followed by feeding and locomotor activity measurements or for 90 min (21:30-23:00) followed by c-Fos examination. In another experiment, animals were intracerebroventricularly (icv) injected with an Oxt-receptor antagonist (OVT, right before dark phase, 19:30) and exposed to the light and 90 min later for 2 h (21:00-23:00) followed by feeding and locomotor activity measurements. Immunohistochemistry for c-Fos and Oxt was performed using a diaminobenzidine (DAB) immunostaining and immunofluorescent staining. A retrograde tracer study was carried out by injecting cholera toxin subunit B (CTB) into PVN followed by the confocal microscopic observation in SCN. The measurement of $[Ca^{2+}]_i$ in PVN Oxt neurons was performed using the ratiometric fura-2 fluorescent imaging, and the slice patch clamp recording on mRFP1 Oxt neurons in PVN was conducted using an Axopatch 200B patch-clamp amplifier.

Results

In the present study, it was observed that light exposure acutely suppressed food intake and elevated c-Fos expression in the SCN AVP and PVN Oxt neurons. The light-induced

suppression of food intake was abolished by the administration of the Oxt receptor antagonist. Retrograde tracer analysis confirmed the projection of SCN AVP neurons to PVN. Furthermore, intracerebroventricular (icv) injection of AVP suppressed food intake and increased c-Fos in PVN Oxt neurons. Intra-PVN injection of AVP exerted an acute anorexigenic effect than icv injection. Moreover, AVP induced intracellular Ca^{2+} signaling and increased firing frequency of PVN Oxt neurons *in vitro*.

Discussion

Light exposure during dark phase rapidly suppressed food intake and increased c-Fos expressions in SCN AVP neurons and PVN Oxt neurons. Although it is known that light activates SCN neurons including those containing AVP, this is firstly suggested that light activates PVN Oxt neurons. Furthermore, icv injection of an Oxt-R antagonist abolished light-induced suppression of food intake, suggesting that Oxt is involved in this process. Icv injection of AVP increased c-Fos expression in PVN Oxt neurons and suppressed food intake, mimicking the effects of light exposure. Intra-PVN injection of AVP exerted feeding suppression. Moreover, AVP increased $[Ca^{2+}]_i$ and firing frequency in PVN Oxt neurons, indicative of a direct action of AVP on PVN Oxt neurons.

Light exposure during dark phase acutely inhibited food intake is consistent with the previous reports. Regarding the possible mechanism underlying the light-associated feeding inhibition, the present study showed that light-induced feeding inhibition paralleled with the activation of PVN Oxt neuron. Moreover, icv injection of Oxt receptor antagonist abolished the light action to suppress feeding, but partially attenuated the light action to suppress locomotor activity. These results suggest that light-induced feeding inhibition is not simply secondary to suppression of locomotor activity, but may be associated with the activation of Oxt neurons. However, it should be considered that an Oxt-R antagonist (OVT) used in this

study is not a selective antagonist to Oxt-R but also could possibly bind to the AVP receptors. Therefore, the effect of OVT to counteract light exposure on feeding and locomotor activity suppression might be also due to the blockade of AVP action.

The current study confirms the projection of SCN AVP neurons to PVN, in consistent with the previous studies in hamsters, rats, and humans. Thus, SCN neurons could directly act on PVN neurons. Furthermore, it is found that AVP induced Ca^{2+} signaling and increased firing frequency in PVN Oxt neurons *in vitro*. These results extended the previous report that AVP excited PVN neurons *in vitro* and identified the Oxt neuron as a target.

The present study suggests a plausible role of the SCN to PVN neurocircuit in feeding regulation. However, previous studies have mainly suggested the implication of this circuit in glucose metabolism. Hence this recent finding, together with these previous reports, may gain an evidence that the SCN to PVN neurocircuit is involved in the regulation of energy homeostasis with a wider spectrum covering feeding and glucose metabolism.

Conclusion

The current study suggests a neurocircuit from SCN AVP to PVN Oxt that relays light reception to inhibition of feeding behavior.

1. INTRODUCTION

Feeding behavior is regulated by complex processes involving both central and peripheral mechanisms (3, 49). In rodents, feeding behavior exhibits a diurnal pattern, being highly promoted during dark phase and diminished during light phase (45, 50). This normal rhythm of feeding is essential for maintaining energy and metabolic homeostasis (41, 42). The feeding rhythm could be disrupted by irregular light exposure (9, 15, 16). The disrupted feeding rhythm has been suggested to promote metabolic dysregulation and thereby induces obesity and related diseases including diabetes and hypertension (2, 4, 8, 21, 27, 31, 51). Importantly, these metabolic disorders are also observed in humans who experience extended and/or irregular light exposure, due to the modern lifestyle (14, 18, 30, 44, 48, 59). These findings suggest that light could entrain feeding behavior and thereby influence metabolism. However, how light could entrain feeding behavior, including feeding inhibition in rodents, remains unclear.

The master clock suprachiasmatic nucleus (SCN) in the hypothalamus functions as the endogenous circadian pacemaker of the body (47). SCN activity oscillates in synchrony with light-dark cycle (7, 12) and thereby drives the various behavioral and physiological rhythms including feeding rhythm (47). It has been shown that irregular or extended light exposure impairs the rhythm of SCN activity (15, 16, 58). Moreover, SCN lesion and blockade of SCN neurotransmission promote arrhythmic feeding in rodents (38, 46). These findings suggest a role of SCN in light-entrained feeding behavior.

Light reception in the retina is converted to nerve impulses and conducted to the SCN via the optic nerves. The light signal through retinohypothalamic tract directly activates the neurons in the ventrolateral part of SCN predominantly expressing vasoactive intestinal polypeptide (VIP) (12, 55). This signal in the ventrolateral part cascadingly activates the

neurons in the dorsomedial part expressing arginine vasopressin (AVP) (6, 12). The SCN AVP neurons project to several brain regions including hypothalamus (11, 36).

It is likely that the light-controlled SCN neurons regulate feeding behavior through the feeding centers located primarily in the hypothalamus (34, 40, 50), where orexigenic and anorexigenic neurons sense and integrate the central and peripheral signals to generate the appetite or satiety responses (19, 28, 29, 32, 33, 43). SCN neurons are known to project to several feeding-related nuclei in hypothalamus including paraventricular nucleus (PVN) (10, 11). PVN, one of the pivotal feeding centers, is also implicated in some physiological processes. PVN relays the light-entrained SCN signal to pineal gland to induce the release of melatonin (25). PVN also produces corticotropin-releasing hormone (CRH), which drives the release of adrenocorticotropic hormone (ACTH) and glucocorticoid (8, 20). Furthermore, it has been found that the expressions of the anorexigenic nesfatin-1 and oxytocin (Oxt) in PVN oscillate in a diurnal pattern (40, 49). These documents collectively rise a question whether the SCN-PVN pathway could be involved in the light-induced inhibition of feeding.

The present study aimed to elucidate the neurocircuit in the hypothalamus that could mediate light-induced inhibition of feeding behavior. Rats were exposed to light during the dark phase, followed by measurements of food intake and c-Fos expression in the hypothalamic areas involved in light reception and feeding regulation, with particular attention to the SCN and PVN neurons. Furthermore, the neural projection from SCN to PVN was analyzed using retrograde tracer. The results suggest a possible role of the SCN AVP to PVN Oxt neurocircuit in the light-induced inhibition of feeding behavior.

II. MATERIALS AND METHODS

2. 1. Animals

Male Wistar rats (6-9 weeks old; purchased from SLC, Shizuoka, Japan) and the transgenic rats bearing an Oxt-monometric red fluorescent protein 1 fusion transgene (Oxt-mRFP1 rats) (26) and AVP-enhanced green fluorescent protein (AVP-eGFP rats) (56) (6-7 weeks old; obtained from University of Occupational and Environmental Health, Kitakyushu, Japan) were reared in the normal light-dark cycle (light on at 07:30 and off at 19:30) and fed with the standard food chow (CE-2, CLEA, Osaka, Japan) and water *ad libitum*. All of the procedures for animals were accordant with the regulation of Jichi Medical Unversity Institute of Animal Care and Use Committee.

2. 2. Cannulations

Animals were anesthetized with avertin (tribromoethanol, 200 mg/kg, intraperitonial) prior to stereotaxic surgery. Furthermore, a 26-gauge guide cannula (ICM-23G; Intermedical, Osaka, Japan) was inserted unilaterally into lateral ventricle for intracerebroventricular (icv) cannulation (at 0.5 mm caudal to bregma, 1.5 mm lateral from midline and 3.3 mm below the skull surface) or into the PVN for intra-PVN cannulation (at 1.8 mm caudal to bregma, 0.3 mm lateral from midline and 7.3 mm below the skull surface). Animals were allowed to recover from the operation and habituated to the handling for 7-10 days before used for the experiments. The placement of cannula into lateral ventricle was subsequently confirmed after the experiments by injecting the dye (methylene blue; 5μl) through the cannula on the euthanized animals followed by the brain slicing using a razor blade and direct examination of the dye in the lateral ventricle. The placement of cannula into PVN was confirmed by

histological examination using Nissl staining [34]. Data obtained from the incorrectly cannulated rats were excluded from the analysis.

2. 3. Light exposure experiments

Rats were habituated for a week under the normal light-dark cycle. On the day of the experiment, animals were randomly divided into two groups. Both groups were firstly reared in normal dark condition (light off at 19:30), furthermore one group was exposed to the white light (200 lux; white fluorescent lamp, Panasonic, Osaka, Japan) for 2 h (21:30-23:30) and another group was kept in normal dark condition as the control. Food intake was measured each hour using a digital balance and locomotor activity was measured using infrared light-beam censor for activity monitoring system (ACTIMO-100; Shinfactory, Fukuoka, Japan). In another experiment for c-Fos examination, animals were exposed to the light for 90 min (21:30-23:00) followed by transcardial perfusion using 4% paraformaldehyde (PFA) and brain sampling for the immunohistochemical examinations.

2. 4. Central injection of AVP and Oxt receptor (Oxt-R) antagonist

AVP injection: cannulated rats were fasted for 2 h before the dark phase (17:30-19:30). Prior to dark phase (19:15), different groups of animals were injected with AVP (Peptide Institute, Osaka, Japan) via icv (AVP 200 pmol/5µl) or intra-PVN (AVP 20, 60 pmol/0.5µl) and with saline (NaCl 0.9%) as the control. Furthermore, animals were refed immediately and food intake was measured from 0.5 to 12 h after injection. In another experiment, animals were icv injected with AVP (200 pmol/5µl) or saline without refed, followed by transcardial perfusion with PFA 4% 90 min after injection. The brain samples were fixed and processed for the immunohistochemical experiments.

Oxt-R antagonist injection: animals were icv injected with Oxt-R antagonist, $d(CH_2)_5^1$, $Tyr(Me)^2$, Orn^8)-oxytocin (Ornithine vasotocin, OVT, Bachem, Torrance, CA, USA; 9 nmol/5µl) or saline (control) right before the dark phase (19:30). Ninety min post injection, animals were exposed to the light for 2 h (21:00-23:00) while the other groups were kept in the dark as the control, followed by the measurement of food intake and locomotor activity. Hence, in this experiment, light exposure was applied 30 min earlier (at 21:00) as compared to the other light exposure experiments (at 21:30 in feeding and c-Fos expression experiments). This scenario was performed in order to expose the animals to the light precisely at 90 min after Oxt-R antagonist injection.

2. 5. Immunohistochemical staining

The fixed brain samples were cut and processed for immunostaining accordingly to the previous report (29). Single and dual-diaminobenzidine (DAB) immunostaining for c-Fos immunoreactive (IR) and Oxt-IR neurons was carried out by following the procedures as described elsewhere (28-29, 34). A rabbit anti c-Fos antiserum (sc-52, Santa Cruz, CA, USA; 1 : 2000) and rabbit anti Oxt (Millipore, Temecula, CA, USA, 1: 1000) were used as the first antibody for c-Fos and Oxt, respectively, and a biotinylated goat anti-rabbit IgG (Vector Lab, Burlingame, CA, USA; 1: 500) was used as the second antibody. In the immunofluorescent staining for c-Fos, a rabbit anti c-Fos antiserum was used as the first antibody and Alexa fluor 594 donkey anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA; 1:400; red fluorescence) or Alexa fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA-USA; 1:400; green fluorescence) was used as the second antibody. The coronal brain sections containing the representative nuclei of hypothalamus and brain stem were arranged from frontal to caudal region on the slides. Furthermore, the numbers of c-Fos-IR neurons and peptide-IR (or peptide-positive) neurons were counted manually on the bilateral

sides of each nucleus per section (four sections/rat) based on the photographs taken using photomicroscope (Olympus DP71, Olympus, Tokyo, Japan and Fluoreview FV1000-TO; Olympus, Tokyo, Japan). The data were presented as the average value of all sections per group.

2. 6. Retrograde tracer study

A retrograde tracer cholera toxin subunit B (CTB) Alexa Fluor 488 (Invitrogen, Carlsbad, CA-USA; 2% CTB in PBS/0.5µl) was injected unilaterally into PVN of the avertin anesthetized-Wistar rats (7-8 weeks old). The coordinate of PVN positioning was the same as described previously (in animal cannulation's section). Five days later animals were transcardially perfused using 4% PFA. The fixed brain samples were cut and processed for the immunofluorescent staining of AVP using the procedures as described elsewhere (29). A rabbit anti AVP (Millipore, Temecula, CA, USA; 1:5000) was used as the first antibody and a donkey anti-rabbit Alexa 594 IgG (1:400) as the second antibody. The confocal fluorescent images for CTB-labeled neurons and AVP-IR neurons in the SCN were obtained using a confocal laser-scanning microscope (Fluoreview FV1000-TO; Olympus, Tokyo, Japan).

2. 7. Measurement of cytosolic calcium concentration ($[Ca^{2+}]_i$) and immunocytochemical staining for Oxt in isolated PVN neurons

Isolation of single neurons: single neurons were prepared from PVN according to the procedures described previously [28, 34]. Briefly, rats were anesthetized with an ip injection of urethane (ethyl carbamate; 1 g/kg) followed by the brain removal and microdissection of PVN under a dissecting microscope. The PVN slices were washed with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB with 1 mM glucose) and incubated for 15

min at 36^{o}C in HKRB containing 0.75 mg/ml BSA, 0.015 mg/ml DNAase and 20 units/ml papain (Sigma Chemical, St.Louis, MO, USA). The sample was subsequently washed with HKRB and triturated mechanically followed by centrifugation (7x100 rpm at 15^{o}C for 5 min). The yielded pellet was re-suspended in HKRB and distributed onto coverslips followed by incubation in humidified chamber at 30^{o}C for 30 min. The cells were kept at room temperature until $[Ca^{2+}]_i$ measurements.

Measurement of $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ in isolated single PVN neurons was measured by ratiometric fura-2 fluorescent imaging with Argus system (Hamamatsu Photonics, Hamamatsu, Japan) as previously reported (28, 34). Briefly, single neurons were superfused at 1 ml/min with 10 mM HKRB containing 1 mM glucose kept at 33^{0}C for 7 min, and AVP (10^{-9}M) was administered for 5 min. The criteria for $[Ca^{2+}]_i$ responses followed the previous report (34).

Immunocytochemical staining: Immunocytochemical identification of Oxt-IR neurons following $[Ca^{2+}]_i$ measurements was performed as previously described (28, 34). Rabbit anti-Oxt (Millipore, Temecula, CA, USA, 1: 1000) was used as the first antibody and biotinylated goat-anti-rabbit IgG (Vector Lab, 1:400) was used as the second antibody. The correlation of $[Ca^{2+}]_i$ and immunocytochemical data was performed by comparing the phase-contrast photographs of the cells subjected to $[Ca^{2+}]_i$ measurements and the result of immunostaining.

2. 8. Slice patch clamp recording on Oxt-mRFP1 neurons in PVN

The coronal brain slices (300 μm) containing the PVN were prepared from Oxt-mRFP1 rats. After at least 45 min recovery at room temperature in artificial cerebrospinal fluid (aCSF) gassed with 95% O_2 and 5% CO_2, brain slices were transferred to a recording chamber continuously perfused at 2 ml/min with gassed aCSF solution (containing in mM 125 NaCl, 21 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose; pH 7.4). Patch pipettes were filled with internal solution (in mM 128 K-glutamate, 10 KCl, 10

HEPES, 0.1 ethylene glycol tetraacetic acid EGTA, 2 $MgCl_2$, 0.3 NaGTP; pH 7.3 adjusted with KOH). Experiments were carried out at 22-25^{o}C. Brain slice was viewed with a microscope fitted with infrared differential interference contrast (IR-DIC) videomicroscopy (Olympus, Tokyo, Japan). Whole-cell recordings were made using an Axopatch 200B patch-clamp amplifier (Axopatch 200B; Molecular Devices, Foster, CA-USA). Patch pipettes had resistances of 3- 5 ΩM when filled with internal solution. Data were filtered and sampled using digidata 1440A and pCLAMP 10 software. Data were analyzed by Clampfit and Origin 9 software.

2. 9. Data presentation and statistical analysis

Data are presented as mean ± SEM. The ANOVA followed by Bonferroni multiple comparisons post hoc test was applied to compare the multiple test groups and unpaired Student's t-test for two independent groups. The significant value was set at $P < 0.05$.

III. RESULTS

3. 1. Light exposure suppressed food intake and locomotor activity, and increased c-Fos in SCN AVP and PVN Oxt neurons

The present study explores the effect of light exposure during dark phase on food intake, locomotor activity, and c-Fos expression. Light exposure, compared to the control without light, reduced food intake at 1 h ($P < 0.01$; Fig. 1A) and locomotor activity at 1 and 2 h of treatment ($P < 0.01$; Fig. 1B). Furthermore, light also significantly increased the number of c-Fos-IR neurons 2.5 times in SCN ($P < 0.01$; Fig. 1C-D, K), two times in PVN ($P < 0.01$; Fig. 1E-F, K), and 1.5 times in supraoptic nucleus (SON) and nucleus tractus solitarius (NTS) ($P < 0.05$; Fig. 1G-K), compared to control.

In the study of immunohistochemistry, light exposure, compared to control, increased c-Fos expression in SCN AVP-positive neurons in the dorsomedial part of SCN and in non-AVP neurons in the ventrolateral part of SCN (Fig. 2A-L, M). The number of AVP neurons was not significantly different between two groups (Fig. 2N). Light exposure increased the incidence of c-Fos-IR neurons among AVP neurons three times ($P < 0.01$; Fig. 2O), but did not significantly alter the incidence of AVP neurons among c-Fos-IR neurons (Fig. 2P).

Light exposure also increased c-Fos in Oxt neurons of PVN (Fig. 3A-F) and SON (Fig. 3G-L). The number of Oxt neurons was not different between two groups in PVN and SON (Fig. 3M). Light exposure significantly increased the incidence of c-Fos-IR neurons among Oxt neurons 3.6 times in PVN and two times in SON ($P < 0.01$, Fig. 3N), but did not significantly alter the incidence of Oxt neurons among c-Fos-IR neurons in PVN and SON (Fig. 3O).

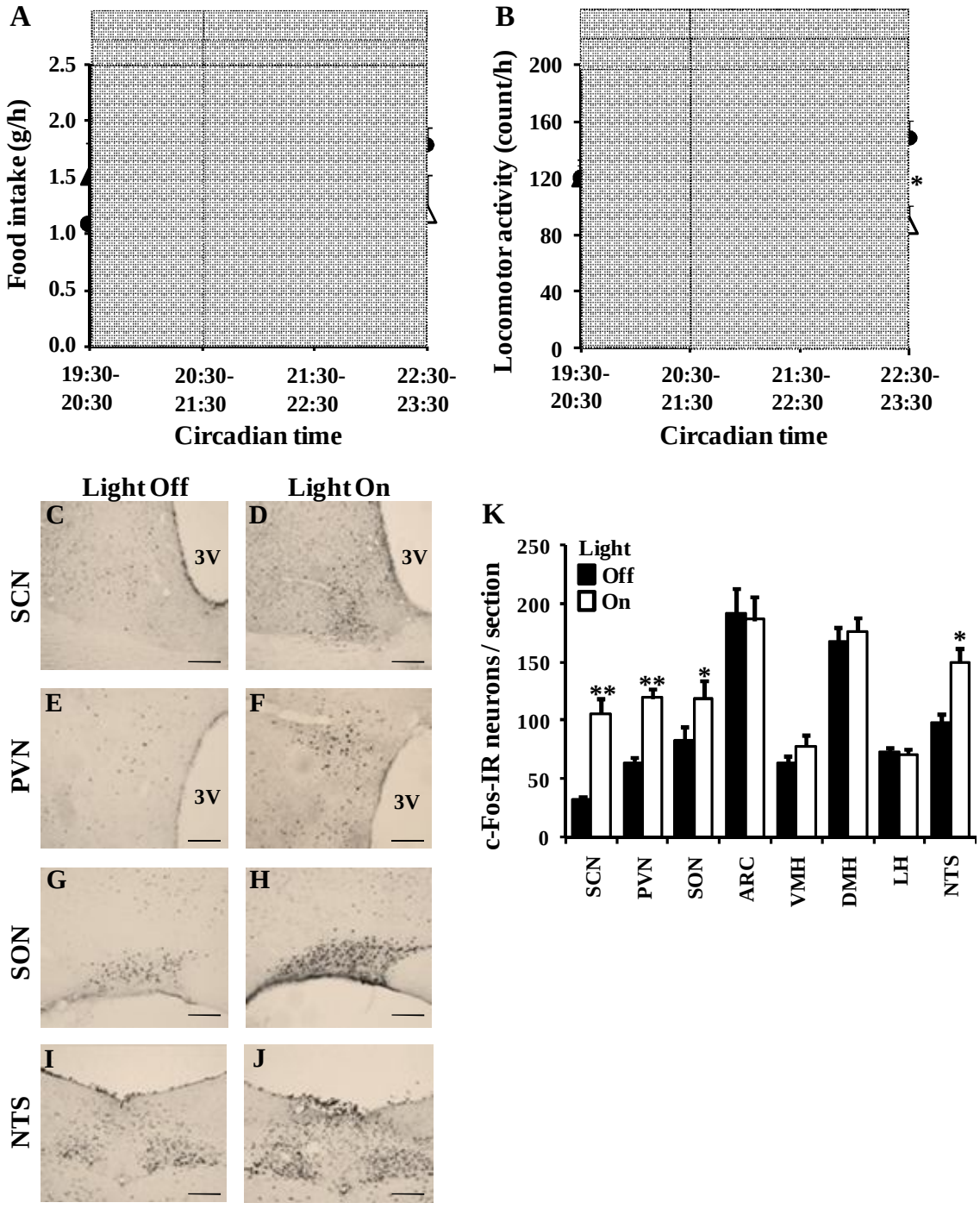

Figure 1. Effect of light exposure during dark phase on food intake, locomotor activity and c-Fos expression in the hypothalamus and brain stem of Wistar rats. ***A-B,*** Food intake (A) and locomotor activity (B) measured for each hour in control group (dark) and light exposure group (2 h light exposure; 21:30-23:30). ***C-J***, Representative pictures of c-Fos expression in the SCN (C-D), PVN (E-F), SON (G-H), and NTS (I-J) both in control group (left panels) and light exposure group (right panels). ***K***, Average number of c-Fos-IR neurons in control (black bar) and light exposure group (white bar) obtained total 4 sections/each nucleus/animal. Bars in A and J represent mean ± SEM. ** $P < 0.01$, * $P < 0.05$ by ANOVA followed by Bonferroni post hoc test for data in A and B, and by Student's t-test for data in K. Scale bars represent 100 µm. n = 5-6 in A and B, n = 5 in K.

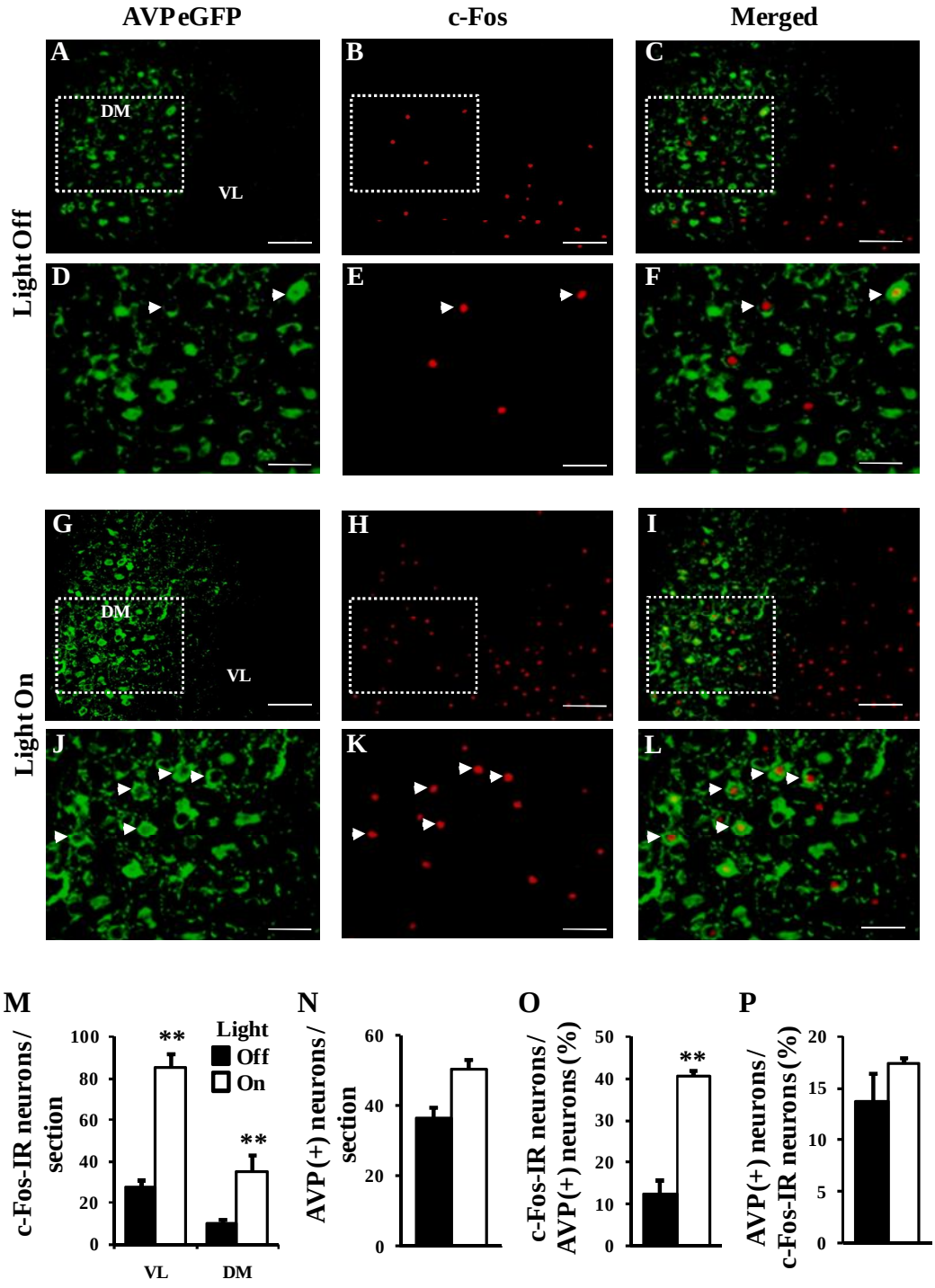

Figure 2. **Effect of light exposure on c-Fos expression in SCN AVP neurons of AVP-eGFP rats.** ***A-L***, Representative pictures depicting fluorescence of AVP-positive neurons (green) and c-Fos-IR neurons (red) in SCN in control (A-F) and light exposure (G-L) groups. ***M***, Number of c-Fos-IR neurons per section in ventrolateral part (VL) and dorsomedial part (DM) of SCN. ***N***, Number of AVP-positive neurons per section. ***O***, Incidence of c-Fos-IR neurons in AVP-positive neurons. ***P***, Incidence of AVP-positive neurons in c-Fos-IR neurons. The white square areas in A-C and G-I represent the expanded portion in D-F and J-L, respectively. The white arrows indicate the colocalization of AVP-positive and c-Fos-IR neurons. Scale bars represent 50 μm in A-C and G-I, and 25 μm in D-F and J-L. Bars represent mean ± SEM. ** $P < 0.01$ by Student's t test. $n = 4$.

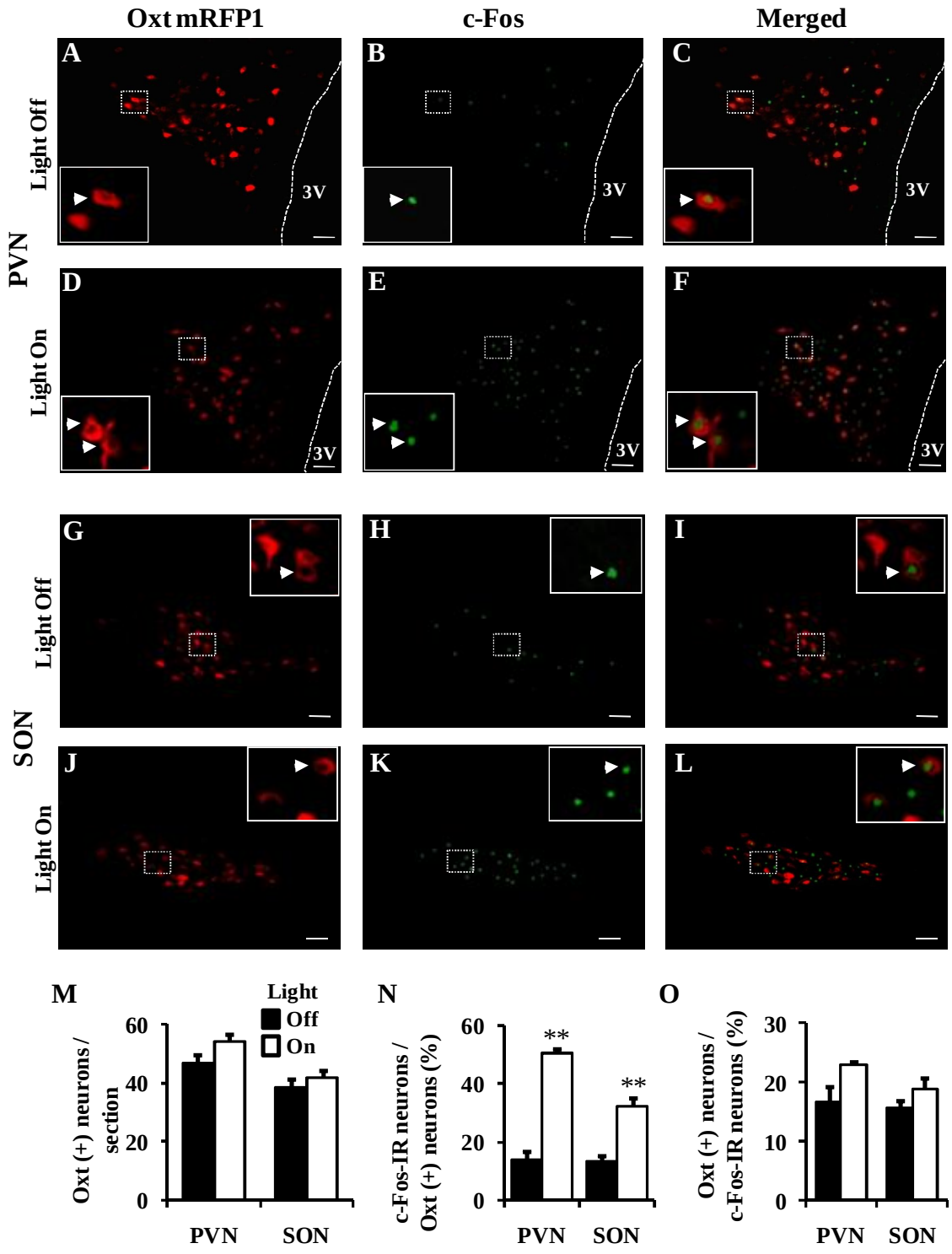

Figure 3. **Effect of light exposure on c-Fos expression in Oxt neurons of Oxt-mRFP1 rats.** ***A-L***, Representative pictures depicting fluorescence of Oxt-positive neurons (red) and c-Fos-IR neurons (green) in PVN (A-F) and SON (G-L) for control and light exposure groups. ***M***, Number of Oxt-positive neurons per section in PVN and SON. ***N***, Incidence of c-Fos-IR neurons in Oxt-positive neurons. ***O***, Incidence of Oxt-positive in c-Fos-IR neurons. Small white square areas in A-L represent the expanded portion as depicted in bigger panels at the corner of each figure. White arrows indicate the colocalization of c-Fos-IR and Oxt-positive neurons. Scale bars represent 50 μm in A-L. Bars represent mean ± SEM. ** $P < 0.01$ by Student's t test. n = 4.

3. 2. Light exposure suppressed food intake in Oxt-dependent manner

To examine whether Oxt is involved in light-induced inhibition of feeding, animals were pretreated with an Oxt-R antagonist (icv; OVT 9 nmol; 19:30) and exposed to the light 90 min later for two hours followed by measurement of feeding and locomotor activity. The result showed that OVT alone did not significantly alter food intake and locomotor activity. Furthermore, in OVT-injected group, light exposure failed to suppress feeding (Fig. 4A) but still partially suppressed locomotor activity (Fig. 4B).

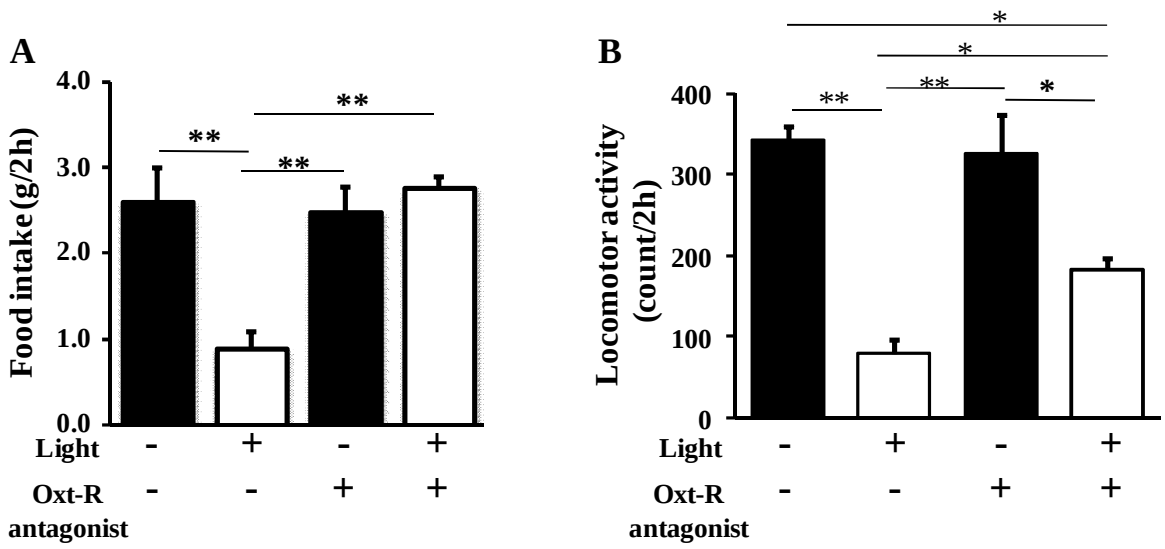

Figure 4. Effect of icv injection of Oxt-R antagonist OVT on food intake and locomotor activity under the light exposure during dark phase. ***A-B,*** Food intake (g/2 h) (A) and locomotor activity (count/2 h) (B) after icv injection of OVT **(**9 nmol/5µl at 19:30, right before the dark phase**)** or saline in dark groups (black bars) and light exposure groups (white bars). Light was applied for 2 h starting at 90 min after OVT injection (21:00-23:00). Bars represent mean ± SEM. ** $P < 0.01$, * $P < 0.05$ by ANOVA followed by Bonferroni post hoc test. n = 5-7 in A and 5 in B.

3. 3. Icv AVP suppressed food intake and increased c-Fos in PVN Oxt neurons

The results that light exposure activated AVP and Oxt neurons as well as suppressed food intake raised a question whether AVP activates PVN Oxt neurons and thereby suppresses food intake. To examine this, the effects of icv AVP (200 pmol/5µl) on food intake and c-Fos expression were examined. Icv injection of AVP, compared to saline, tended to reduce cumulative food intake at 1 h and significantly reduced it at 2 h and 6 h after injection (Fig. 5A). AVP injection also increased the number of c-Fos-IR neurons in the PVN (Fig. 5B vs. C), SCN and NTS (Fig. 5D). In the dual immunostaining experiments, icv AVP induced c-Fos expression in the PVN Oxt-IR neurons (Fig. 5E vs. F). AVP injection did not change the number of Oxt-IR neurons (Fig. 5G), but significantly increased the incidence of c-Fos-IR neurons among Oxt-IR neurons 2.5 times ($P < 0.01$; Fig. 5H) and Oxt-IR neurons among c-Fos-IR neurons two times ($P < 0.01$; Fig. 5I).

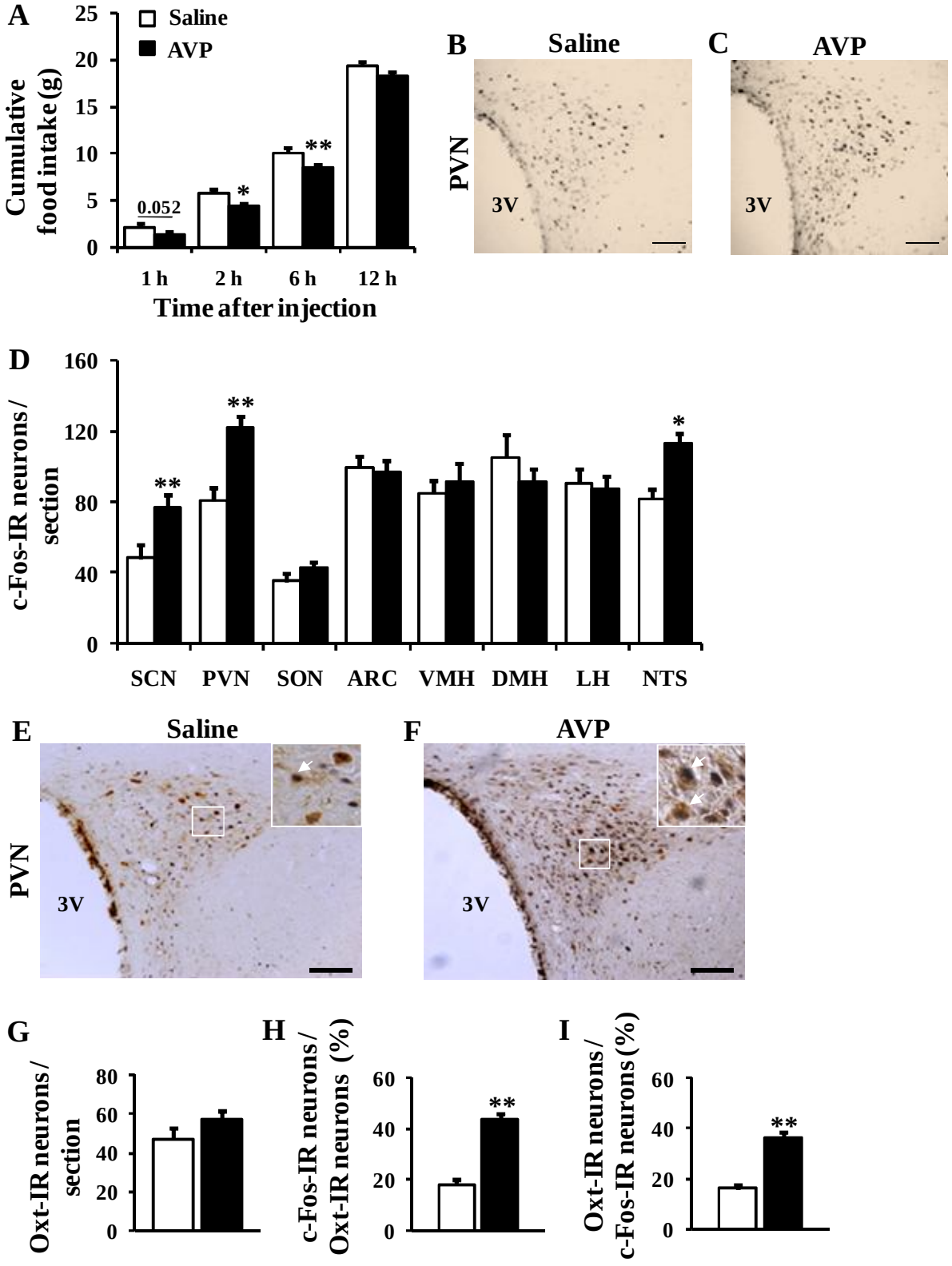

Figure 5. Effects of icv injection of AVP on food intake and c-Fos expression. ***A***, Cumulative food intake in rats after icv injection of saline (control, white bars) and AVP (black bars; 200 pmol/5 µl at 19:30). ***B-C***, Representative pictures showing c-Fos expression in PVN after icv injection of saline (B) or AVP (C). ***D***, Number of c-Fos-IR neurons per section in the hypothalamic nuclei and brain stem in saline group (white bars) and AVP

group (black bars). ***E-F***, Representative pictures showing dual immunostaining for c-Fos-IR and Oxt-IR neurons in the PVN. ***G***, Number of Oxt-IR neurons per section in the PVN. ***H***, Incidence of c-Fos-IR among Oxt-IR neurons in the PVN. ***I***, Incidence of Oxt-IR among c-Fos-IR neurons in the PVN. White square area in E and F represents the expanded portion in upper right panels. White arrows in E and F indicate dual IR neuron for c-Fos and Oxt. Scale bars represent 100 μm. Bars represent mean ± SEM. ** $P < 0.01$, * $P < 0.05$ by ANOVA followed by Bonferroni post hoc test in A, and by Student's t-test in D, G-I. $n = 7$ in A, $n = 5$ in D, G-I.

3. 4. The neural projection from SCN AVP to PVN

To confirm the anatomical projection of SCN AVP neurons to PVN, a retrograde tracer CTB was injected into PVN (Fig. 6A) and subsequently observed its fluorescence in SCN. The CTB fluorescence was detected in the SCN neurons 5 days after injection (Fig. 6B). In the same samples, some of AVP-IR neurons (Fig. 6C) overlapped with the CTB-labeled cells (Fig. 6D). Inversely, as many as 38% of CTB-labeled neurons were AVP-IR neurons (Fig. 6E). These results suggested that SCN AVP neurons project to PVN. To explore the functional role of this AVP projection to PVN, the effect of intra-PVN injection of AVP (Fig. 6F) on food intake was studied. AVP injection reduced food intake at 0.5 h to 6 h in a dose-dependent manner (Fig. 6G).

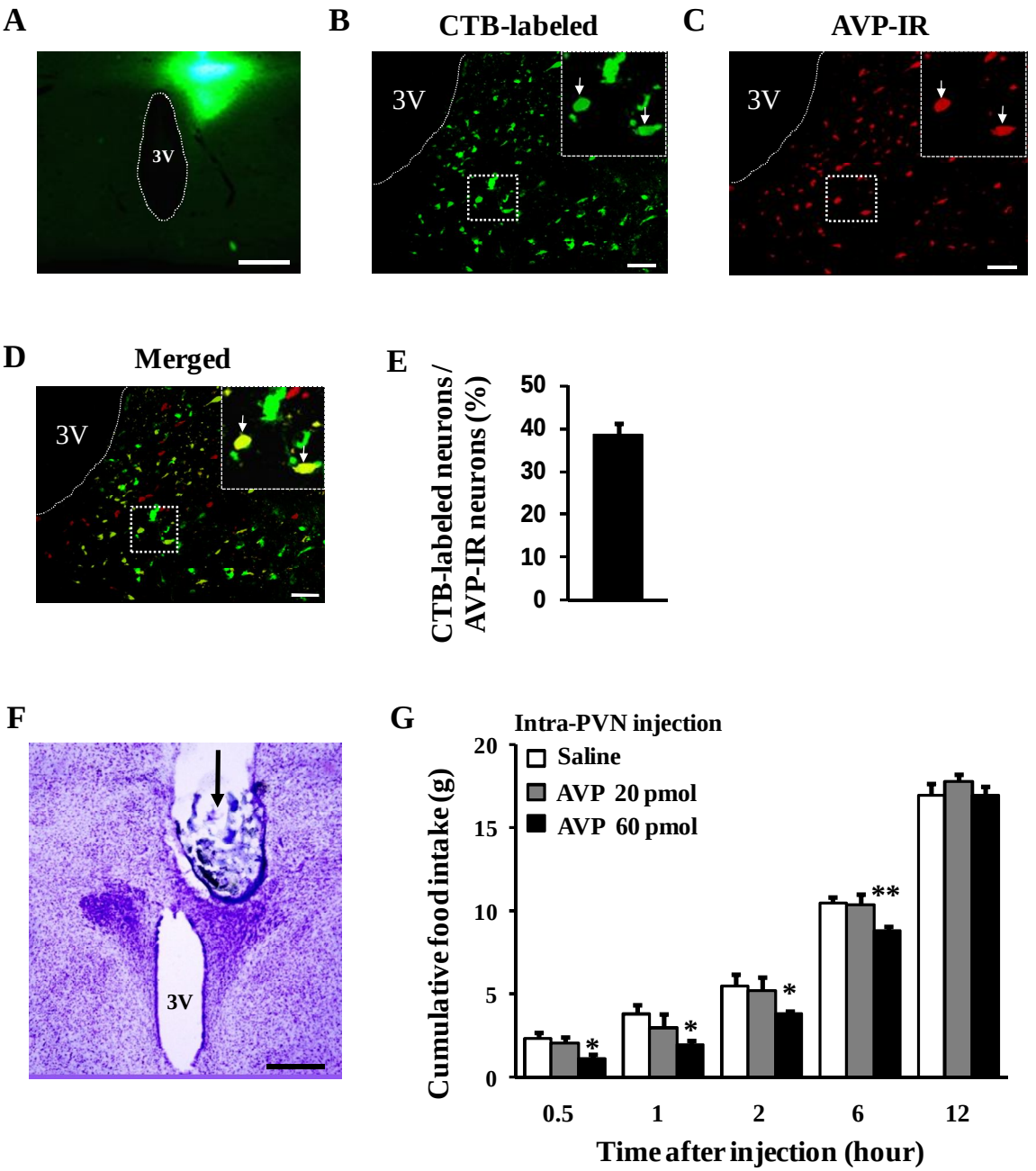

Figure 6. Projection of SCN AVP neurons to PVN, and the anorexigenic effect of intra-PVN injection of AVP in Wistar rats. ***A,*** Representative picture depicting injection site of CTB in the PVN. ***B***, Immunofluorescence of CTB-labeled neurons (green) in SCN examined using confocal microscope 5 days after CTB injection. ***C***, AVP-IR neurons (red) in the SCN of the same sample as in B. ***D***, merged picture of CTB-labeled and AVP-IR showing colocalized neurons (yellow). ***E***, Incidence of CTB-labeled neurons in AVP-IR neurons in the SCN. ***F***, Representative picture of intra-PVN cannulation based on histological examination using Nissl staining. ***G***, Cumulative food intake after intra-PVN injection of saline (white bars) or AVP 20 pmol/ 0.5 µl (gray bars) and AVP 60 pmol pmol/0.5 µl (black bars). Scale bars in A and F represent 50 µm, B-D 25 µm. The small white-marked areas in B-D represent the expanded portion in upper right panels. White arrows in B-D represent the colocalization of CTB-labeled and AVP-IR neurons. Black arrow in F indicates the cannulation site intra

PVN. Bars in E and G represent mean ± SEM. ** $P < 0.01$, * $P < 0.05$ by two-way ANOVA followed by Bonferroni post hoc test in G. n = 3 in E and 6 in G.

3. 5. AVP increased $[Ca^{2+}]_i$ and firing frequency in PVN Oxt neurons *in vitro*

To explore whether AVP directly activates PVN Oxt neurons, the effect of AVP on $[Ca^{2+}]_i$ in isolated PVN neurons from Wistar rats was examined. AVP at 10^{-9} M increased $[Ca^{2+}]_i$ in single PVN neurons that were subsequently shown to be IR to Oxt by immunocytochemistry (Fig. 7A). Forty-two of 118 (36%) PVN neurons examined responded to AVP with $[Ca^{2+}]_i$ increases (Fig. 7B), and a greater fraction, 16 of 33 (48%), of Oxt-IR neurons, responded to AVP (Fig. 7C).

In the slice patch clamp recording, administration of 10^{-8} M AVP increased firing frequency in PVN Oxt neurons from mRFP rats (Fig. 7D). Administration of AVP, compared to aCSF, significantly increased the average of firing frequency in Oxt neurons ($P < 0.05$; Fig. 7E) and depolarized 9 of 13 (69%) Oxt neurons examined (Fig. 7F).

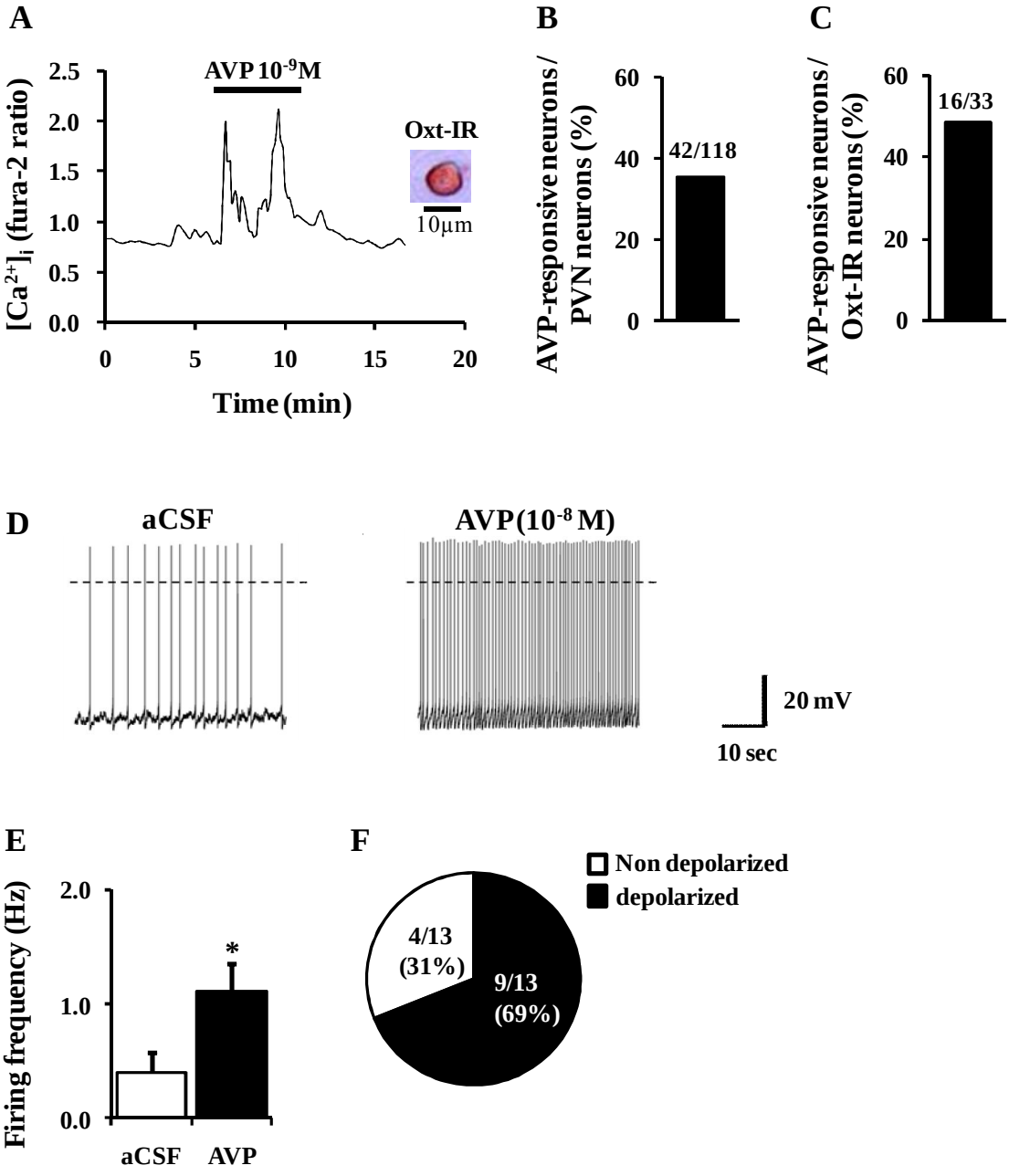

Figure 7. Effects of AVP on the activity of PVN Oxt neurons *in vitro*. *A*, Representative recording of $[Ca^{2+}]_i$ increase under the addition of AVP (10^{-9} M in HKRB containing 1mM glucose) in isolated PVN Oxt neurons of Wistar rats that were subsequently identified as Oxt-IR neurons by the immunocytochemical staining as depicted on the right panel. ***B***, Incidence of AVP-responsive neurons among those of examined PVN neurons. ***C***, Incidence of AVP-responsive neurons among those of Oxt-IR neurons. ***D***, Firing frequency recorded by slice patch clamp in PVN Oxt- mRFP1 neuron under the application of aCSF alone (left panel) and an addition of AVP 10^{-8} M (right panel). ***E***, The average of firing frequency in PVN Oxt neurons under the application of aCSF alone or in addition of AVP 10^{-8} M. ***F***, Proportion of depolarized and non-depolarized PVN Oxt neurons under the application of AVP examined by slice patch clamp recording, presented in percentage. Bars represent mean ± SEM. * $P < 0.05$ by Student's t-test in E. The number above each bar in B and C indicates the number of neurons that responded out of those examined.

IV. DISCUSSION

The present study suggests a possible role of neurocircuit from SCN AVP to PVN Oxt neurons in light-induced feeding inhibition, based on the following results. Light exposure during dark phase rapidly suppressed food intake and elevated c-Fos expressions in SCN AVP neurons and PVN Oxt neurons. Although it has been reported that light activates SCN neurons including those containing AVP, this is firstly suggested that light activates PVN Oxt neurons. Furthermore, icv injection of an Oxt-R antagonist abolished light-induced suppression of food intake, suggesting that Oxt is involved in this process. Based on retrograde tracer study, SCN AVP neurons substantially projected to PVN. Icv injection of AVP increased c-Fos expression in PVN Oxt neurons as well as suppressed food intake, mimicking the effects of light exposure. Moreover, intra-PVN injection of AVP exerted a stronger anorexigenic effect than icv injection. AVP increased $[Ca^{2+}]_i$ and firing frequency in PVN Oxt neurons, indicative of direct action of AVP on PVN Oxt neurons.

The present result that light exposure during dark phase acutely inhibited food intake is consistent with the previous reports (38, 49) that food intake in normal rodents is suppressed during light phase compared to dark phase. Regarding the possible mechanism underlying the light-associated feeding inhibition, the present study showed that the light exposure-induced feeding inhibition paralleled with the activation of PVN Oxt neurons. Moreover, administration of an Oxt-R antagonist abolished the light action to suppress feeding, but partially attenuated the light action to suppress locomotor activity. These results suggest that light-induced feeding inhibition is not simply secondary to suppression of locomotor activity, but may be associated with the activation of Oxt neurons, one of the potent anorexigenic components in the brain (5, 34, 50, 60). However, it should be considered that an Oxt-R antagonist (OVT) used in this study is not a highly selective

antagonist to Oxt-R but could possibly bind to AVP receptors. It has been known that Oxt-R and AVP receptors have similar structures (19). Therefore, the effect of OVT to counteract light-induced suppression of feeding and locomotor activity might be also due to the blockade of AVP action. Further study using a highly selective Oxt-R antagonist is required to clarify this issue. Likewise, the correlation and independence between feeding and locomotor responses to light exposure remain to be clarified more in detail.

The light phase-associated rise of Oxt in the brain and its link to suppression of feeding were previously reported. Icv injection of a non selective Oxt-R antagonist, OVT, significantly increased food intake in mice specifically in light phase (34, 40) but not dark phase (60). Expression of Oxt in the brain tends to be in synchrony with the light-dark cycle (1, 32), being particularly high during light phase compared to dark phase in rodents (40). The present study has extended these previous findings and suggests that Oxt plays a role in coupling the light exposure to feeding suppression.

The current study observed the projection of SCN AVP neurons to PVN, in consistent with the previous anatomical studies in hamsters (37) and humans (11), and with electrophysiological study in rats (10). However, the percentage of SCN AVP neurons that project to PVN was higher as compared to the previous reports. This quantitative difference in SCN AVP projection may be due to species variance between Wistar rats used in this study and hamsters in the previous study (37). Alternatively, this dense population may also include a subset of SCN AVP neurons innervating the subparaventricular zone (SPVZ), an area closely located below PVN and vastly receiving SCN AVP projection (57). A portion of retrograde tracer molecules injected into PVN might also unavoidably spread to SPVZ, due to the close location, and consequently increased the retrograde labeling in SCN AVP neurons.

AVP neurons are also located in the PVN and SON, as well as SCN. Moreover, SON neurons project to PVN (53). Hence, AVP neurons in PVN and SON may also participate in

the light-induced activation of PVN Oxt neurons and feeding suppression. In this study, it was found that light exposure increased c-Fos expression in AVP neurons of PVN and SON to a lesser extent than in SCN (data not shown). Since SCN is directly innervated by retinohypothalamic tract from the eyes, AVP neurons in SCN may be primarily activated by the light signal as compared to those in PVN or SON. Moreover, previous reports have demonstrated that depletion of SCN AVP neurons impaired the activity of PVN neurons in rats (20), and that grafts of SCN in SCN-lesioned mice successfully restored the neuronal activity rhythm of PVN neurons (54). This current finding and the previous reports, taken together, suggest that AVP neurons in SCN may play a major role in light-induced activation of PVN neurons and feeding suppression, while AVP neurons in PVN and/or SON might additionally contribute to these processes.

This study showed that AVP induced $[Ca^{2+}]_i$ signaling and increased firing frequency in PVN Oxt neurons *in vitro*. These results extended the previous report that AVP excited PVN neurons *in vitro* (23) and suggest the Oxt neuron as a target. The AVP type 1A receptor (V1a-R), which is expressed in PVN (6, 22, 32, 39), may mediate the effect of AVP on PVN Oxt neurons. Alternatively, Oxt-R expressed in PVN may facilitate AVP action on Oxt neurons since that AVP is also reported to be affinitive to Oxt-R (19). Further investigation is required to confirm this issue.

In the present study, intra-PVN injection of AVP dose-dependently suppressed feeding, in which a relatively high dose of AVP was required. An expected minimum effective dose of AVP (20 pmol) failed to significantly suppress food intake. The discrepancy in the effective dose could be due to the unilateral, instead of bilateral, intra-PVN injection of AVP, used in this current study. Consequently, AVP might have interacted with only a half of PVN neurons. Therefore, a higher dose of AVP (60 pmol) was required to exert the

significant effect on feeding by unilateral injection. Further study using bilateral injection is needed to examine this speculation.

In the present study, it was observed that light exposure activated several neurons other than SCN AVP and PVN Oxt neurons. Light exposure markedly increased c-Fos expression in neurons located in the ventrolateral part of SCN, possibly including neurons expressing VIP (12). A fraction of SCN VIP neurons project to PVN (11, 37). Hence, the projection from SCN VIP neurons to PVN could be an alternative neurocircuit responding to light exposure. However, this circuit may only additionally participate in light-induced feeding inhibition, since icv injection of VIP alone exerted only a weak anorexigenic effect even at a much higher dose (10 nmol) (17) than that for AVP (200 pmol) used in this study. Pituitary adenylate cyclase-activating polypeptide (PACAP) is also implicated in the SCN activity under the light exposure (13). Moreover, it has been previously reported that PACAP regulates anorexigenic neurons in the PVN (35), suggesting that this putative neurocircuit possibly contributes to feeding suppression. These findings suggest that VIP and/or PACAP could partly contribute to the light-responsive SCN function to inhibit feeding. This might also underly the current results that the magnitude of feeding suppression by intra-PVN injection of AVP was weaker than that by light exposure.

Both light exposure and icv injection of AVP increased c-Fos expression in the NTS of the brain stem. NTS consists of several anorexigenic neurons, including proopiomelanocortin (POMC) neurons (50). It has been previously shown that the PVN oxytocinergic projection to NTS POMC neurons plays a role in suppressing feeding (34). Hence, c-Fos expression in NTS neurons, possibly including POMC neurons, might reflect secondary activation via projection from PVN Oxt neurons.

The present study suggests a role of the SCN to PVN neurocircuit in feeding regulation. However, previous studies have mainly suggested the implication of this circuit in

glucose metabolism. SCN neurons directly modulate the activity of PVN neurons that are connected to the liver via autonomic nervous system, regulating plasma glucose levels (10, 24). SCN and/or PVN lesions cause hepatic insulin insensitivity (8, 9, 13). Mice deficient in AVP receptor V1R exhibit glucose intolerance, possibly due to impaired SCN AVP action on PVN neurons (39). Hence this current finding, together with these previous reports, may gain an evidence that the SCN to PVN neurocircuit is involved in the regulation of energy homeostasis with a wider spectrum covering feeding and glucose metabolism.

In rodents, under the natural circadian setting, light phase is associated with continuous suppression of feeding, compared to dark phase. In the present study, food intake in dark phase was suppressed by light exposure dramatically at 1 h and mildly at 2 h, showing a rather transient, but not continuous, suppression. This difference in the time course of food intake suppression might have resulted from different physiological states of the body at different Zeitgeber time. As a speculated mechanism, expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP), the powerful orexigenic peptides (49), are continuously elevated at the circadian phase associated with dark phase. Hence, the rise in Oxt induced by light exposure during dark phase may be initially efficacious in inhibiting feeding but later counteracted by the action of tonically elevated NPY and AgRP activity (3). However, future study is definitely required to address this issue.

V. CONCLUSION

The present study suggests that the neurocircuit from SCN AVP to PVN Oxt couples light reception to inhibition of food intake. This circuit may be implicated in light-entrained feeding behavior. Moreover, this circuit could be affected by irregular light exposure and consequently induce feeding disorders and associated metabolic diseases.

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