Title: Paraventricular dynorphin A neurons mediate LH pulse suppression induced by hindbrain glucoprivation in female rats

Authors: Hitomi Tsuchida, Parvin Mostari, Koki Yamada, Sae Miyazaki, Yuki Enomoto, Naoko Inoue, Yoshihisa Uenoyama, Hiroko Tsukamura

Institution: Laboratory of Animal Reproduction, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.

Short title: Dynorphin mediates glucoprivic LH suppression

Keywords: dynorphin A receptor; *Kiss1*; malnutrition

***Corresponding author*:** Hiroko Tsukamura, PhD, Laboratory of Animal Reproduction, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan. E-mail: htsukamura@nagoya-u.jp

Financial Support: This work was supported in part by a Grant-in-Aid for a Research Fellow of the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers 18H03973 (to H. T.), 19H03103 (to N. I.) and 20H03127 (to Y. U.).

Disclosure Summary: The authors have nothing to disclose.

Abstract

Malnutrition suppresses reproductive function in mammals, which is considered to be mostly due to the inhibition of pulsatile gonadotropin-releasing hormone (GnRH)/gonadotropin secretion. Accumulating evidence suggests that kisspeptin neurons in the arcuate nucleus (ARC) play a critical role in the regulation of pulsatile GnRH/gonadotropin release. The present study aimed to examine if the hypothalamic dynorphin A (Dyn) neurons mediate the suppression of GnRH/luteinizing hormone (LH) pulses during malnutrition. Ovariectomized rats treated with a negative feedback level of estradiol-17β-treated (OVX+E2) were administered with peripheral (iv) or fourth cerebroventricle (4V) 2-deoxy-D-glucose (2DG), an inhibitor of glucose utilization, to serve as a malnutrition model. Central administration of a Dyn receptor antagonist blocked the iv- or 4V-2DG-induced suppression of LH pulses in OVX+E2 rats. The 4V 2DG administration significantly increased the number of *Pdyn* (Dyn gene)-positive cells co-expressing *fos* in the paraventricular nucleus (PVN), but not in the ARC and supraoptic nucleus (SON), and the iv 2DG treatment significantly increased the number of *fos*- and *Pdyn*-co-expressing cells in the PVN and SON, but decreased it in the ARC. The E2 treatment significantly increased *Pdyn* expression in the PVN, but not in the ARC and SON. Double *in situ* hybridization for *Kiss1* (kisspeptin gene) and *Oprk1* (Dyn receptor gene) revealed that around 60% of ARC *Kiss1*-expressing cells co-expressed *Oprk1*. These results suggest that the PVN Dyn neurons, at least in part, mediate LH pulse suppression induced by the hindbrain or peripheral glucoprivation, and Dyn neurons may directly suppress the ARC kisspeptin neurons in female rats.

Introduction

Reproductive function has been shown to be suppressed during malnutrition in various mammalian species, such as cows (1), sheep (2), goats (3), rats (4), mice (5), and monkeys (6). This suppression is thought to be a strategic adaptation to save energy by avoiding pregnancy, parturition and consequent lactation during malnutrition. In fact, delayed puberty onset, decreased pregnancy, and lowered fertility rates have been reported in malnourished and fasted rats (7), and pulsatile luteinizing hormone (LH) secretion is inhibited in female rats by 48-h fasting (8).

As an experimental model of malnutrition, 2-deoxy-D-glucose (2DG), an inhibitor of glucose utilization, has been used to investigate the effect of glucoprivation on reproductive function in mammals. Peripheral administration of 2DG combined with fasting disturbed estrus cycles and behavior in Syrian hamsters (9). LH pulse frequency was transiently decreased by a single intravenous (iv) or intracerebroventricle (icv) injection of 2DG in sheep (10). Further, female rats peripherally administered with iv 2DG showed suppression of pulsatile LH release, resulting in a decrease in the mean plasma LH concentrations and frequency and amplitude of LH pulses (11). Importantly, sex steroids negative feedback is suggested to be involved in fasting- or 2DG-induced suppression of LH pulses. Food deprivation for 48 h suppressed LH pulses in intact cycling or estrogen-replaced ovariectomized female rats or androgen-replaced castrated male rats, but not in gonadectomized (GDX) male and female rats (4,12). Further, peripheral 2DG administration strongly suppressed LH pulses in GDX male or female rats when they were treated with estrogen (for females) or androgen (for males), but moderately suppressed in the GDX rats without steroid replacement (11). These results suggest that sex steroids enhance the suppressive effect of fasting or glucoprivation on pulsatile LH secretion in both male and female rats (4,11).

An administration of 2DG into the fourth ventricle (4V) suppressed LH pulses in male rats

(13), and an *in vitro* study by Moriyama et al. (14) showed that lowered glucose availability increased intracellular Ca^{2+} levels in the ependymocytes taken from the hindbrain of rats. An infusion of 5-thio-D-glucose (5TG), another glucose utilization inhibitor, into the 4V increased food intake and blood glucose level in rats, whereas 5TG infusion into the lateral ventricle (LV) failed to induce changes in food intake and blood glucose when the cerebral aqueduct was obstructed with silicon grease, indicating that a glucose sensor in the hindbrain mainly senses lowered glucose availability to control blood glucose levels and feeding behaviors (15). Taken together, these studies suggest that the malnutrition signal, at least in part, sensed by the ependymocytes surrounding the 4V, is transmitted to the hypothalamus to induce counterregulatory responses in feeding, gluconeogenesis and reproduction. The malnutrition signal would be conveyed to the hypothalamic center for gonadotropin-releasing hormone (GnRH) release to inhibit pulsatile LH release via an inhibitory neuronal pathway. Indeed, a recent tracing study showed that wheat germ agglutinin (WGA), a neuronal tracer, was found in the several hypothalamic nuclei, such as the paraventricular nucleus (PVN), supraoptic nucleus (SON), as well as the arcuate (ARC) kisspeptin neurons, a putative center for tonic GnRH/gonadotropin release, when the WGA was injected into the 4V in female rats (16). Further, a subcutaneous administration of 2DG increased expression of Fos, a marker for neural activation, in the PVN (17), suggesting that PVN originating neurons may mediate the glucoprivic suppression of LH secretion. Indeed, previous studies have suggested that the PVN corticotropin-releasing hormone (CRH) neurons mediate fasting-induced or glucoprivic inhibition of LH pulses in female rats (18,19).

Dynorphin A (Dyn), an endogenous opioid peptide, and its receptor, namely kappa-opioid receptor (KOR), may mediate LH pulse suppression during malnutrition. Dyn neurons are reportedly located in the hypothalamic nuclei, including the PVN, ARC and SON (20,21), and a central KOR antagonism increased LH release in female rats in an estrogen-dependent

manner (22). The KOR is reportedly located in the whole brain including in the hypothalamic nuclei such as the PVN, ARC and SON (23). An intraperitoneal injection of a KOR antagonist, nor-binaltorphimine (nor-BNI), increased plasma LH levels in 72-h fasted male rats (24), suggesting that Dyn may be involved in suppression of LH secretion during malnutrition. A part of the ARC Dyn neurons are known to co-express kisspeptin and neurokinin B (NKB) in rats, goats and ewes, therefore, they are referred to as KNDy neurons (25–27). These KNDy neurons have been suggested to be involved in GnRH/LH pulse generation and estrogen negative feedback on GnRH/LH pulses (28–32) and a study reported that KOR-immunoreactivities were found in the KNDy neurons in ewes (33). For further understanding of the central mechanism responsible for the suppression of reproductive function during malnutrition, it is worthwhile to clarify if central Dyn-KOR signaling mediates malnutrition-induced suppression of pulsatile LH release via affecting the ARC KNDy neurons.

The present study, therefore, aims to determine whether Dyn-KOR signaling mediates the glucoprivic suppression of pulsatile LH secretion in ovariectomized rats treated with a negative feedback level of estradiol-17β (OVX + low E2). To address this issue, we examined if third ventricle (3V) administration of nor-BNI, a KOR antagonist, blocks the suppression of pulsatile LH secretion induced by iv treatment of 2DG in OVX + low E2 rats to determine the involvement of central Dyn-KOR signaling in the peripheral glucoprivic suppression of LH pulse. We also investigated whether the 3V nor-BNI administration reverses the LH pulse suppression induced by 2DG infusion into the 4V in OVX + low E2 rats to clarify the role of KOR signaling in mediating LH pulse suppression specifically induced by hindbrain glucoprivation. Then, we investigated the effect of iv or 4V 2DG administration on Fos gene (*fos*) expression in the Dyn gene (*Pdyn*)-expressing cells in the hypothalamic nuclei, such as the PVN, ARC, and SON to determine if these Dyn neurons are activated by the peripheral or hindbrain glucoprivation in OVX + low E2 rats. We also investigated whether 4V 2DG

administration affects kisspeptin gene (*Kiss1*) and kisspeptin protein expression in the ARC and whether low E2 treatment affects *Pdyn* expression in the PVN, ARC, and SON of OVX rats. Further, we performed double *in situ* hybridization (ISH) for the *Kiss1* and KOR gene (*Oprk1*) in the hypothalamus to investigate if the ARC kisspeptin neuron is a possible site of Dyn action in rats.

Materials and methods

Animals

Adult Wistar-Imamichi strain female rats (8-12 weeks old, 190-230 g; Institute for Animal Reproduction, Kasumigaura, Japan) were housed in a controlled environment (14-h light, 10-h darkness with lights on at 0500 h, 22 ± 3°C) and had free access to food (CE-2; CLEA Japan, Tokyo, Japan) and water until the day of brain or blood sampling. Female rats having shown at least two consecutive estrous cycles were bilaterally ovariectomized. Some of these rats were implanted with subcutaneous Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 25 mm in length; Dow Corning, Midland, MI, USA) containing E2 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in peanut oil (Sigma-Aldrich) at 20 μg/ml to serve as OVX + low E2 rats. The E2 treatment was confirmed to show negative feedback to LH pulses but not to induce LH surge (34). These surgical procedures, if not otherwise specified, were performed under ketamine (27 mg/kg)/xylazine (5.3 mg/kg) mixture and inhalant 1-2% isoflurane (Pfizer Japan, Tokyo, Japan) anesthesia. The present study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Blood sampling to determine the effect of central KOR antagonism on peripheral/hindbrain glucoprivic suppression of LH pulses

OVX + low E2 rats were stereotaxically implanted with stainless-steel guide cannulae (22G;

Plastics ONE, Roanoke, VA) into the 3V (n=19) or both the 3V and 4V (n=24) according to the rat brain atlas (35) as follows: at 0.8 mm posterior and 7.5 mm ventral to the bregma at midline for the 3V and 12.5 mm posterior and 7.9 mm ventral to the bregma at midline for the 4V seven days before the day of blood sampling to determine the effect of 3V administration of the KOR antagonist on the suppression of LH pulses induced by iv or 4V 2DG administration. The OVX + low E2 rats subjected to blood sampling were inserted with a silicon cannula (inner diameter, 0.5 mm; outer diameter, 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) into the right atrium through the jugular vein on the day before blood sampling. The animals were deprived of food for 3-h during the blood sampling period to ensure that plasma LH and glucose concentrations would not be affected by food intake. The free-moving conscious rats were administrated with nor-BNI (Sigma-Aldrich, a selective KOR antagonist), dissolved in ultrapure water at 10 μg/μl, into the 3V at the flow rate of 1 μl/min for 2 min by a microsyringe pump (EICOM, Kyoto, Japan) through an internal cannula (28G; Plastics ONE) immediately after the first blood sampling collected via the jugular vein cannula. Nor-BNI was dissolved immediately before used, and ultrapure water only was used in vehicle-treated control rats. Immediately after the nor-BNI administration, 2DG (400 mg/kg BW, Sigma-Aldrich) dissolved at 200 mg/ml in saline, or equimolar hypertonic concentration of xylose (366 mg/kg BW, Katayama Chemical Industries, Osaka, Japan), an indigestible sugar for rats, dissolved at 183 mg/ml in saline were intravenously injected and 100 μl of blood samples were collected for 3 h at 6 min intervals (n=4 for 2DG (iv)-nor-BNI (3V)-injected group; n=5 for the other groups). The doses of 2DG and xylose were chosen according to a previous study (11), showing that LH pulses were significantly suppressed by iv 2DG (400 mg/kg BW) treatment in OVX + E2 rats. As for the hindbrain glucoprivation, the rats were infused with 2DG (30 mg/kg BW, ~6 mg/animal dissolved in saline) or equimolar hypertonic concentration of xylose as a control (27.45 mg/kg BW, ~5.49 mg/animal dissolved in saline) into the 4V (n=6

for each group) at the flow rate of 0.2 µl/min for 1 h (12 µl in total volume for individuals) with a microsyringe pump according to a previous study (13), indicating that the 4V 2DG infusion significantly suppressed pulsatile LH release in testosterone-implanted castrated male rats and that the flow rate did not allow the drag solution flow to the forebrain cerebral ventricles. The 2DG dose was chosen based on our preliminary study, in which 2DG at 30 mg/kg BW was confirmed to suppress LH pulses in OVX + low E2 rats. Blood samples (100 µl) were collected just before the 3V nor-BNI and 4V 2DG administration and then collected every 6 min for 3 h. Red blood cells taken from donor rats and washed with saline were replaced at each blood collection to keep the hematocrit level constant. Plasma samples (50 µl) were obtained by immediate centrifugation and stored at -20°C until assayed for LH. Plasma glucose concentrations were measured in an additional volume (50 µl) of blood samples obtained every 12 min during the first 1 h and every 30 min during the last 2 h of the blood sampling period, as previously described (36). Immediately after the end of the blood sampling, animals were allowed to access food for 0.5 h and then the amount of food intake was measured to determine the effect of 2DG administration on feeding.

Assays for plasma LH and glucose concentrations

Plasma LH concentrations were measured by a double-antibody radioimmunoassay (RIA) as previously described (37) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA) and were expressed in terms of NIDDK rat LH-RP-3. The least detectable level in 50-µl plasma samples was 0.078 ng/ml, and the intra- and inter-assay coefficients of variation were 7.3% and 10.4% at 0.89 ng/ml, respectively. Plasma glucose concentrations were measured by the glucose oxidase method using a commercial kit (Glucose C-Test; Wako Pure Chemical Corporation, Osaka, Japan), as previously described (36). The least detectable level in 1.5-µl plasma samples was

0.25 mg/ml, and the intra- and inter-assay coefficients of variation were 2.6% and 6.4% at 1.244 mg/ml, respectively.

Brain sampling for histological analysis

To determine the effects of 4V or iv administration of 2DG on *fos* expression in hypothalamic Dyn neurons, some OVX + low E2 rats (n=8) were implanted with a stainless-steel guide cannula into the 4V and then inserted with iv cannula into the right atrium as described above. Seven days after the brain surgery free-moving conscious OVX + low E2 rats were infused with 2DG (~6 mg/12 µl saline, 0.2 µl/min for 1 h) or xylose (~5.49 mg/12 µl saline, 0.2 µl/min for 1 h) into the 4V (4V 2DG, n=4; 4V xylose, n=4). Some OVX + low E2 rats without brain surgery were acutely injected with iv 2DG (400 mg/kg BW) or xylose (366 mg/kg BW) (iv 2DG, n=3; iv xylose, n=3) through iv cannula attached on the previous day. Animals were deprived of their food after the 4V or iv 2DG/xylose administration until the brain sampling to avoid the effect of feeding on expression of the genes mentioned above. The animals were then deeply anesthetized 1 h after the 4V or iv 2DG/xylose administration with sodium pentobarbital (40 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and perfused with 4% paraformaldehyde (Sigma-Aldrich) in 0.05 M phosphate buffer (PB). The brain was immediately removed from the skulls and postfixed with the same fixative at 4ºC overnight then immersed in 30% sucrose in 0.05 M PB at 4ºC. Frozen sections (50-µm thickness) of the hypothalamus including the PVN, ARC and SON were obtained with a cryostat. The brain sections taken from OVX + low E2 rats treated with iv or 4V 2DG/xylose were subjected to a double ISH for *Pdyn*/*fos*. Hypothalamic sections taken from OVX + low E2 rats infused with 4V 2DG or xylose were subjected to a double ISH for *Kiss1*/*Oprk1* (2DG, n=3; xylose, n=3). The hypothalamic sections of OVX + low E2 rats treated with 4V 2DG or xylose were also subjected to a single ISH for *Kiss1* (2DG, n=4; xylose, n=4) or immunohistochemistry for

kisspeptin (2DG, n=4; xylose, n=4). Further, brain sections of OVX rats with or without low E2 treatment (OVX, n=3; OVX + low E2, n=3) were also collected as the same manner and subjected to a single ISH for *Pdyn* in order to investigate whether *Pdyn* expression in the hypothalamus is affected by the E2 treatment.

Double ISH for *Pdyn*/*fos* or *Kiss1*/*Oprk1,* and single ISH for *Kiss1* or *Pdyn* in the rat hypothalamus

Free-floating double ISH for *Pdyn* and *fos* was performed using every fourth section (=every 200 μm) of the hypothalamus including the PVN, ARC and SON as previously described (38). The *fos*-specific digoxigenin (DIG)-labeled complementary RNA (cRNA) probe (position 573-1193; GenBank accession number NM_022197) was designed and synthesized from the rat whole hypothalamic cDNA by using a DIG-labeling kit (Roche Diagnostics, Basel, Switzerland). The *Pdyn*-specific fluorescein isothiocyanate (FITC)-labeled cRNA probe (position 315-731; GenBank accession number NM_019374) were designed and synthesized from the rat whole hypothalamic cDNA using a FITC-labeling kit (Roche Diagnostics). The brain sections were hybridized with 1 μg/ml anti-sense cRNA probes at 60^{o}C overnight. Hybridized sections were incubated with a peroxidase (POD)-conjugated anti-FITC antibody (Roche Diagnostics) (39) and tyramide signal amplification (TSA) Plus FITC Kit (1:100; Perkin Elmer, Waltham, MA) to detect the *Pdyn* probe. After inactivation of the peroxidase by incubating the sections in 0.1 N hydrochloric acid for 30 min, the *fos* probe was detected using the POD-conjugated anti-DIG antibody (40), TSA Plus Biotin Kit (1:100) and Dylight 594-conjugated streptavidin.

Double ISH for *Kiss1* and *Oprk1* was performed using every fourth section of the ARC in the same manner. The *Oprk1*-specific DIG-labeled cRNA probes (position 66-768, 805-1956; GenBank accession number NM_017167) and *Kiss1*-specific FITC-labeled cRNA probe

(position 33-348; GenBank accession number NM_181692) were designed and synthesized as described above. After the hybridized FITC-labeled *Kiss1* probe was visualized with the POD-conjugated anti-FITC antibody, TSA Plus FITC Kit, the hybridized DIG-labeled *Oprk1* probe was visualized with the POD-conjugated anti-DIG antibody and TSA Plus Biotin Kit and Dylight 594-conjugated streptavidin. No positive signals for *fos*, *Oprk1* and *Pdyn* mRNA were detected in the brain sections hybridized with the corresponding sense probe as a negative control (data not shown) and we previously confirmed that no *Kiss1* positive signal was detected in the brain sections hybridized with the corresponding sense probe (41). Fluorescence images were obtained on an ApoTome fluorescence microscope (Carl Zeiss, Oberkochen, Germany), and the number of each gene-expressing cells were counted unilaterally in the PVN (every 200 μm from 0.96 to 1.92 mm posterior to the bregma, totally 4 sections), ARC (every 200 μm from 1.72 to 4.36 mm posterior to the bregma, totally 13 sections) and SON (every 200 μm from 1.20 to 1.80 mm posterior to the bregma, totally 3 sections) on the photographs according to the rat brain atlas (35). The cell number was counted twice in each section by 2 investigators and then the total number of each gene-expressing cells in each nucleus was averaged in the groups.

A free-floating single ISH for *Kiss1* or *Pdyn* was performed by using every fourth hypothalamic section including the ARC and PVN as previously described (37). The *Kiss1*- or *Pdyn*-specific DIG-labeled cRNA probe was designed and synthesized as described above. The hybridized DIG-labeled probe was detected with an alkaline phosphatase-conjugated anti-DIG antibody (42) (1:1000; Roche Diagnostics) for 2 h for *Kiss1* or *Pdyn* at 37°C. The sections were treated with a chromogen solution (338 μg/ml 4-nitroblue tetrazolium chloride, 175 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate) until a visible signal was detected. The brain sections were then mounted and the signals were examined by an optical microscope (BX53; Olympus, Tokyo, Japan). The number of *Kiss1*-expressing cells in the ARC and *Pdyn*-expressing cells in

the PVN, ARC and SON were counted unilaterally by 2 investigators in duplicate as described above and averaged. The intensity of *Kiss1* or *Pdyn* mRNA signals in a digital photomicrograph of the brain sections of each rat were quantitatively analyzed using ImageJ software (version 1.50i; http://imagej.nih.gov/ij/). Each of the nuclei was outlined in the gray-scale image and processed for intensity measurement. Nonspecific background intensity was eliminated using the same threshold for each rat. The intensity was calculated as integrated density, i.e., the sum of the 8-bit gray-scale values of all the pixels in the threshold area.

Immunohistochemistry for kisspeptin

Every fourth section through the ARC (from 1.72 to 4.36 mm posterior to the bregma) taken from the OVX + low E2 rats administered with 4V 2DG or xylose, were subjected to immunohistochemistry for kisspeptin. Brain sections were incubated with the anti-kisspeptin rabbit polyclonal antibody (C2, 1:2000, kindly donated by Dr. H. Okamura) (43–45) for 48 h at 4ºC, followed by an incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (46) (1:200; Thermo Fisher Scientific). The specificity of the anti-kisspeptin antibody was previously described (43). The sections were mounted and fluorescence images were obtained with the ApoTome fluorescence microscope. Quantitative analysis for intensity of kisspeptin-immunoreactive signals in a digital photomicrograph of the ARC of each rat was performed using ImageJ software as described above.

Statistical analysis

LH pulses were identified by the PULSAR computer program (47) as previously described (48), the mean LH concentrations and the frequency and amplitude of LH pulses for the 3-h blood sampling period were calculated for each individual and then for the group. Statistical differences in the mean LH concentrations, the frequency and amplitude of LH pulses, the area

under the curve (AUC) of plasma glucose level and amount of food intake between the 2DG (iv or 4V)- and xylose (iv or 4V)-treated groups administered with nor-BNI (3V) or vehicle (3V) were determined by two-way (2DG and nor-BNI treatments as main effects) ANOVA followed by analysis of simple main effects using SAS University Edition (https://www.sas.com/). Mean LH concentrations and the frequency and amplitude of LH pulses were log transformed prior to analysis to normalize their distribution. Statistical differences in plasma glucose level between the groups were determined by three-way repeated measures (2DG, nor-BNI treatments and time as main effects) ANOVA followed by analysis of simple main effects. Statistical differences in the number of the *Pdyn*-expressing cells with or without *fos* expression, total *fos*-expressing cells and *fos*-expressing *Pdyn*-negative cells, and the percentage of the *Pdyn*- and *fos*-co-expressing cells out of the total *Pdyn*-expressing cells in the PVN, ARC and SON between 2DG- and xylose-treated rats were determined by the Welch *t*-test. Statistical differences in the number of *Kiss1*-expressing cells and the *Kiss1*- and *Oprk1*-co-expressing cells, the intensity of *Kiss1* mRNA signals and kisspeptin immunoreactivity in the ARC between 2DG- and xylose-treated rats were determined by the Welch *t*-test. Statistical differences in the number of *Pdyn*-expressing cells and/or intensity of *Pdyn* mRNA signals in the PVN, ARC and SON between OVX and OVX + low E2 rats were determined by the Welch *t*-test.

Results

Central KOR antagonism blocked the suppression of pulsatile LH secretion induced by peripheral 2DG administration

Figure 1A shows LH profiles in representative OVX + low E2 rats receiving an iv injection of 2DG or xylose and 3V injection of nor-BNI, a KOR antagonist, or vehicle. Pulsatile LH release were apparent in xylose (iv)-vehicle (3V)-injected control rats, while LH pulses in the

2DG (iv)-vehicle (3V)-injected group were profoundly suppressed in comparison with the control group. The 3V nor-BNI injection blocked the 2DG (iv)-induced suppression of pulsatile LH release and restored apparent LH pulses. The nor-BNI (3V) treatment also increased LH release in iv xylose-treated rats. Two-way ANOVA revealed that 2DG (iv) treatment (a main effect) significantly decreased the mean plasma LH concentration ($F(1,15) = 15.16$, †, $P = 0.0014$, n=4-5) and nor-BNI (3V) treatment (a main effect) significantly increased the mean plasma LH concentration ($F(1,15) = 24.08$, *, $P < 0.001$, Fig. 1B). No significant interaction was found between the main effects on the mean plasma LH concentration. Two-way ANOVA revealed that there was a significant interaction between the main effects on the frequency of LH pulses ($F(1,15) = 13.87$, $P = 0.0020$). Specifically, the frequency in 2DG (iv)-vehicle (3V) group was significantly lower than that in the xylose (iv)-vehicle (3V)-treated group (‡, $P = 0.0085$, Fig. 1C), whereas the frequency in 2DG (iv)-nor-BNI (3V) group was significantly higher than that in the 2DG (iv)-vehicle (3V)-treated group (§, $P = 0.0348$, Fig. 1C) and the levels were comparable to xylose (iv)-vehicle (3V)-treated control rats. The frequency in xylose (iv)-nor-BNI (3V) group was significantly lower than that in xylose (iv)-vehicle (3V)-treated controls (§, $P = 0.0096$, Fig. 1C). There were no significant main effects and interaction on the amplitude of LH pulses (Fig. 1D).

Central KOR antagonism blocked the 4V 2DG-induced LH pulse suppression

Figure 2A shows LH profiles in representative OVX + low E2 rats receiving 4V injection of 2DG or xylose and 3V injection of nor-BNI or vehicle. Pulsatile LH release was profoundly suppressed by the 4V 2DG treatment, but the 3V nor-BNI injection blocked the 2DG (4V)-induced suppression of LH pulse. The nor-BNI (3V) treatment also increased plasma LH levels in 4V xylose-treated rats. Two-way ANOVA revealed that nor-BNI (3V) treatment (a main effect) significantly increased the mean plasma LH concentration ($F(1,20) = 21.32$, *, $P <$

0.001, n=6, Fig. 2B) and the frequency of LH pulses (F (1,20) = 6.45, *, P = 0.0195, Fig. 2C) compared with vehicle (3V)-treated group. No significant interactions were found between the main effect on both the mean plasma LH concentration and the frequency of LH pulses. There were no significant main effects and interaction on the amplitude of LH pulses (Fig. 2D).

Central KOR antagonism failed to affect the glucoprivic increase in plasma glucose concentrations and food intake

Figures 3A and 3B show changes in plasma glucose concentrations for the 3-h blood sampling period in OVX + low E2 rats receiving an iv (n=4-5, Fig. 3A) or 4V (n=6, Fig. 3B) injection of 2DG or xylose and 3V injection of nor-BNI or vehicle. The iv as well as 4V 2DG administration acutely increased plasma glucose concentrations in both 3V nor-BNI- and vehicle-treated animals. Three-way ANOVA analyses revealed that 2DG (iv or 4V) treatment (a main effect) significantly increased the plasma glucose concentrations (iv, F (1,15) = 42.09, *, P < 0.001, Fig. 3A; 4V, F (1,20) = 101.08, *, P < 0.001, Fig. 3B) and that there are significant interactions between the main effects (iv or 4V 2DG treatment and time) on the plasma glucose concentration (iv, F (9,135) = 13.07, P < 0.001; 4V, F (9,180) = 29.99, P < 0.001). Specifically, plasma glucose concentrations in iv 2DG-treated rats were significantly higher compared with iv xylose-treated controls during 12 to 180 min after iv 2DG injection (*, P < 0.001, Fig. 3A). Similarly, plasma glucose concentrations in 4V 2DG-treated rats were significantly higher compared with 4V xylose-treated controls during 24 to 180 min after 4V 2DG infusion (*, P < 0.001, Fig. 3B). Two-way ANOVA analysis revealed that the AUC of the plasma glucose concentrations was significantly higher in 2DG (iv or 4V)-treated groups (a main effect) compared with xylose (iv or 4V)-treated groups (iv, F (1,15) = 36.68, †, P < 0.001; 4V, F (1,20) = 121.98, †, P < 0.001, Figs. 3C and 3D). No significant main effect for the nor-BNI treatment and interaction between the nor-BNI treatment and time were found on the plasma glucose

concentration.

The amount of food intake for 0.5 h after the 3-h blood sampling period was significantly higher (iv, $F(1,15) = 31.31$, †, $P < 0.001$; 4V, $F(1,20) = 21.21$, †, $P < 0.001$; two-way ANOVA) in 2DG (iv or 4V)-treated groups (a main effect) compared with xylose (iv or 4V)-treated controls (Figs. 3E and 3F). No significant main effect for the nor-BNI treatment and interaction between the main effects were found on the food intake.

Peripheral and 4V 2DG administration increased Fos gene expression in the PVN Dyn neurons

Figure 4 shows the expression of *Pdyn* and *fos* mRNA in the hypothalamic nuclei, such as the PVN (A), ARC (B) and SON (C), in representative OVX + low E2 rats treated with iv 2DG or xylose. The number of *Pdyn*-expressing cells with *fos* co-expression in the PVN of iv 2DG-treated rats was significantly higher compared with that in iv xylose-treated controls (*, $P = 0.0128$; Welch *t*-test, Fig. 4D). The percentage of the *Pdyn*- and *fos*-co-expressing cells out of the total *Pdyn*-expressing cells in the PVN of iv 2DG-treated rats (44 ± 0.3 %, n=3) was also significantly higher compared with that of iv xylose-treated controls (12 ± 2.6 %, n=3) ($P = 0.0084$; Welch *t*-test). The number of total *fos*-expressing cells (†, $P = 0.0181$) and *fos*-expressing *Pdyn*-negative cells (§, $P = 0.0058$) in the PVN of iv 2DG-treated rats (n=3) was significantly higher compared with that in iv xylose-treated controls (n=3) (Welch *t*-test, Fig. 4D). In the ARC, the number of *Pdyn*-expressing cells with *fos* co-expression in iv 2DG-treated rats (n=3) was significantly lower compared with that in iv xylose-treated controls (n=3) (*, $P = 0.0217$; Welch *t*-test, Fig. 4E), while there was no significant difference in the percentage of *Pdyn*-expressing cells with *fos* co-expression (xylose, 9.9 ± 0.4 %; 2DG, 4.6 ± 1.5 %) and the number of total ARC *fos*-expressing and *fos*-expressing *Pdyn*-negative cells between iv 2DG-treated (n=3) and iv xylose-treated control groups (n=3, Fig. 4E). The number of *Pdyn*-

394 expressing cells with *fos* co-expression in the SON of iv 2DG-treated rats was significantly
395 higher compared with that of iv xylose-treated controls (*, P = 0.0283; Welch *t*-test, Fig. 4F).
396 The percentage of the SON *Pdyn*- and *fos*-co-expressing cells out of the total *Pdyn*-expressing
397 cells in iv 2DG-treated rats (26 ± 3.1 %, n=3) was also significantly higher compared with that
398 in iv xylose-treated controls (5.7 ± 3.2 %, n=3) (P = 0.0159; Welch *t*-test). The number of total
399 *fos*-expressing cells (†, P = 0.0251) and *fos*-expressing *Pdyn*-negative cells (§, P = 0.0141) in
400 the SON of iv 2DG-treated rats (n=3) was also significantly higher compared with that in iv
401 xylose-treated controls (n=3) (Welch *t*-test, Fig. 4F). No significant difference was found in the
402 number of *Pdyn*-expressing cells between iv 2DG and xylose-injected rats in any nuclei.
403 Figure 5 shows the expression of *Pdyn* and *fos* mRNA in the PVN (A), ARC (B) and SON
404 (C) in representative OVX + low E2 rats treated with 2DG or xylose into the 4V. The number
405 of *Pdyn*-expressing cells with *fos* co-expression in the PVN of 4V 2DG-treated rats was
406 significantly higher compared with that in 4V xylose-treated controls (*, P = 0.0470; Welch *t*-
407 test, Fig. 5D). The percentage of the *Pdyn*- and *fos*-co-expressing cells out of the total *Pdyn*-
408 expressing cells in the PVN in 4V 2DG-treated rats (36 ± 7.3 %, n=4) was also significantly
409 higher compared with that in 4V xylose-treated controls (14 ± 3.1 %, n=4) (P = 0.0458; Welch
410 *t*-test). On the other hand, in the ARC and SON, there was no significant difference in the
411 number and the percentage of *Pdyn*- and *fos*-co-expressing cells between 4V 2DG-treated
412 (ARC, 2.3 ± 0.5 %; SON, 11 ± 4.0 %) and 4V xylose-treated control groups (ARC, 1.4 ± 0.3 %;
413 SON, 7.6 ± 2.3 %) (Figs. 5E, 5F). The total number of *fos*-expressing cells in the PVN of 4V
414 2DG-treated rats (n=4) was significantly higher compared with that in 4V xylose-treated
415 controls (n=4) (†, P = 0.0171; Welch *t*-test, Fig. 5D). The number of total *fos*-expressing cells
416 (†, P = 0.0225) and *fos*-expressing *Pdyn*-negative cells (§, P = 0.0208) in the ARC of 4V 2DG-
417 treated rats (n=4) were also significantly higher compared with that in 4V xylose-treated
418 controls (n=4) (Welch *t*-test, Fig. 5E). On the other hand, in the SON, there was no significant

difference in the number of total *fos*-expressing cells and *fos*-expressing *Pdyn*-negative cells between 4V 2DG-treated (n=4) and 4V xylose-treated control groups (n=4, Fig. 5F). No significant difference was found in the number of *Pdyn*-expressing cells between 4V 2DG and xylose-infused rats in any nuclei.

Comparison of the number of ARC *Kiss1*-expressing cells and kisspeptin immunoreactivity between the 4V 2DG- and xylose-treated female rats

Figure 6A shows *Kiss1* expression in the ARC of representative OVX + low E2 rats treated with 2DG or xylose into the 4V. A number of *Kiss1*-expressing cells were detected in the ARC of OVX + low E2 rats treated with 4V 2DG or xylose (Fig. 6A). Kisspeptin-immunoreactive cell bodies and fibers were also found in the ARC of OVX + low E2 rats treated with 4V 2DG or xylose (Fig. 6B). No significant differences were found in the number of *Kiss1*-expressing cells and the intensity of *Kiss1* mRNA signals and kisspeptin immunoreactivity in the ARC between 4V 2DG- and xylose-treated groups (n=4 for each group, Figs. 6C and 6D).

Expression of KOR gene in the ARC *Kiss1* neurons

Oprk1 mRNA expression was found in the majority of the *Kiss1*-expressing cells and some *Kiss1*-negative cells in the ARC of OVX + low E2 rats regardless of 4V 2DG or xylose treatments (Fig. 7A). Quantitative analysis revealed that 62% (4V xylose) and 64% (4V 2DG) of ARC *Kiss1*-expressing cells showed *Oprk1* expression in the ARC of 4V xylose-treated (n=3) and 4V 2DG-treated OVX + low E2 rats (n=3), respectively, and the percentage was comparable between groups. There was no significant difference in the number of *Kiss1* and *Oprk1*-co-expressing cells between 4V xylose- and 2DG-treated groups (Fig. 7B).

Effects of low E2 treatment on the *Pdyn* expression in the PVN, ARC and SON in OVX

rats

The effect of negative feedback levels of E2 on *Pdyn* mRNA expression was examined in the PVN, ARC and SON in OVX rats, as shown in the photomicrographs of representative OVX or OVX + low E2 rats (Figs. 8A-8C). A number of *Pdyn*-expressing cells were found in the PVN of OVX + low E2 rats, while the cell number was fewer in OVX rats (Fig. 8A). The intensity (*, P = 0.0016; Welch *t*-test) and the number (*, P = 0.0434; Welch *t*-test) of *Pdyn*-expressing cells in the PVN were higher in OVX + low E2 rats compared with OVX group (n=3, Fig. 8D). There was no significant difference in the intensity and the number of the *Pdyn*-expressing cells in the ARC between OVX and OVX + low E2 groups (n=3, Fig. 8E). The intensity of *Pdyn* mRNA signals were comparable in the SON between OVX and OVX + low E2 groups (Fig. 8F). Note that the cell numbers in the SON could not be counted due to the overlap of the cells.

Discussion

The present study demonstrated that the Dyn neurons mediate, at least in part, the glucoprivic suppression of pulsatile LH secretion in female rats, because the current central administration of nor-BNI, a KOR antagonist, blocked the suppression of pulsatile LH secretion induced by both peripheral and 4V 2DG treatments. The number of Dyn neurons co-expressing *fos* in the PVN was significantly increased by the 4V or iv 2DG administration, but the 4V 2DG administration failed to affect the number of Dyn neurons co-expressing *fos* in the ARC and SON, suggesting that the PVN Dyn neurons were specifically activated by the hindbrain glucoprivation. Thus, it is likely that the PVN Dyn neurons mainly mediate the hindbrain glucoprivic suppression of LH pulses. Importantly, the current study showed that the negative feedback level of E2 specifically enhanced *Pdyn* expression in the PVN but not in the ARC and SON and that the majority of the ARC *Kiss1*-positive neurons expressed KOR mRNA

(*Oprk1*) in female rats. Further, the ARC kisspeptin neurons (KNDy neurons) are suggested to be involved in GnRH/LH pulse generation in mammals including rodents and goats (26,32). Taken together, these results suggest that the malnutrition signal sensed in the hindbrain activates the PVN Dyn neurons, resulting in the suppression of ARC KNDy neuronal activity and consequent suppression of pulsatile GnRH/LH secretion in female rats. The ARC kisspeptin neurons are suggested to be also involved in the GnRH/LH surge generation in sheep (49), suggesting that glucoprivic activation of Dyn neurons would be also involved in GnRH/LH surge suppression during malnutrition in this species (2). It should be noted that KOR was expressed in GnRH neurons in female rats (33), suggesting that Dyn neurons activated by glucoprivation may also directly suppress GnRH neurons during malnutrition.

Peripheral or 4V administration of 2DG has been shown to suppress LH pulses in female and male rats (11,13) and that the ependymocytes surrounding the 4V have a role to sense a low glucose availability (14). Additionally, the 4V administration of 3-hydroxybutyrate (3HB), a ketone body, whose plasma concentration increases during malnutrition, suppressed pulsatile LH release in female rats (50). Noradrenergic neurons originated from the nucleus tractus solitarius (NTS), located in the vicinity of ependymocytes of the 4V, are reported to project to the PVN (51), and noradrenaline release increased in the PVN by iv 2DG or 4V 3HB treatment in female rats (50,52). Furthermore, previous studies showed that an administration of a catecholamine synthesis inhibitor into the PVN canceled the fasting- or iv 2DG-induced suppression of LH pulse in female rats (18,52). Deletion of brainstem catecholamine neurons by an injection of dopamine-β-hydroxylase-saporin into the PVN blocks the inhibiting effect of subcutaneous 2DG injection on estrous cycles in rats (53). Our iv and 4V 2DG administrations similarly induced *fos* expression in the PVN Dyn neurons. Taken together with the current study, these results suggest that malnutritional signals sensed by the 4V ependymocytes are transmitted to the PVN via hindbrain noradrenergic neurons, resulting in

494 an activation of PVN Dyn neurons to mediate the glucoprivic or malnutritional suppression of
495 LH pulse in rats.
496 Regarding of *fos* expression in the present study, iv 2DG injection significantly decreased
497 the number of *fos*-expressing Dyn-positive cells in the ARC. It is speculated that a part of these
498 cells might be KNDy neurons and the decrease in the number of ARC *fos*- and Dyn-co-
499 expressing cells would be associated with the suppression of KNDy neuron activity induced
500 by peripheral glucoprivation. In the SON, unlike with 4V 2DG administration, iv 2DG
501 treatment increased the number of *fos*-expressing Dyn-positive cells as well as the total number
502 *fos*-expressing cells. This suggests that these SON neurons were activated by peripheral
503 glucoprivation sensed by other glucosensor(s) rather than the hindbrain sensor. Previous studies
504 have shown that an intraperitoneal 2DG administration increased Fos-immunoreactivity in
505 nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive neurons in the
506 SON of OVX + E2 rats (54) and that vasopressin and oxytocin neurons in the SON contain
507 NADPH-d in male rats (55). Thus, taken together with the current study, it can be speculated
508 that Dyn, vasopressin and oxytocin neurons in the SON may be activated to maintain metabolic
509 homeostasis under peripheral glucoprivic conditions. Further studies are required to address
510 the role of the SON Dyn and non-Dyn neurons activated during peripheral glucoprivation.
511 The action site(s) of Dyn involved in the glucoprivic LH suppression could be mainly the
512 ARC KNDy neurons, because the *Oprk1* mRNA expression was evident in a majority of the
513 ARC *Kiss1*-expressing cells in the present study. This notion is consistent with our recent
514 tracing study showing the existence of the neural pathway from the 4V ependymocytes to the
515 PVN neurons and ARC kisspeptin neurons: WGA was detected in the NTS, PVN as well as
516 ARC kisspeptin neurons when WGA was administered to the 4V (16). Further, another study
517 by Tóth and Palkovits (56) using a tract-tracer also showed that a relatively dense network was
518 detected between the PVN and ARC. Thus, it is likely that the PVN Dyn neurons may receive

519 malnutrition signals from the NTS noradrenergic neurons to suppress the ARC kisspeptin
520 neurons through KOR localized in the kisspeptin neurons, and consequently suppress pulsatile
521 LH secretion during malnutrition. Interestingly, the current 4V 2DG administration failed to
522 affect the intensity of the ARC kisspeptin immunoreactivity and *Kiss1* mRNA signals and the
523 number of *Kiss1*-expressing cells in the ARC. This result suggests that the current acute
524 glucoprivation originating in the 4V 2DG administration may cause an immediate suppression
525 of KNDy neuronal activity, and consequently suppressed pulsatile GnRH/LH release without
526 affecting *Kiss1* and kisspeptin expression and synthesis in the ARC. It would be worth
527 clarifying if the *Kiss1* mRNA expression per neuron could be affected by glucoprivation,
528 because the number of *Kiss1*-expressing cells and the intensity of *Kiss1* mRNA signals in the
529 ARC may not always reflect the *Kiss1* mRNA expression level in each cell.
530 The current results showed that the low E2 treatment profoundly increased *Pdyn* expression
531 in the PVN but not the ARC or SON, suggesting that the estrogen-dependent suppression of
532 LH pulses by fasting or glucoprivation (4,11) could be mediated by the PVN Dyn neurons
533 upregulated by a diestrous level of estrogen. This notion is consistent with previous reports,
534 indicating that fasting suppressed LH pulses when E2 was implanted into the PVN of OVX
535 rats (57,58). The E2-induced upregulation of PVN Dyn expression suggests that the PVN Dyn
536 neurons also mediate estrogen negative feedback effect on GnRH/LH release under normal
537 nutritional conditions. This is in agreement with previous studies in rats and goats: 3V
538 administration of nor-BNI significantly increased LH secretion in OVX + low E2 rats but not
539 in OVX rats (22); iv infusion of a KOR antagonist increased the pulsatile LH release and
540 frequency of MUA volley, an indicator for GnRH pulse generator activity, in OVX + low E2
541 goats (59). Indeed, the current study showed that the central administration of nor-BNI also
542 increased LH release in iv or 4V xylose-injected OVX + low E2 control rats. Lopez et al. (60)
543 showed that a central administration of nor-BNI increased LH pulses in E2-treated OVX lambs

544 at prepubertal age, when sensitivity to estrogen negative feedback is higher than at postpubertal
545 age, and few Dyn cells were found in the ARC of the prepubertal OVX + E2 lambs. The
546 estrogen-dependent enhancement of PVN Dyn tone might be involved in the prepubertal
547 suppression of LH release in ewes. The results of Goodman et al. (61) suggested that Dyn
548 neurons mainly mediate negative feedback effect of progesterone on GnRH/LH pulses by
549 affecting GnRH neurons in sheep. In this context, Dyn (probably originated from the PVN)-
550 KOR (probably in the ARC KNDy neurons and GnRH neurons) signaling may be involved in
551 negative feedback effect of sex steroids in ruminants as well as rodents. Further studies are
552 required to address this issue.

553 In the present study, *fos* mRNA expression was also abundantly found in non-Dyn cells in
554 the PVN as well in iv or 4V 2DG-treated rats, suggesting that the malnutrition signals from
555 peripheral and the 4V activated other PVN neurons in addition to the Dyn neurons. Considering
556 the following previous studies, the activated non-Dyn PVN neurons would be CRH neurons:
557 CRH neurons are suggested to mediate malnutrition-induced suppression of LH pulses, since
558 a central administration of a CRH antagonist blocked the suppression of LH pulses induced by
559 fasting or peripheral 2DG administration in female rats (18,19). In addition, a central CRH
560 antagonism blocked LH pulse suppression induced by the PVN noradrenaline administration
561 in OVX + low E2 rats (62). As for the ARC *fos*-positive non-Dyn cells increased by the 4V
562 2DG treatment, a part of them might be neuropeptide Y (NPY) neurons because ARC NPY
563 neuron is known as an orexigenic neuron (63) and the 4V 2DG administration increased food
564 intake. It is reported that the NPY mRNA level was increased by both acute food deprivation
565 and chronic food restriction in rats ARC (63) and administration of NPY to the 3V decreases
566 plasma LH concentration in rats, suggesting the possibility that the ARC NPY neurons would
567 be also involved in suppression of reproductive function during malnutrition (64). Taken
568 together with the present study, these findings suggest that both Dyn and CRH neurons

originated from the PVN and NPY neurons originated from the ARC might be involved in the LH pulse suppression during malnutrition.

The present study showed that central KOR antagonism failed to affect the glucoprivic increase in blood glucose concentrations in both iv or 4V 2DG-treated female rats. Thus, it is unlikely that Dyn-KOR signaling is involved in the counter-regulatory gluconeogenesis in response to glucoprivation. In other words, central Dyn-KOR signaling specifically mediates glucoprivic suppression of GnRH/LH pulses, but not glucoprivic regulation of blood glucose concentrations. This is in agreement with the work of Tudurí and colleagues (65), showing that an administration of a KOR agonist into the lateral cerebral ventricle failed to affect blood glucose levels at glucose tolerance test in mice. Additionally, the glucoprivation by peripheral administration of 2DG induced an increase in plasma corticosterone level in male rats as shown by Sun et al. (66), suggesting that a glucoprivic increase in gluconeogenesis could be mediated by CRH signaling rather than Dyn-KOR signaling.

Concerning the feeding after the 2DG treatment, the central KOR antagonist administration failed to show a significant effect on food intake in the presence or absence of peripheral/central 2DG, suggesting that Dyn neurons are unlikely involved in the central regulation of feeding during glucoprivation. There are controversial reports on a role of Dyn neurons in regulation of feeding: a central Dyn injection significantly increased food intake in male rats (67), suggesting a stimulatory role of Dyn neurons in feeding behavior. Interestingly, the response of Dyn neurons to energetic stimuli varies in a nucleus-dependent manner: Food restriction increased the number of Dyn-immunoreactive cells in the nucleus accumbens, bed nucleus of stria terminalis, lateral hypothalamus, medial preoptic area, and medial hypothalamus, but reduced the number in the central nucleus of amygdala (68); acute food deprivation as well as chronic food restriction caused a reduction of Dyn levels in the hypothalamus (69). Further studies are required to clarify the role of Dyn neurons in controlling feeding.

In conclusion, the present study demonstrates that the Dyn neurons, which most probably originate from the PVN, mediate the suppression of pulsatile LH secretion during central and peripheral glucoprivation in female rats. Further, it is likely that the Dyn neurons, at least in part, directly suppress KNDy neurons via KOR expressed in the neurons and consequently suppress pulsatile GnRH/LH release.

Acknowledgement

We thank the National Hormone and Peptide Program for the rat LH assay kit. The radioimmunoassay was performed at the Nagoya University Radioisotope Centre. We also thank Arisa Sugimoto and Narumi Kawai for their technical support. We thank Dr. Nicola Skoulding for editorial assistance.

References

1. **Imakawa K, Day ML, Zalesky DD, Clutter A, Kittok RJ, Kinder JE.** Effects of 17 beta-estradiol and diets varying in energy on secretion of luteinizing hormone in beef heifers. *J. Anim. Sci.* 1987;64(3):805–815.
2. **Foster DL, Olster DH.** Effect of restricted nutrition on puberty in the lamb: Patterns of tonic luteinizing hormone (LH) secretion and competency of the LH surge system. *Endocrinology* 1985;116(1):375–381.
3. **Matsuyama S, Ohkura S, Ichimaru T, Sakurai K, Tsukamura H, Maeda KI, Okamura H.** Simultaneous observation of the GnRH pulse generator activity and plasma concentrations of metabolites and insulin during fasting and subsequent refeeding periods in shiba goats. *J. Reprod. Dev.* 2004;50(6):697–704.
4. **Cagampang FRA, Maeda KI, Yokoyama A, Ota K.** Effect of food deprivation on the pulsatile LH release in the cycling and ovariectomized female rat. *Horm. Metab.*

Res. 1990;22(5):269–272.

5. **Minabe S, Uenoyama Y, Tsukamura H, Maeda KI.** Analysis of pulsatile and surge-like luteinizing hormone secretion with frequent blood sampling in female mice. *J. Reprod. Dev.* 2011;57(5):660–664.

6. **Schreihofer DA, Amico JA, Cameron JL.** Reversal of fasting-induced suppression of luteinizing hormone (LH) secretion in male rhesus monkeys by intragastric nutrient infusion: evidence for rapid stimulation of LH by nutritional signals. *Endocrinology* 1993;132(5):1890–1897.

7. **Mcclure TJ, Saunders J.** Effects of withholding food for 0-72 h on mating, pregnancy rate and pituitary function in female rats. *J Reprod Fertil* 2006;74(1):57–64.

8. **Maeda KI, Nagatani S, Estacio MA, Tsukamura H.** Novel estrogen feedback sites associated with stress-induced suppression of luteinizing normone secretion in female rats. *Cell. Mol. Neurobiol.* 1996;16(3):311–324.

9. **Schneider JE, Goldman MD, Leo NA, Rosen ME.** Central vs. peripheral metabolic control of estrous cycles in Syrian hamsters. II. Glucoprivation. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 1997;272(1):406–412.

10. **Bucholtz DC, Vidwans NM, Herbosa CG, Schillo KK, Foster DL.** Metabolic interfaces between growth and reproduction. v. pulsatile luteinizing hormone secretion is dependent on glucose availability. *Endocrinology* 1996;137(2):601–607.

11. **Nagatani S, Bucholtz D, Murahashi K, Estacio MA, Tsukamura H, Foster DL, Maeda KI.** Reduction of glucose availability suppresses pulsatile luteinizing hormine release in female and male rats. *Endocrinology* 1996;137(4):1166–1170.

12. **Tsukamura H, Yamada S, Maeda KI.** Fasting-induced changes in pulsatile luteinizing hormone (LH) secretion in male rats: The role of testosterone and the hypothalamic paraventricular nucleus. *J. Reprod. Dev.* 2000;46(4):227–234.

13. **Murahashi K, Bucholtz DC, Nagatani S, Tsukahara S, Tsukamura H, Foster DL, Maeda KI.** Suppression of luteinizing hormone pulses by restriction of glucose availability Is mediated by sensors in the brain stem. *Endocrinology* 1996;137(4):1171–1176.

14. **Moriyama R, Tsukamura H, Kinoshita M, Okazaki H, Kato Y, Maeda KI.** In vitro increase in intracellular calcium concentrations induced by low or high extracellular glucose levels in ependymocytes and serotonergic neurons of the rat lower brainstem. *Endocrinology* 2004;145(5):2507–2515.

15. **Ritter RC, Slusser PG, Stone S.** Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 1981;213(4506):451–452.

16. **Deura C, Minabe S, Ikegami K, Inoue N, Uenoyama Y, Maeda KI, Tsukamura H.** Morphological analysis for neuronal pathway from the hindbrain ependymocytes to the hypothalamic kisspeptin neurons. *J. Reprod. Dev.* 2019;65(2):129–137.

17. **Sanders NM, Ritter S.** Repeated 2-deoxy-D-glucose-induced glucoprivation attenuates Fos expression and glucoregulatory responses during subsequent glucoprivation. *Diabetes* 2000;49(11):1865–1874.

18. **Maeda KI, Cagampang FRA, Coen CW, Tsukamura H.** Involvement of the catecholaminergic input to the paraventricular nucleus and of corticotropin-releasing hormone in the fasting-induced suppression of luteinizing hormone release in female rats. *Endocrinology* 1994;134(4):1718–1722.

19. **Tsukahara S, Tsukamura H, Foster DL, Maeda KI.** Effect of corticotropin-releasing hormone antagonist on oestrogen-dependent glucoprivic suppression of luteinizing hormone secretion in female rats. *J. Neuroendocrinol.* 1999;11(2):101–105.

20. **Khachaturian H, Watson SJ, Lewis ME, Coy D, Goldstein A, Akil H.** Dynorphin immunocytochemistry in the rat central nervous system. *Peptides* 1982;3(6):941–954.

21. **Fallon JH, Leslie FM.** Distribution of dynorphin and enkephalin peptides in the rat brain. *J. Comp. Neurol.* 1986;249(3):293–336.

22. **Mostari P, Ieda N, Deura C, Minabe S, Yamada S, Uenoyama Y, Maeda KI, Tsukamura H.** Dynorphin-kappa opioid receptor signaling partly mediates estrogen negative feedback effect on LH pulses in female rats. *J. Reprod. Dev.* 2013;59(3):266–72.

23. **Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ.** Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: An in situ hybridization study. *J. Comp. Neurol.* 1994;350(3):412–438.

24. **Matsuzaki T, Tungalagsuvd A, Munkhzaya M, Iwasa T, Yano K, Mayila Y, Tokui T, Yanagihara R, Matsui S, Kato T, Kuwahara A, Irahara M.** Neurokinin B receptor agonist and Dynorphin receptor antagonist stimulated luteinizing hormone secretion in fasted male rodents. *Endocr. J.* 2018. doi:10.1507/endocrj.EJ17-0136.

25. **Goodman RL, Lehman MN, Smith JT, Coolen LM, Oliveira CVR De, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ.** Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* 2007;148(12):5752–5760.

26. **Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda KI, Steiner RA, Okamura H.** Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J. Neurosci.* 2010;30(8):3124–3132.

27. **Murakawa H, Iwata K, Takeshita T, Ozawa H.** Immunoelectron microscopic observation of the subcellular localization of kisspeptin, neurokinin B and dynorphin A in KNDy neurons in the arcuate nucleus of the female rat. *Neurosci. Lett.*

694 2016;612:161–166.
695 28. **Maeda KI, Ohkura S, Uenoyama Y, Wakabayashi Y, Oka Y, Tsukamura H,**
696 **Okamura H.** Neurobiological mechanisms underlying GnRH pulse generation by the
697 hypothalamus. *Brain Res.* 2010;1364:103–115.
698 29. **Moore AM, Prescott M, Czieselsky K, Desroziers E, Yip SH, Campbell RE,**
699 **Herbison AE.** Synaptic innervation of the GnRH neuron distal dendron in female
700 mice. *Endocrinology* 2018;159(9):3200–3208.
701 30. **Minabe S, Nakamura S, Fukushima E, Sato M, Ikegami K, Goto T, Sanbo M,**
702 **Hirabayashi M, Tomikawa J, Imamura T, Inoue N, Uenoyama Y, Tsukamura H,**
703 **Maeda KI, Matsuda F.** Inducible Kiss1 knockdown in the hypothalamic arcuate
704 nucleus suppressed pulsatile secretion of luteinizing hormone in male mice. *J. Reprod.*
705 *Dev.* 2020. doi:10.1262/jrd.2019-164.
706 31. **Ikegami K, Goto T, Nakamura S, Watanabe Y, Sugimoto A, Majarune S,**
707 **Horihata K, Nagae M, Tomikawa J, Imamura T, Sanbo M, Hirabayashi M, Inoue**
708 **N, Maeda KI, Tsukamura H, Uenoyama Y.** Conditional kisspeptin neuron-specific
709 Kiss1 knockout with newly generated Kiss1-floxed and Kiss1-Cre mice replicates a
710 hypogonadal phenotype of global Kiss1 knockout mice. *J. Reprod. Dev.* 2020.
711 doi:10.1262/jrd.2020-026.
712 32. **Herbison AE.** The gonadotropin-releasing hormone pulse generator. *Endocrinology*
713 2018;159(11):3723–3736.
714 33. **Weems PW, Witty CF, Amstalden M, Coolen LM, Goodman RL, Lehman MN.** κ-
715 opioid receptor is colocalized in GnRH and KNDy cells in the female ovine and rat
716 brain. *Endocrinology* 2016;157(6):2367–2379.
717 34. **Cagampang FRA, Maeda KI, Tsukamura H, Phkura S, Ota K.** Involvement of
718 ovarian steroids and endogenous opioids in the fasting-induced suppression of

pulsatile LH release in ovariectomized rats. *J. Endocrinol.* 1991;129(3):321–328.

35. **Paxinos G, Watson C.** The rat brain in stereotaxic coordinates - the new coronal set. Cambridge; 2007.

36. **Minabe S, Deura C, Ikegami K, Goto T, Sanbo M, Hirabayashi M, Inoue N, Uenoyama Y, Maeda KI, Tsukamura H.** Pharmacological and morphological evidence of AMPK-mediated energy sensing in the lower brain stem ependymocytes to control reproduction in female rodents. *Endocrinology* 2015;156(6):2278–2287.

37. **Sugimoto A, Tsuchida H, Ieda N, Ikegami K, Inoue N, Uenoyama Y, Tsukamura H.** Somatostatin-somatostatin receptor 2 signaling mediates LH pulse suppression in lactating rats. *Endocrinology* 2019;160(2):473–483.

38. **Assadullah, Ieda N, Kawai N, Ishii H, Ihara K, Inoue N, Uenoyama Y, Tsukamura H.** Co-expression of the calcitonin receptor gene in the hypothalamic kisspeptin neurons in female rats. *Reprod. Med. Biol.* 2018;17(2):164–172.

39. **RRID:AB_840257. https://antibodyregistry.org/AB_840257.**

40. **RRID:AB_514500. https://antibodyregistry.org/AB_514500.**

41. **Yamada S, Uenoyama Y, Kinoshita M, Iwata K, Takase K, Matsui H, Adachi S, Inoue K, Maeda KI, Tsukamura H.** Inhibition of metastin (kisspeptin-54)-GPR54 signaling in the arcuate nucleus-median eminence region during lactation in rats. *Endocrinology* 2007;148(5):2226–2232.

42. **RRID:AB_2734716. https://antibodyregistry.org/AB_2734716.**

43. **Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, Inoue N, Sanbo M, Tomita K, Hirabayashi M, Tanaka S, Imamura T, Okamura H, Maeda KI, Tsukamura H.** Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* 2012;109(20):E1294–E1301.

44. **Okamura H, Yamamura T, Wakabayashi Y.** Mapping of KNDy neurons and immunohistochemical analysis of the interaction between KNDy and substance P neural systems in goat. *J. Reprod. Dev.* 2017;63(6):571–580.
45. **RRID:AB_2832253. https://antibodyregistry.org/AB_2832253.**
46. **RRID:AB_143165. https://antibodyregistry.org/AB_143165.**
47. **Merriam GR, Wachter KW.** Algorithms for the study of episodic hormone secretion. *Am. J. Physiol. - Endocrinol. Metab.* 1982;243(4):E310–E318.
48. **Maeda KI, Tsukamura H, Uchida E, Ohkura N, Ohkura S, Yokoyama A.** Changes in the pulsatile secretion of LH after the removal of and subsequent resuckling by pups in ovariectomized lactating rats. *J. Endocrinol.* 1989;121(2):277–283.
49. **Smith JT, Li Q, Pereira A, Clarke IJ.** Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. *Endocrinology* 2009;150(12):5530–5538.
50. **Iwata K, Kinoshita M, Susaki N, Uenoyama Y, Tsukamura H, Maeda KI.** Central injection of ketone body suppresses luteinizing hormone release via the catecholaminergic pathway in female rats. *J. Reprod. Dev.* 2011;57(3):379–384.
51. **Saphier D.** Electrophysiology and neuropharmacology of noradrenergic projections to rat PVN magnocellular neurons. *Am. J. Physiol.* 1993;264(5):R891–R902.
52. **Nagatani S, Tsukamura H, Murahashi K, Bucholtz D, Foster DL, Maeda KI.** Paraventricular norepinephrine release mediates glucoprivic suppression of pulsatile luteinizing hormone secretion. *Endocrinology* 1996;137(8):3183–3186.
53. **I'Anson H, Sundling LA, Roland SM, Ritter S.** Immunotoxic destruction of distinct catecholaminergic neuron populations disrupts the reproductive response to glucoprivation in female rats. *Endocrinology* 2003;144(10):4325–4331.

54. **Briski KP, Sylvester PW.** Site-specific induction of Fos immunoreactivity in preoptic and hypothalamic NADPH-positive neurons during glucoprivation. *Neuroendocrinology* 1999;69(3):181–190.

55. **Hatakeyama S, Kawai Y, Ueyama T, Senba E.** Nitric oxide synthase-containing magnocellular neurons of the rat hypothalamus synthesize oxytocin and vasopressin and express Fos following stress stimuli. *J. Chem. Neuroanat.* 1996;11(4):243–256.

56. **Tóth ZE, Palkovits M.** Distributions of periventricular projections of the paraventricular nucleus to the median eminence and arcuate nucleus. *Brain Res.* 1998;802(1–2):294–297.

57. **Nagatani S, Tsukamura H, Murahashi K, Maeda KI.** A rapid suppressive effect of estrogen in the paraventricular nucleus on pulsatile LH release in fasting-ovariectomized rats. *J. Neuroendocrinol.* 1996;8(4):267–273.

58. **Nagatani S, Tsukamura H, Maeda KI.** Estrogen feedback needed at the paraventricular nucleus or A2 to suppress pulsatile luteinizing hormone release in fasting female rats. *Endocrinology* 1994;135(3):870–875.

59. **Sasaki T, Ito D, Sonoda T, Morita Y, Wakabayashi Y, Yamamura T, Okamura H, Oishi S, Noguchi T, Fujii N, Uenoyama Y, Tsukamura H, Maeda KI, Matsuda F, Ohkura S.** Peripheral administration of κ-opioid receptor antagonist stimulates gonadotropin-releasing hormone pulse generator activity in ovariectomized, estrogen-treated female goats. *Domest. Anim. Endocrinol.* 2019;68:83–91.

60. **Lopez JA, Bedenbaugh MN, McCosh RB, Weems PW, Meadows LJ, Wisman B, Coolen LM, Goodman RL, Hileman SM.** Does dynorphin play a role in the onset of puberty in female sheep? *J. Neuroendocrinol.* 2016;28(12).

61. **Goodman RL, Coolen LM, Anderson GM, Hardy SL, Valent M, Connors JM, Fitzgerald ME, Lehman MN.** Evidence that dynorphin plays a major role in

mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. *Endocrinology* 2004;145(6):2959–2967.

62. **Tsukamura H, Nagatani S, Cagampang FRA, Kawakami S, Maeda KI.** Corticotropin-releasing hormone mediates suppression of pulsatile luteinizing hormone secretion induced by activation of alpha-adrenergic receptors in the paraventricular nucleus in female rats. *Endocrinology* 1994;134(3):1460–1466.

63. **Bi S, Robinson BM, Moran TH.** Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 2003;285(5):R1030–R1036.

64. **Mcdonald J k.** Role of neuropeptide Y in reproductive function. *Ann. N. Y. Acad. Sci.* 1990;611(1):258–272.

65. **Tudurí E, Beiroa D, Stegbauer J, Fernø J, López M, Diéguez C, Nogueiras R.** Acute stimulation of brain mu opioid receptors inhibits glucose-stimulated insulin secretion via sympathetic innervation. *Neuropharmacology* 2016;110:322–332.

66. **Sun CL, Thoa NB, Kopin IJ.** Comparison of the effects of 2-deoxyglucose and immobilization on plasma levels of catecholamines and corticosterone in awake rats. *Endocrinology* 1979;105(1):306–311.

67. **Gosnell BA, Levine AS, Morley JE.** The stimulation of food intake by selective agonists of mu, kappa and delta opioid receptors. *Life Sci.* 1986;38(12):1081–1088.

68. **Berman Y, Devi L, Carr KD.** Effects of chronic food restriction on prodynorphin-derived peptides in rat brain regions. *Brain Res.* 1994;664(1–2):49–53.

69. **Johansson A, Fredriksson R, Winnergren S, Hulting AL, Schiöth HB, Lindblom J.** The relative impact of chronic food restriction and acute food deprivation on plasma hormone levels and hypothalamic neuropeptide expression. *Peptides* 2008;29(9):1588–1595.

819

820 **Figure legend**

821 **Figure 1.** Effects of central administration of nor-binaltorphimine (nor-BNI), a kappa opioid
822 receptor antagonist, on the suppression of luteinizing hormone (LH) pulses induced by
823 peripheral (intravenous, iv) injection of 2-deoxy-D-glucose (2DG) in estradiol-17β (E2)-
824 treated ovariectomized (OVX + low E2) rats. Plasma LH profiles in representative OVX + low
825 E2 rats, which were treated with the third ventricle (3V) injection of nor-BNI (or vehicle) and
826 iv injection of 2DG (or xylose) (A). The 3V nor-BNI or vehicle (timing indicated by arrows)
827 was injected just before the iv 2DG (or xylose) injection and blood samples were collected
828 every 6 min for 3 h. Arrowheads indicate the peaks of LH pulses identified by the PULSAR
829 computer program. The mean plasma LH concentrations (B), the frequency (C) and amplitude
830 (D) of LH pulses in each group. Values are means ± SEM. The numbers in each column indicate
831 the number of animals used. * Significant main effect of nor-BNI (3V) treatment ($P < 0.05$,
832 two-way ANOVA). † Significant main effect of 2DG (iv) treatment ($P < 0.05$, two-way
833 ANOVA). ‡ Significant difference between 2DG (iv)- and xylose (iv)-treated rats within
834 vehicle (3V)-injected groups ($P < 0.05$, the simple main effect of the two-way ANOVA). §
835 Significant difference between nor-BNI (3V)- and vehicle (3V)-injected rats within 2DG (iv)-
836 or xylose (iv)-treated groups ($P < 0.05$, the simple main effect of the two-way ANOVA).

837

838 **Figure 2.** Effects of 3V administration of nor-BNI on the suppression of LH pulses induced by
839 4V 2DG treatment in OVX + low E2 rats. Plasma LH profiles in representative OVX + low E2
840 rats, which were injected with nor-BNI (or vehicle) in the 3V and infused with 2DG (or xylose)
841 into the fourth ventricle (4V) for the first 60 min (A). The nor-BNI or vehicle (arrows) were
842 injected into the 3V just before the onset of the 4V 2DG (or xylose) infusion. The gray colored
843 area indicates the duration of 2DG (or xylose) infusion into the 4V. The mean plasma LH

concentrations (B), and the frequency (C) and amplitude (D) of LH pulses in each group. Values are means ± SEM. * Significant main effect of nor-BNI (3V) treatment ($P < 0.05$, two-way ANOVA). See Fig. 1 for details.

Figure 3. Effects of the 3V nor-BNI administration on plasma glucose levels and food intake after the iv or 4V treatment of 2DG in OVX + low E2 rats. Changes in the mean plasma glucose concentrations in OVX + low E2 rats bearing the 3V nor-BNI (or vehicle) and iv 2DG (or xylose) injection at onset of the blood sampling (A). Changes in the mean plasma glucose concentration in OVX + low E2 rats bearing the 3V nor-BNI (or vehicle) injection and 4V 2DG (or xylose) infusion for the first 1 h (B). * Significant difference between 2DG- and xylose-treated groups within iv (A) or 4V (B) 2DG/xylose-treated groups ($P < 0.05$, the simple main effect of the three-way ANOVA). The area under the curve (AUC) of plasma glucose concentrations for 3 h after iv injection of 2DG or xylose (C) or after 4V infusion of 2DG or xylose (D) in the rats treated with 3V nor-BNI or vehicle. Amount of food intake in OVX + low E2 rats bearing the 3V nor-BNI (or vehicle) and iv or 4V 2DG (or xylose) treatment (E, F). Food intake of each group was measured for 0.5 h just after the 3-h blood sampling period for plasma LH and glucose analysis. Values are means ± SEM. The numbers in each column indicate the number of animals used. † Significant main effect of 2DG treatment ($P < 0.05$, two-way ANOVA).

Figure 4. Effect of iv 2DG administration on *fos* and dynorphin A gene (*Pdyn)* mRNA expression in the hypothalamic nuclei, such as the paraventricular nucleus (PVN), arcuate nucleus (ARC) and supraoptic nucleus (SON) of OVX + low E2 rats. *Pdyn* and *fos* expression in the hypothalamus was determined by double ISH. *Pdyn*-expressing (green) and *fos*-expressing cells (magenta) in the PVN (A), ARC (B) and SON (C) of representative OVX +

low E2 rats at 1 h after the iv 2DG or xylose injection. The insets indicate *fos* expression in the *Pdyn*-expressing cells indicated by white arrowheads at higher magnification. Scale bars, 100 μm. The number of *Pdyn*-expressing cells with (green and magenta column) or without (green column) *fos* expression and the number of *fos*-expressing cells with (green and magenta column) or without (magenta column) *Pdyn*-expression in the PVN (D), ARC (E) and SON (F). Schematic illustrations of the hypothalamic nuclei showing the position of representative photomicrographs for each nucleus (G). Values are means ± SEM. Open circles indicate the individual data points of the total number of *Pdyn*-positive cells (left columns) and *fos*-positive cells (right columns). Solid circles indicate the individual data points of the *Pdyn*- and *fos*-positive cells (left columns) and *fos*-positive and *Pdyn*-negative cells (right columns) in each nucleus. n=3 for each group. * Significant difference in the number of *Pdyn*-expressing cells with *fos*-expression between iv 2DG- and xylose-injected rats ($P < 0.05$, Welch *t*-test). † Significant difference in the total number of *fos*-expressing cells between iv 2DG- and xylose-injected rats ($P < 0.05$, Welch *t*-test). § Significant difference in the number of *fos*-expressing *Pdyn*-negative cells between iv 2DG- and xylose-injected rats ($P < 0.05$, Welch *t*-test). No significant difference was found in the number of *Pdyn*-expressing cells between iv 2DG and xylose-injected rats in any nuclei.

Figure 5. Effect of 4V 2DG administration on *fos* and *Pdyn* mRNA expression in the PVN, ARC and SON of OVX + low E2 rats. *Pdyn*-expressing (green) and *fos*-expressing cells (magenta) in the PVN (A), ARC (B) and SON (C) in representative OVX + low E2 rats infused with 2DG or xylose into the 4V for 1 h. The insets indicate *Pdyn*-expressing cells indicated by white arrowheads at higher magnification. Scale bars, 100 μm. The number of *Pdyn*-expressing cells with (green and magenta column) or without (green column) *fos*-expression and the number of *fos*-expressing cells with (green and magenta column) or without (magenta column)

894 *Pdyn*-expression in the PVN (D), ARC (E) and SON (F). Values are means ± SEM. Open circles
895 indicate the individual data points of the total number of *Pdyn*-positive cells (left columns) and
896 *fos*-positive cells (right columns). Solid circles indicate the individual data points of the *Pdyn*-
897 and *fos*-positive cells (left columns) and *fos*-positive and *Pdyn*-negative cells (right columns)
898 in each nucleus. n=4 for each group. * Significant difference in the number of *Pdyn*-expressing
899 cells with *fos* expression between 4V 2DG- and xylose-infused rats ($P < 0.05$, Welch *t*-test). †
900 Significant difference in the total number of *fos*-expressing cells between 4V 2DG- and xylose-
901 infused rats ($P < 0.05$, Welch *t*-test). § Significant difference in the number of *fos*-expressing
902 *Pdyn*-negative cells between 4V 2DG- and xylose-infused rats ($P < 0.05$, Welch *t*-test). No
903 significant difference was found in the number of *Pdyn*-expressing cells between 4V 2DG and
904 xylose-infused rats in any nuclei. See Fig. 4 for details.
905
906 **Figure 6.** Expression of *Kiss1* mRNA and intensity of kisspeptin immunoreactivity in the ARC
907 in OVX + low E2 rats treated with the 4V 2DG (or xylose) infusion. *Kiss1* expression
908 visualized by ISH (A) and kisspeptin immunoreactivity (B) in the ARC of representative OVX
909 + low E2 rats infused with 2DG or xylose into the 4V for 1 h. The insets indicate *Kiss1*-
910 expressing cells (black arrowheads) or kisspeptin-immunoreactive cell body and fibers (white
911 arrowheads) at higher magnification. Intensity of *Kiss1* mRNA signals and kisspeptin
912 immunoreactivity were determined by the ImageJ software. Scale bars, 100 μm. The intensity
913 of *Kiss1* mRNA signals, the number of *Kiss1*-expressing cells (C) and the intensity of
914 kisspeptin immunoreactivity (D) in the ARC of the rats infused with 2DG or xylose into the
915 4V. Values are means ± SEM. Open circles indicate the individual data points in each group.
916 n=4 for each group. The numbers in each column indicate the number of animals used. No
917 significant difference was found in the intensity of *Kiss1* mRNA signals, the number of *Kiss1*-
918 expressing cells and the intensity of kisspeptin immunoreactivity between 4V 2DG- or xylose-

infused groups (Welch *t*-test).

Figure 7. Expression of *Kiss1* and *Oprk1* (Dyn receptor gene) in the ARC of OVX + low E2 rats determined by double ISH. *Kiss1*-expressing (green) and *Oprk1*-expressing cells (magenta) in the ARC of representative OVX + low E2 rats infused with xylose- or 2DG into the 4V (A). The insets indicate *Kiss1* and *Oprk1*-co-expressing cells (white arrowheads) at higher magnification. Scale bars, 100 μm. The number of *Kiss1*-expressing cells with (green and magenta column), or without (green column) *Oprk1*expression in the ARC of the OVX + low E2 rats treated with 4V xylose or 2DG (B). Values are means ± SEM. Open circles indicate the individual data points of the total number of *Kiss1*-positive cells and solid circles indicate the individual data points of the *Kiss1*- and *Oprk1*-positive cells in the ARC. n=4 for each group. The number in each column indicates the number of animals used.

Figure 8. Effect of negative feedback level of E2 treatment on hypothalamic *Pdyn* expression in OVX rats. *Pdyn*-expressing cells in the PVN (A), ARC (B) and SON (C) in representative OVX and OVX + low E2 rats. The insets indicate *Pdyn*-expressing cells (black arrowheads) at higher magnification. The E2 treatment increased the intensity of *Pdyn* mRNA signals and the number of *Pdyn*-expressing cells in the PVN (D), but not in the ARC (E) and SON (F) of OVX rats. Scale bars, 100 μm. Values are means ± SEM. Open circles indicate individual data points in each group. n=3 for each group. The numbers of each column indicate the number of animals used. * Significant difference between OVX and OVX + low E2 rats ($P < 0.05$, Welch *t*-test).

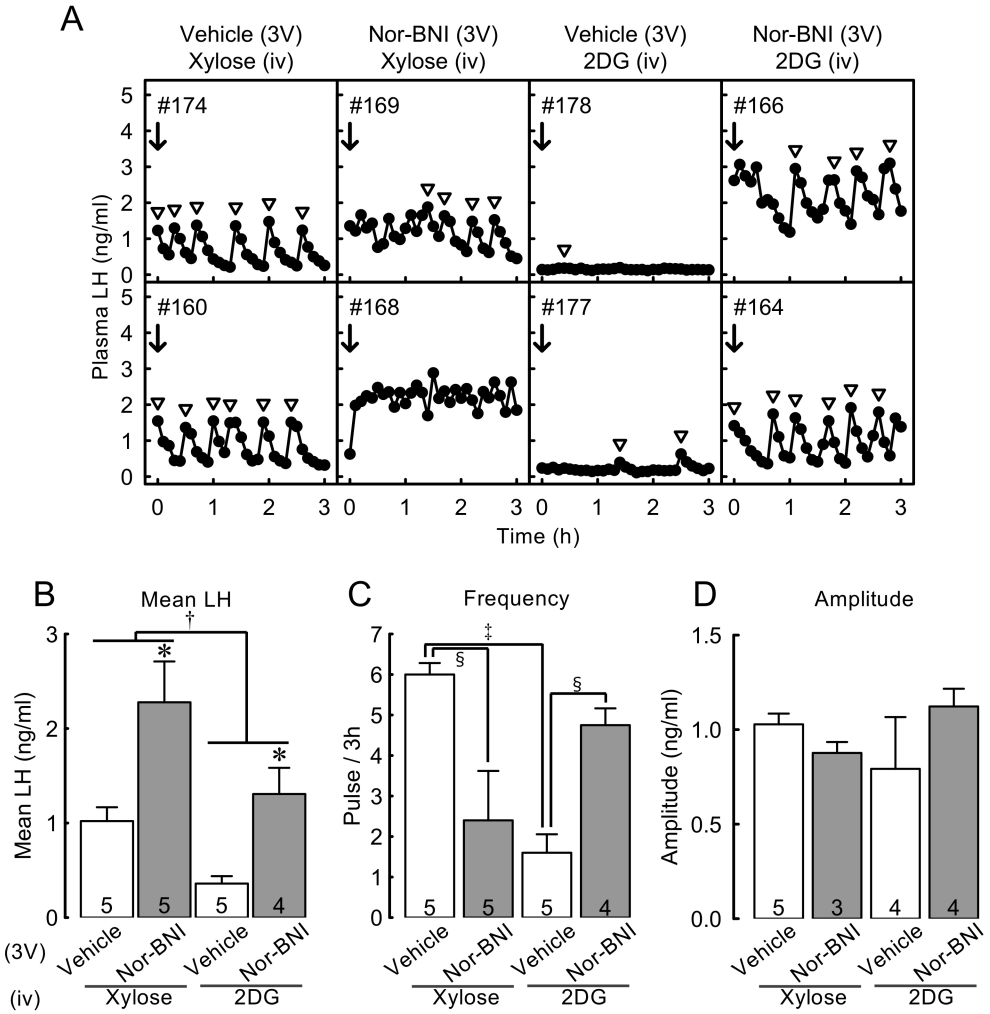

Figure 1. Tsuchida *et al*.

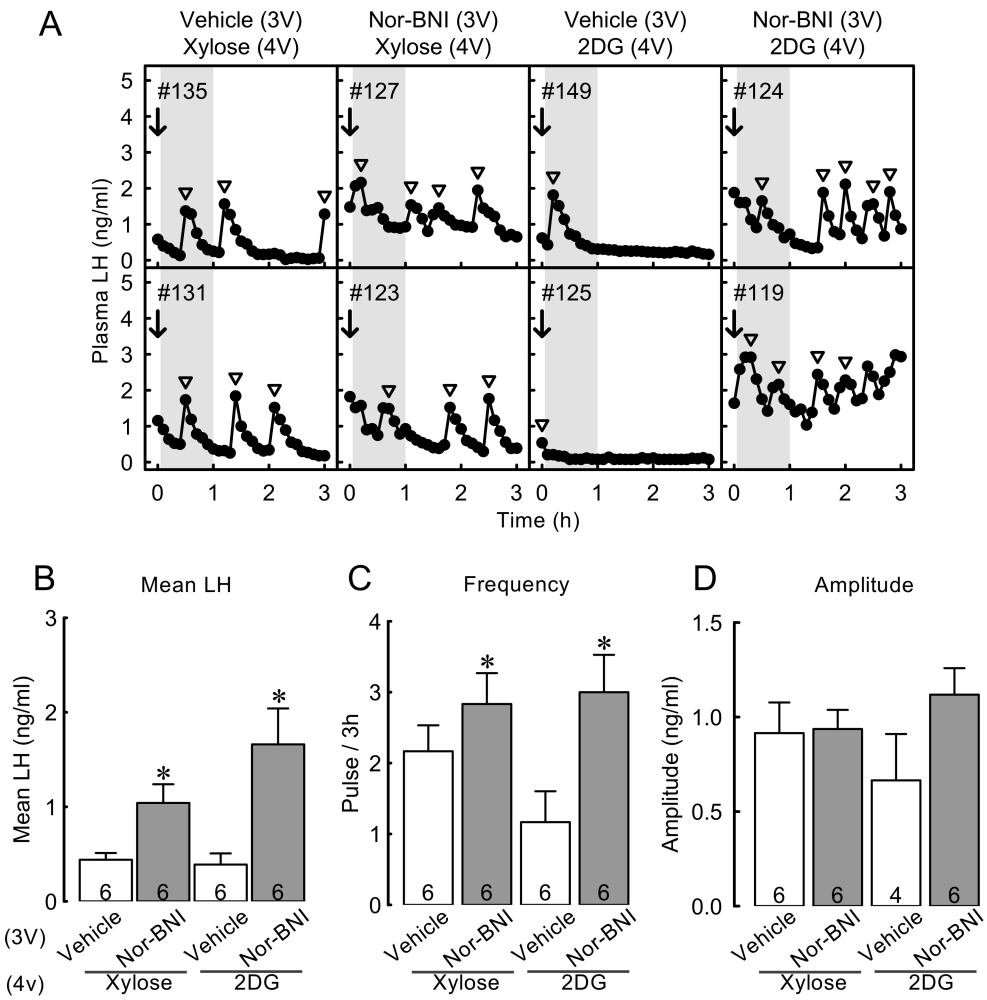

Figure 2. Tsuchida *et al*.

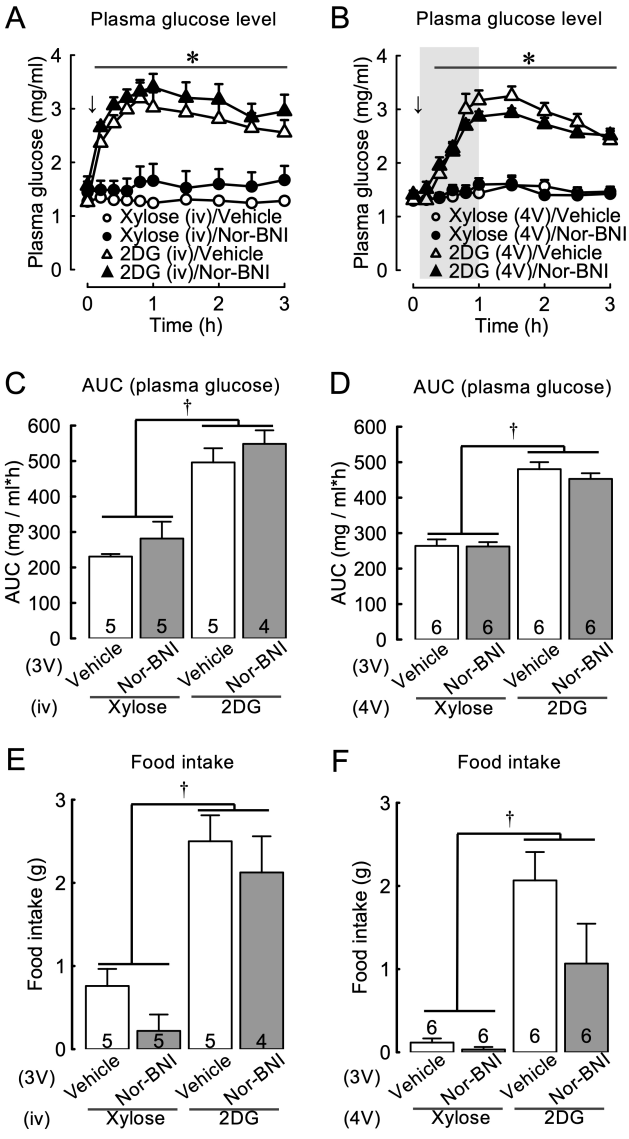

Figure 3. Tsuchida *et al.*

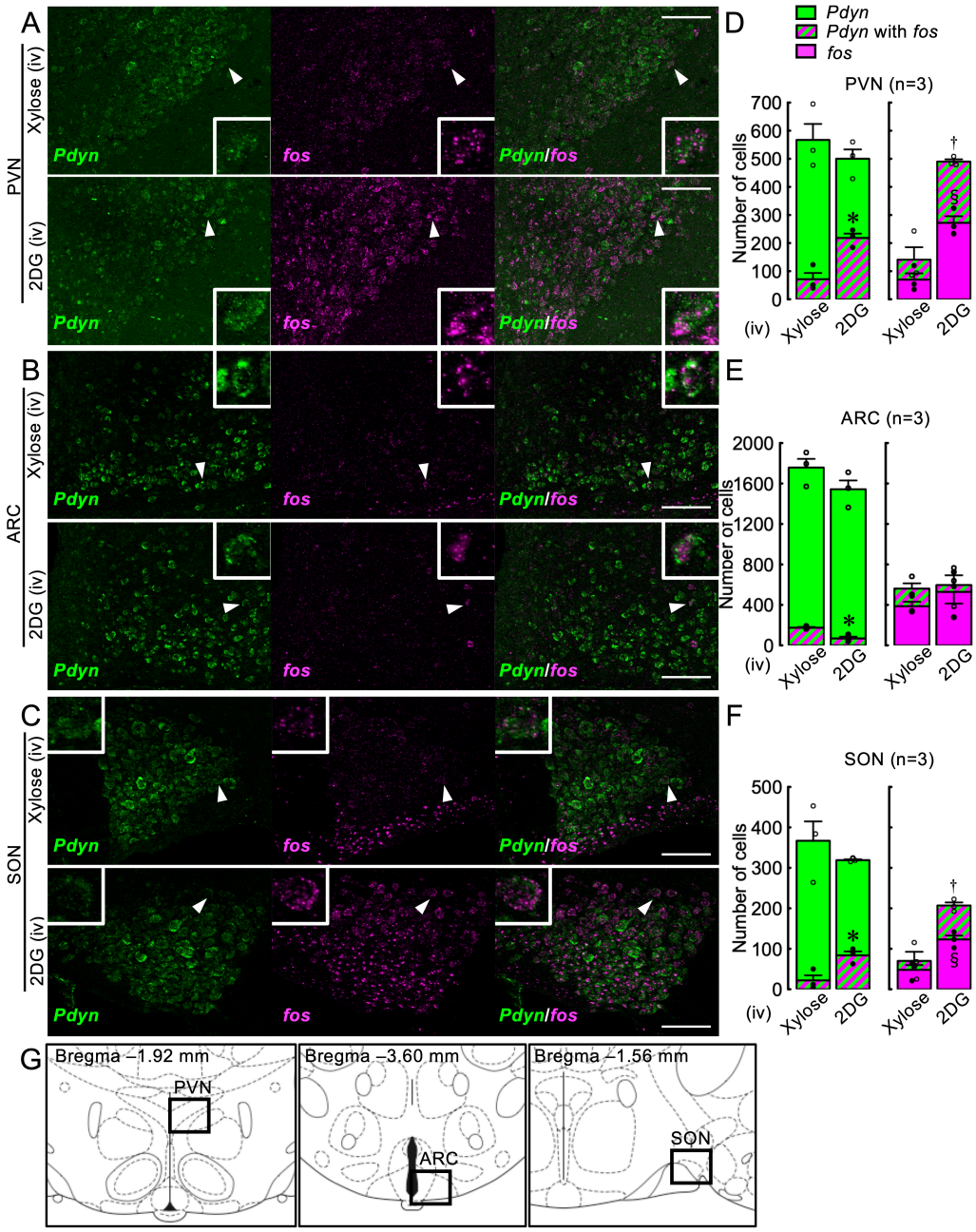

Figure 4. Tsuchida *et al.*

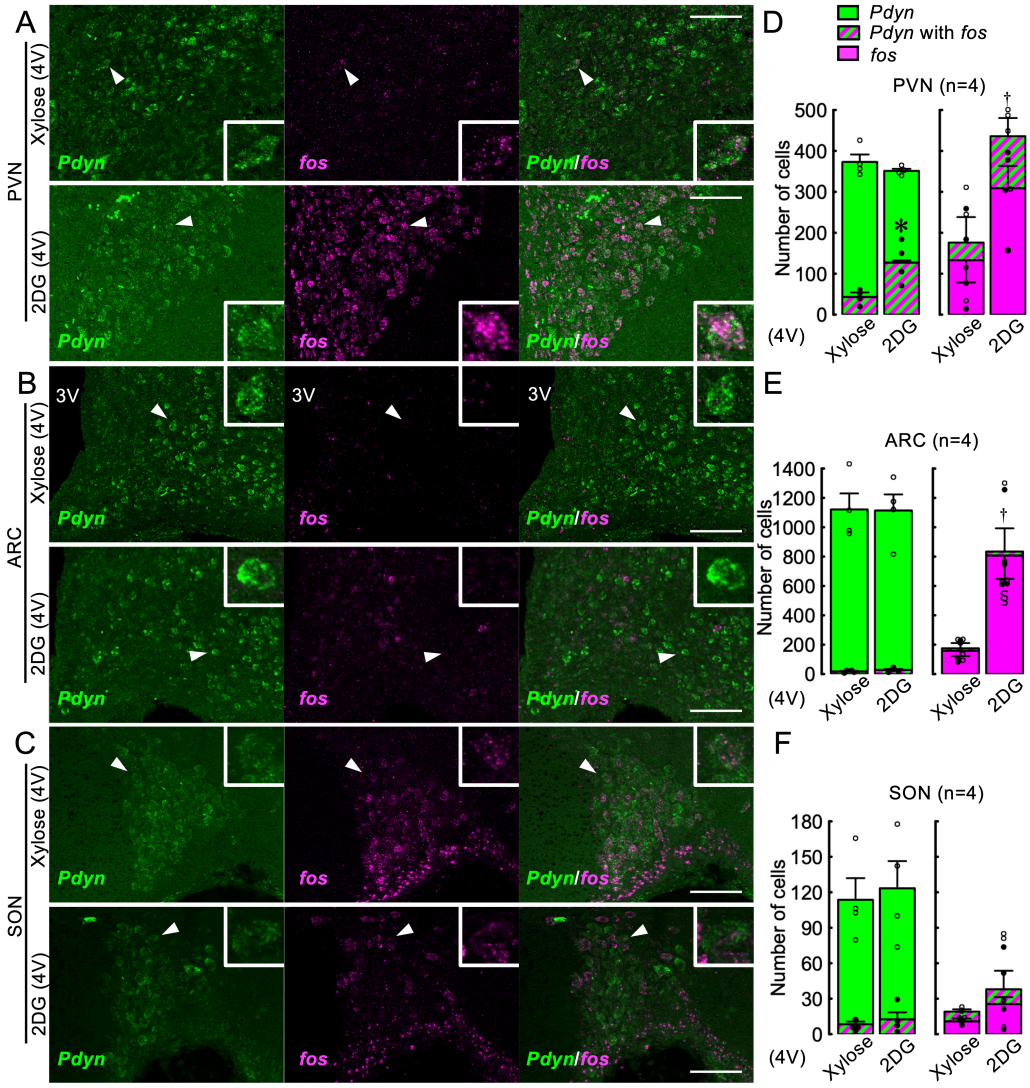

Figure 5. Tsuchida *et al*.

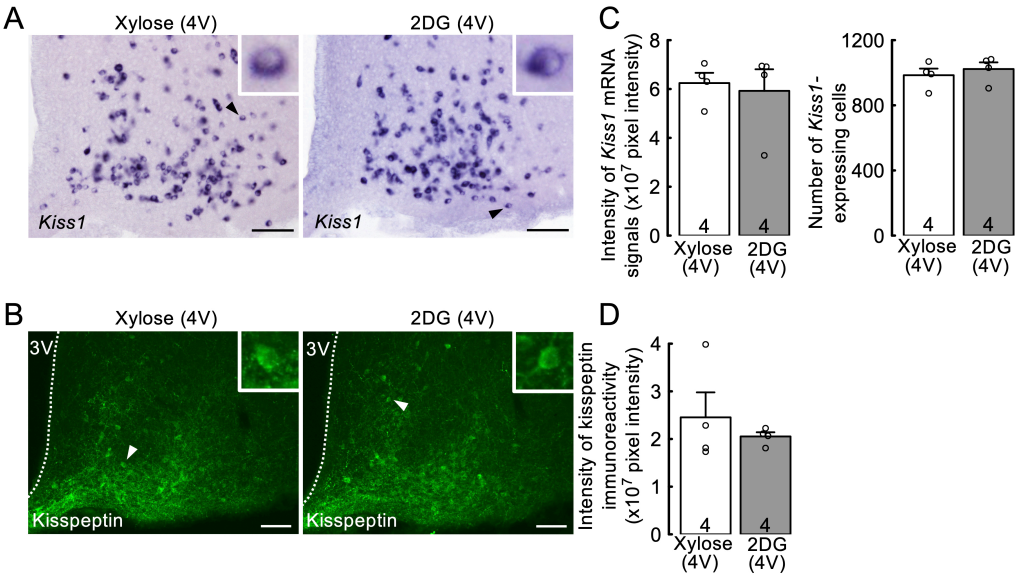

Figure 6. Tsuchida *et al*.

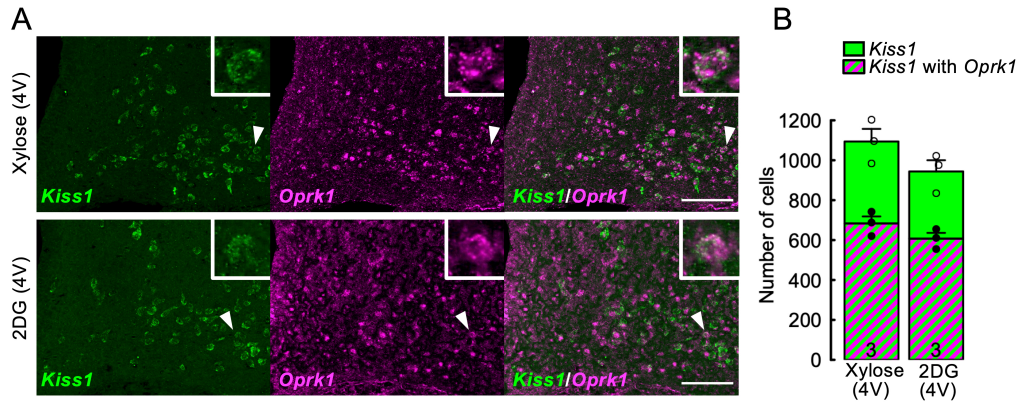

Figure 7. Tsuchida *et al*.

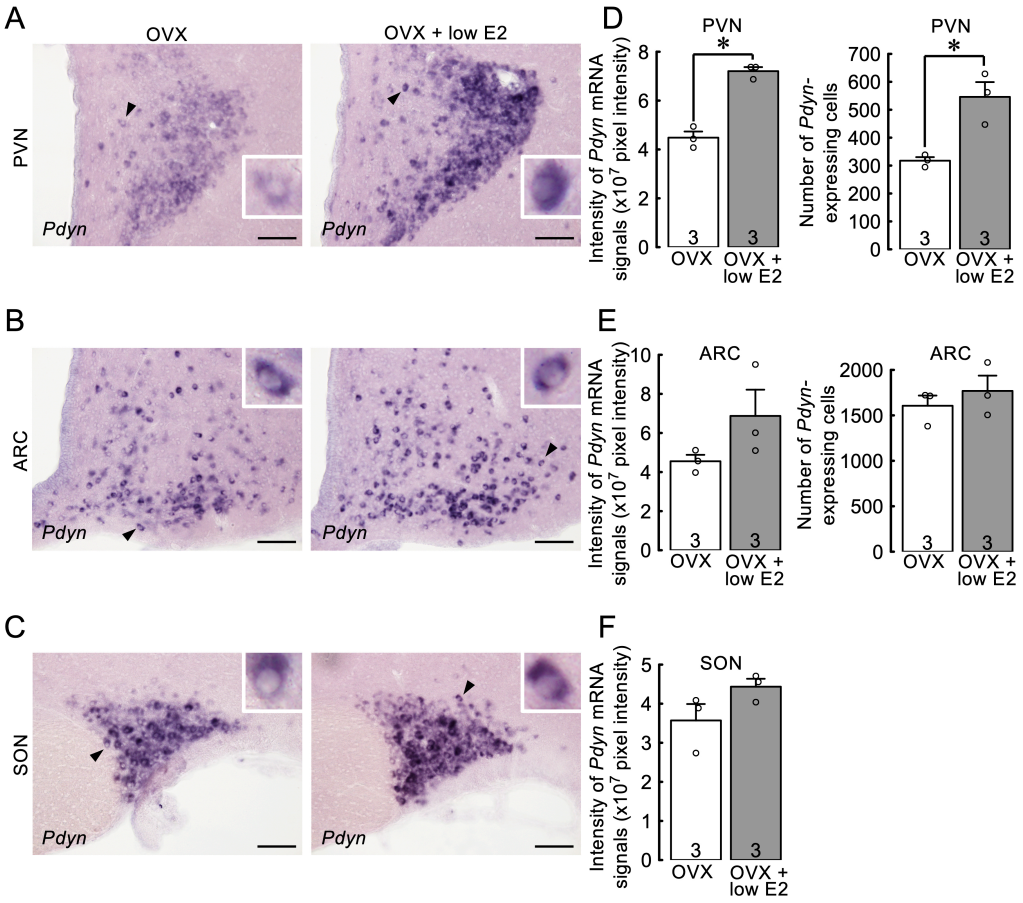

Figure 8. Tsuchida *et al*.