Neuropeptide W: An Anorectic Peptide Regulated by Leptin and Metabolic State

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Neuropeptide W (NPW) is an anorectic peptide produced in the brain. Here, we showed that NPW was present in several hypothalamic nuclei, including the paraventricular hypothalamic nucleus, ventromedial hypothalamic nucleus, lateral hypothalamus, and hypothalamic arcuate nucleus. NPW expression was significantly up-regulated in leptin-deficient ob/ob and leptin receptor-deficient db/db mice. The increase in NPW expression in ob/ob mice was abrogated to control levels after leptin replacement. Leptin induced suppressors of cytokine signaling-3 after phosphorylation of signal transducer and activator of transcription-3 in NPW-expressing neurons. In addition, we demonstrated that NPW reduces feeding via the melanocortin-4-receptor signaling pathway. We also showed that NPW activates proopiomelanocortin and inhibits neuropeptide Y neurons using loose-patch extracellular recording of these neurons identified by promoter-driven green fluorescent protein expression. This study indicates that NPW may play an important role in the regulation of feeding and energy metabolism under the conditions of leptin insufficiency. (Endocrinology 151: 2200–2210, 2010)

The fine regulation of feeding requires a complicated interaction of multiple orexigenic and anorectic signals produced in the brain and peripheral tissues. Identification of substances involved in feeding regulation has provided new insight into the molecular and cellular basis of energy homeostasis. A variety of substances involved in feeding regulation have previously been identified. α-MSH, derived from proopiomelanocortin (POMC), is one of the key molecules involved in regulation of feeding. α-MSH released from axon terminals binds to and activates the melanocortin-4-receptor (MC4-R), reducing food intake and body

Abbreviations: aCSF, Artificial cerebrospinal fluid; AGRP, agouti-related protein; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ARC, hypothalamic arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; DAB, 3,3'-diaminobenzidine tetrahydrochloride; EGFP, enhanced green fluorescent protein; JAK, Janus-activated kinase; iox, intracerebroventricular; LH, lateral hypothalamus; MC4-R, melanocortin-4-receptor; NPW, neuropeptide W; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular hypothalamic nucleus; Rseal, seals of 4- to 11-MΩ resistance; sIPSCs, spontaneous inhibitory postsynaptic current; SOCS3, suppressors of cytokine signaling-3; STAT3, signal transducer and activator of transcription-3; TRH, TSH-releasing hormone; VMH, ventromedial hypothalamic nucleus; VMHC, VMH central.
weight (1). POMC neurons are activated by leptin (2), whereas they are inhibited by neighboring orexigenic substances, neuropeptide Y (NPY) or agouti-related protein (AGRP) (3).

Neuropeptide W (NPW), a hypothalamic peptide, was previously identified as an endogenous ligand for GPR7 and GPR8 by our research group (4). A single intracerebroventricular (icv) administration of NPW to fasting rats or free-feeding rats before dark phase (feeding phase) suppressed food intake and increased both heat production and body temperature (5). Continuous icv infusion of NPW also suppressed feeding and body weight gain over the infusion period, suggesting that NPW functions as an endogenous catabolic signaling molecule. By contrast, there were some reports that icv administered NPW in light phase increased food intake (4, 6, 7). The GPR7 is robustly expressed in the suprachiasmatic nucleus, an area critical for the regulation of circadian rhythm. This suggests that the differences of data of NPW-related feeding may occur through diurnal variation of a functional relationship between NPW and its receptor present in the suprachiasmatic nucleus.

Many of the neuropeptides that regulate feeding are closely associated with leptin signaling pathways in the hypothalamus (8, 9). To investigate whether NPW is under the control of leptin, we examined the distribution of NPW-containing neurons in the hypothalamus. Interestingly, NPW neurons were found in hypothalamic nuclei known to be involved in feeding regulation, including the ventromedial hypothalamic nucleus (VMH), in which neuropeptides had not previously been identified. To address the role of endogenous NPW in leptin signaling, we evaluated NPW mRNA expression in the hypothalamus of leptin-deficient (ob/ob) mice with or without leptin replacement, ob/ob mice pair-fed with the leptin-treated ob/ob mice, ob/ob mice made Euglycemic with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) treatment, and leptin receptor-deficient db/db mice. Unexpectedly, NPW expression increased with disruption of leptin signaling and returned to control levels with leptin replacement. Additionally, NPW expression increased in the hypothalamus of diet-induced obese rats. We examined the mechanism by which leptin regulates NPW gene expression by examining the phosphorylation of signal transducer and activator of transcription-3 (STAT3) and the induction of suppressors of cytokine signaling-3 (SOCS3) in the hypothalamic neurons. We also used immunoelectron microscopy, physiological and electrophysiological experiments to investigate a neuronal circuit involved in NPW-induced feeding reduction.

Materials and Methods

Experimental animals

Animals were individually housed in plastic cages at a constant room temperature in a 12-h light, 12-h dark cycle (0800–2000 h light). Male Wistar rats weighing 320–350 g and C57Bl1/6 (12-wk-old males; Charles River Japan, Inc., Shiga, Japan), ob/ob (12-wk-old males; Charles River Japan, Inc.), and db/db (12-wk-old males; Charles River Japan, Inc.) mice were given standard laboratory chow and water ad libitum. C57Bl1/6 mice were divided into two groups. The first was given ip injections of saline as a control group (n = 6). The second group was given ip injections of murine recombinant leptin (1 μg/g initial body weight, twice daily, 0900 and 1900 h) for 5 d (n = 6). Ob/ob mice (12-wk-old male; Charles River Japan, Inc.) were divided into three groups. The first group received ip injections of either murine recombinant leptin (1 μg/g initial body weight, twice daily at 0900 and 1900 h) (n = 6) or saline (n = 6) for 10 d, then fed ad libitum. The second group was pair-fed with the leptin-treated mice (n = 6). The third group was given a sc injection of AICAR (250 mg/kg initial body weight; Toronto Research Chemical, Inc., Toronto, Ontario, Canada) for 7 d (n = 6). Body weight and blood glucose were measured each morning before leptin or AICAR administration.

Twenty Wistar rats (3-wk-old males; Charles River Japan, Inc.) were individually housed and fed standard laboratory chow for the first week. Ten rats were at random placed on a high-fat diet (5.2 kcal/g) containing 60% fat, 20% carbohydrate, and 20% protein (Research Diets, Inc., New Brunswick, NJ). The remaining rats (n = 7) were placed on a low-fat diet (3.8 kcal/g) containing 10% fat, 70% carbohydrate, and 20% protein (Research Diets, Inc.). Body weight and energy intake were monitored once a week. After 10 wk on the high-fat diet, the seven rats whose body weights exceeded the average of those of the control rats were assigned to the diet-induced obesity group (body weight gain: control group, 481 ± 16 g; high-fat diet group, 644 ± 15 g; P < 0.0001). The remaining three rats with high-fat diet whose body weights were similar to or less than the average of the control group. Therefore, we discarded them from this study.

We implanted icv cannulae into the lateral cerebral ventricles of rats after anesthesia with ip sodium pentobarbital injection (40 mg/kg; Abbot Laboratories, Chicago, IL). Proper placement of the cannula was verified by dye administration at the end of the experiment. Only animals exhibiting progressive weight gain after surgery were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society’s guidelines for animal care. This protocol was approved by the Ethics Review Committee for Animal Experimentation of the Faculty of Medicine, University of Miyazaki.

Preparation and characterization of antiserum

To generate a monoclonal antibody recognizing the C terminus of NPW23, we immunized 6- to 8-wk-old female BALB/c mice with porcine thyroglobulin-conjugated [1Cys] human NPW-[11-23]. Spleen cells isolated from immunized mice 3 d after iv immunogen injection were fused with mouse myeloma cells, P3X63Ag8·653, as described (10). ANPW23-C (IgG1, κ), a monoclonal antibody specific for the C terminus of NPW23, was purified from ascitic fluid using an immobilized protein A column (Seikagaku Co., Tokyo, Japan). To confirm the biding.
specification of ANPW23-C to rat NPW23, we investigated cross-relativities of ANPW23-C to rat NPW30 and rat neuropeptide B, which show sequence similarity to rat NPW23 at the N terminus using ELISA system. The binding of ANPW23-C to biotinylated [15Cys] human NPW-[11-23] was efficiently inhibited by rat NPW23 in dose-dependent manner, whereas both of rat NPW30 and neuropeptide B did not inhibit the binding. These data indicate that ANPW23-C strictly recognizes the C-terminal region of rat NPW23.

**NPW mRNA expression in the hypothalamus of rats**

Total RNA was extracted from five male Wister rats. The paraventricular hypothalamic nuclei (PVN) were punched out of brain slices 8.1–7.4 mm rostral to the interaural line (11). We also punched out the lateral hypothalamus (LH), VMH, and hypothalamic arcuate nucleus (ARC) of slices 7.2–4.8 mm rostral to the interaural line. The punched out diameters of the PVN, LH, VMH, and ARC were 0.9, 1.7, 1.0, and 1.1 mm, respectively. We also removed a part of the cerebral cortex to use as a control. Total RNAs were extracted from the punched out tissues and cerebral cortex using TRIZOL Reagent (Invitrogen Corp.). First-strand cDNA was synthesized from 1.5 μg RNA with an oligo (deoxynzymid) 1 μl primer and Superscript III polymerase (Invitrogen Corp.); resulting samples were subjected to PCR amplification using primers specific for rat NPW (sense primer, 5′-TGGCGCTCGCAGCAGAGACTAC-3′ and antisense primer, 5′-ACCCACTGTGTGATAGC-3′), corresponding to nucleotides 113–133 and 241–261 of accession no. AB084278 in GenBank. The PCR products were electrophoresed on a 2% agarose gel (FMC BioProducts, Rockland, ME).

**Immunohistochemical study**

Three male Wistar rats were used for immunohistochemical studies. To enhance NPW-producing neuron immunostaining, we injected colchicine (100 μg/rat) into the lateral ventricle 30 h before perfusion. Rats were first perfused transcardially with 0.1 M phosphate buffer (pH 7.4), then with 4% paraformaldehyde in 0.1 M phosphate buffer. The hypothalamus was sectioned into 40-μm slices at −20 °C using a cryostat. Hypothalamic sections were incubated for 2 d at 4°C with ANPW23-C antiserum diluted 1:3000. Sections were stained according to the avidin-biotin complex method (12).

**Quantitative RT-PCR**

The three groups of ob/ob mice, db/db mice, control mice, and C57Bl/6j mice treated for 5 d with leptin were killed at 1000 h. Rat fed with high-fat or low-fat diet were also killed at 1000 h. The operated rats were administered leptin (150 μg/kg body weight) or saline icv at 1930–2000 h, then killed 2 h after injection (n = 8 in each group). Total RNAs were extracted from the brain or hypothalamus of these mice or rats, respectively, using TRIZOL Reagent (Invitrogen Corp.). To get the hypothalamus, we first obtained brain slices 7.7–4.2 mm rostral to the interaural line, then removed the cerebral cortex and hippocampus from these slices. TaqMan PCR analysis was used to quantitate the relative levels of NPW mRNA levels using a standard method (13). Detection of NPW mRNAs was performed using proprietary predeveloped TaqMan primers (5′-GCTTCGCTGTGAGAGCTT-3′ and 5′-ACAGGATCCGGAAAAGATGA- 

**TTT-3′, corresponding to nucleotides 544-561 and 587-608 of accession no. AB084278 in GenBank, respectively) and a 6-carboxy-fluorescein-labeled probe (5′-TGCCCAGCAGATGGTCCTGCA-3′, corresponding to nucleotides 564-587 of accession no. AB084278 in GenBank, Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Glyceraldehyde-3-phosphate dehydrogenase mRNA levels, which were measured using a TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase Control Regent (VIC Dye; Applied Biosystems), were used to normalize the data.

NPW (30 μg/kg body weight) or saline was administered icv to rats at 1930–2000 h (n = 8 in each group). Two hours after administration, total RNAs were extracted from hypothalami that had been removed from the killed animals. Quantitative RT-PCR for NPY, AGRP, POMC, or cocaine- and amphetamine-regulated transcript (CART) was conducted on a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) using a LightCycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics GmbH) with the following primer sets specific for rat NPY: 5′-CTGTGGAGCTGACCCTGCGTCAT-TAT-3′ and 5′-CATGTCCTCTGTTGCGCGTCTCG-3′ (corresponding to nucleotides 96-120 and 182-206 of accession no. NM012614 in GenBank), rat AGRP: 5′-TCTGAGAGAGACGGACGACCGCA-3′ and 5′-AGCCACGCGGAGAACGACT-3′ (corresponding to nucleotides 345-369 and 434-454 of accession no. XM574228 in GenBank), rat POMC: 5′-GAGCTACCCGAGAAGGAAACCTG-3′ and 5′-ACTTC-CGGGGATTTTCACTGAAAG-3′ (corresponding to nucleotides 106-129 and 229-253 of accession no. AF510391 in GenBank), or rat CART: 5′-ATGGAGAGCTCCGGCGTCG-3′ and 5′-CAGCTCTCCTCTCAATGGAG-3′ (corresponding to nucleotides 20-37 and 179-186 of accession no. U10071 in GenBank). RNA numbers were obtained by comparison with standard curves generated from known amounts of NPY, AGRP, POMC, and CART cDNA. Rat rRNA levels were also measured as internal controls.

Rats with implanted cerebroventricular cannulae were subjected to a 52-h fast. Fasting decreases endogenous leptin levels, which enabled us to observe the effect of exogenous leptin. At 12-h intervals, rats that had fasted 52 h were administered leptin (10 μg/kg body weight) or saline icv beginning the onset of fasting (n = 8 in each group). At the end of the 52-h experiment period, total RNA was extracted from VMH. Quantitative RT-PCR for SOCS3 was performed on a LightCycler system (Roche Diagnostics GmbH) as described above. The primer set for rat SOCS3 was 5′-GGTACCCCGGAGAGCTTACTA-3′ and 5′-CTCTTTAAAGTGGAGCATCCTAGT-3′ (corresponding to nucleotides 491-514 and 699-723 of accession no. NM053365 in GenBank). Known amounts of SOCS3 cDNA were used to obtain a standard curve. Rat rRNA levels were used as internal controls.

**STAT3 phosphorylation and SOCS3 expression**

Primary neurons were obtained from the VMH, PVN, ARC, or LH of five rats at 5–6 wk of age. After collagenase and papain digestion (14, 15), isolated neurons were cultured for 5 d at 37 C in 5% CO2 on polyethyleneimine-coated Lab-Tek chamber slides in complete DMEM (with 25 mM glucose) supplemented with 10 mM HEPES (Life Technologies, Inc., Grand Island, NY), 5% newborn calf serum (Life Technologies, Inc.), 5% horse serum (Life Technologies, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO), 4 mM
Generation of POMC-enhanced green fluorescent protein (EGFP) and NPY-EGFP mice

All mice used in this study were hetero- or homozygous for the transgene in which expression of an EGFP fusion protein is directed by POMC or NPY genomic elements and selectively expressed in POMC or NPY producing neurons (POMC-EGFP or NPY-EGFP) (2, 17) in animals of a C57Bl/6j background. Mice of both male and female fed ad libitum were used in this study. They were housed in a rodent facility on a 12-h light, 12-h dark cycle, with lights on at 0715 h, and had ad libitum access to water and food (fat 4.5%; Lab Diet 5001, Rodent Diet; PMI Nutrition International, LLC, Brentwood, MO). All mice were weaned at least 6 d before recording and decapitated at age of 30–70 d old between 1130–1200 h. These animal experiments were conducted in accordance with the Oregon Health and Science University Animal Care and Use Committee.

Slice preparation

Young adult (30–70 d old) POMC-EGFP or NPY-EGFP mice were deeply anesthetized with halothane before decapitation. The brain was entirely removed and immediately submerged in ice-cold, gassed (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF), containing (in mM): 133.2 NaCl; 3.1 KCl; 2 CaCl₂; 1 MgCl₂; 1 NaH₂PO₄; 26.2 NaHCO₃; 10 glucose; 320 mosm/kg, pH 7.4 when gassed with 95% O₂; and 5% CO₂. A brain block of the hypothalamus containing ARC was then made to cut coronal slices of 200-μm thickness using a vibrating slicer (VT1000S; Leica Microsystems, Wetzlar, Germany). After slice preparation was complete, the slices were incubated at 30 C for at least 1 h before being transferred to recording chamber.

Electrophysiology

Before commencement of recording, a slice was transferred to the recording chamber (~0.7 ml in volume of submersion type) and allowed to equilibrate for 10–20 min where it was bathed with the 2–3 ml min⁻¹ of oxygenated warm (30–31 C) aCSF. EGFP-fluorescent neurons were unambiguously identified and patched using combined epifluorescence and infrared-differential interference contrast optics. Fluorescent neurons of healthy infrared-differential interference contrast optics appearance but of every level of fluorescence brightness were chosen for electrophysiological recording. In this study, loose patch extracellular recording was mainly used to record the action potential activity of neurons (18) and whole-cell patch clamp recordings were mainly used to obtain information about membrane potentials and currents. During loose patch extracellular recording, patch pipettes which were filled with recording aCSF and had resistances of approximately 1.6–2.8 MΩ were used to form seals of 4–11-MΩ resistance. The patched membrane was drawn into the pipette. When this increase was large, additional strain was prevented by applying a small amount of positive pressure from a short column of water attached via tubing to the pipette holder. Whole-cell recordings were performed using patch pipettes of 2.2- to 5-MΩ resistance when filled with a solution containing (in mM): 125 K gluconate; 8 KCl; 5 MgCl₂; 10 HEPES; 5 NaOH; 4 Na₂ATP; 0.4 Na₃GTP; 15.4 sucrose; and 7 KOH, which resulted in a pH approximately 7.23 and osmolality of 295–300 mosm/kg.

Data were acquired at 10 kHz using a MultiClamp 700A amplifier (×2000 gain; –3dB filter freq, 5 kHz) and Clampex 8.2 software (Axon Instruments, Union City, CA). Data were analyzed using Mini Analysis Program 5.6.28 (Synaptosoft, Decatur, GA), GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA) and Excel 2000 (Microsoft Corp., Bellevue, WA). The pairs of data obtained from control and treated groups were compared against one another by paired Student’s t test, except where indicated, and presented as mean ± SEM. Significance level was set to 0.05 for all tests.

Immunoelectron microscopy

Three colchicine-treated Wistar rats were perfused as described above. Isolated brains were cut into 30- to 40-μm sections using an Oxford vibratome (Oxford Instruments, Abingdon, UK). Electron microscopy immunohistochemistry was performed using ANPW23-C and then stained with the 3,3’-diaminobenzidine tetrahydrochloride (DAB)-silver-gold-intensification method. After this, second immunostainings were performed with rabbit anti-POMC (1:8000, a gift from S. Tanaka, Shizuoka University) antiserum (19), or rabbit anti-NPY antiserum (1:50,000; DiaSorin, Inc., Stillwater, MN) on the same sections as previously described (20).

Feeding experiments

Rats were administered vehicle alone (saline or normal rabbit IgG, n = 6) or a MC4-R antagonist, AGRP (16 μg/kg, n = 6; Peptide Institute, Osaka, Japan) or SHU9119 (1.2 μg/kg, n = 6; Sigma Chemical Co.) icv 1 h before injection of NPW. These doses are known to increase food intake and cancel mSHI-induced feeding reduction (21, 22). After that, either NPW (40
μg/kg) or saline was administered icv to rats, and 2-h food intake was then measured. The rats fasted in between the two injections.

**Statistical analysis**

Except for electrophysiological studies, we analyzed groups of data (means ± SEM) using ANOVA and post hoc Fisher’s tests. 
P values less than 0.05 were considered to be significant (two-tailed tests).

**Results**

**NPW expression in the hypothalamus**

To identify the mechanism of NPW-induced feeding regulation, we investigated the distribution of NPW in the hypothalamus. Both an NPW transcript and NPW-immunoreactive neurons were observed in the parvocellular division of the PVN, the central division of the VMH, the perifornical region of the LH, and the ARC (Fig. 1A–G). NPW mRNA expression was not found in the cerebral cortex (Fig. 1A). In this immunohistochemical study, five sections containing these hypothalamic nuclei of three rats each were observed. Neurons expressing NPW were also seen in the lateral septal nucleus, ventral pallidum, bed nucleus of the stria terminalis, medial preoptic area, posterior hypothalamic area, dorsal raphe nucleus, and periaqueductal gray (data not shown). These cells were of varied morphology, with sizes ranging 20–30 μm in diameter. As controls, no immunoreactivity for NPW could be detected in these tissues when either normal rabbit serum or antiserum preabsorbed with excessive NPW was used (Fig. 1, H and I).

**NPW mRNA expression in diet-induced obese rats and ob/ob or db/db mice**

To examine the effect of leptin on NPW expression, we quantitated NPW mRNA expression in the hypothalami of ob/ob mice. Expression of NPW mRNA was significantly increased in ob/ob mice with or without AICAR treatment in comparison with either control mice or leptin-replaced ob/ob mice (Fig. 2A). NPW mRNA was also up-regulated in db/db mice and in ob/ob mice pair-fed with leptin-replaced ob/ob mice (Fig. 2A). There were no differences in NPW mRNA levels between control and leptin-replaced ob/ob mice (Fig. 2A). There were no changes in the NPW mRNA levels in the hypothalamus after ip administration of leptin to wild-type mice for 5 d or a single icv injection of leptin into rats (Fig. 2, B and C). To verify a physiological role of NPW, we also investigated NPW mRNA expression in the hypothalamus of diet-induced obese rats. Expression of NPW mRNA was significantly increased in diet-induced obese rats compared with rats bred with normal chow (Fig. 2D).

**Leptin-dependent STAT3 phosphorylation and SOCS3 expression**

Ob-Rb stimulates the Janus-activated kinase (JAK)-STAT signaling pathway, leading to increases in the phosphorylated form of STAT3. We therefore investigated whether STAT3 is phosphorylated in leptin-treated NPW neurons of the VMH (Fig. 3, A–D), PVN, ARC, or LH (Supplemental Fig. 2). Before starting this experiment, we confirmed that NPW and ob-Rb staining colocalized in extensive numbers of neurons (Supplemental Fig. 1). STAT3 phosphorylation was strongly detected in both the cytoplasm and nucleus of the majority of NPW-expressing neurons after a 20-min treatment with leptin in comparison with saline (Fig. 3, A–D). Approximately 37% (44 of 119) of neurons of the VMH with phosphorylated STAT3 induced by leptin were NPW-immunoreactive neurons. STAT3 phosphorylation was observed in nearly all NPW neurons of the VMH. SOCS3, a direct target of STAT3, was intensively detectable in NPW-expressing neurons af-
The effect of NPW on NPY, AGRP, POMC, and CART

To investigate the mechanism of NPW-induced feeding reduction, we analyzed NPY, AGRP, POMC, and CART mRNA expression in the rat hypothalamus 2 h after ivc administration of NPW by quantitative RT-PCR. NPW significantly decreased AGRP but did not affect NPY mRNA expression (Fig. 4, A and B). Furthermore, using loose patch recording from NPY-GFP neurons located in the ARC, we found that bath application of 50–100 nm of NPW significantly decreased action potential firing activity of eight ARC NPY neurons from 2.1 ± 0.4 Hz in control to 1.03 ± 0.2 Hz (n = 8, P < 0.05; Fig. 4, C and D). NPW also significantly increased POMC but did not affect CART mRNA expression (Fig. 5, A and B). Using CsCl electrodes, we performed whole-cell recording of POMC-GFP neurons in the ARC (n = 14). Bath applications of 50–100 nm of NPW significantly decreased frequency of spontaneous inhibitory postsynaptic current (sIPSCs) recorded from ARC POMC neurons from 8.7 ± 0.8 Hz in control to 6.3 ± 0.9 Hz (n = 14, P < 0.01; Fig. 5, C–E). NPW had no significant effects on the amplitude of the sIPSCs (n = 14; Fig. 5, C, D, and F).

Immunoelectron microscopy

We also examined the anatomical relationship between POMC or NPY/AGRP neurons and NPW-containing fibers. NPW-immunoreactive neuronal axons and terminals contained large dense-core synaptic vesicles, as indicated by DAB-silver-gold-intensification reaction products (Fig. 6, A–D). POMC or NPY expression was visualized by DAB labeling, seen as a light-to-dark gray (Fig. 6, A–D). POMC-immunoreactive neuronal dendritic processes often received synapses from NPW-immunoreactive axon terminals (Fig. 6A). We ob-

FIG. 2. A, NPW mRNA levels in control mice (C57B1/6j), db/db mice, ob/ob mice pair-fed with leptin-replaced ob/ob mice, ob/ob mice receiving 7 d of AICAR (250 mg/kg, ip), or ob/ob mice treated with leptin (1 μg/g, ip, twice daily) or saline for 10 d (n = 6 in each group). *, P < 0.005; **, P < 0.0001 vs. control. B, NPW mRNA levels in the hypothalami of C57B1/6j mice receiving either leptin (1 μg/g, ip) or saline for 5 d (n = 6 in each group). C, NPW mRNA levels in the hypothalami of rats were measured 2 h after receiving either leptin (150 μg/kg, icv) or saline (n = 8 in each group). D, NPW mRNA levels in the hypothalami of diet-induced obese rats (n = 7 in each group). *, P < 0.05.

FIG. 3. Induction of STAT3 phosphorylation or SOCS3 expression in leptin-treated NPW-expressing neurons. A, Phospho-STAT3-immunoreactivity in neurons of the VMH 20 min after saline treatment. B, Phospho-STAT3 was intensified in neurons of the VMH 20 min after leptin treatment (10−10 μ) (red, phospho-STAT3). C, NPW-immunoreactive neurons of the same sample as that shown in B (green, NPW). D, Overlap of B and C revealed STAT3 phosphorylation in leptin-treated NPW-expressing neurons (neurons coexpressing phospho-STAT3 and NPW are yellow). E, SOCS3-immunoreactivity in neurons of the VMH 60 min after saline treatment. F, SOCS3 expression in neurons of the VMH was intensified 60 min after leptin treatment (10−10 μ) (red, SOCS3). G, NPW-immunoreactive neurons of the same sample as that shown in F (green, NPW). H, Overlap of F and G revealed that SOCS3 expression is induced in leptin-treated NPW neurons (yellow indicates neurons coexpressing SOCS3 and NPW). I, SOCS3 mRNA levels of the VMH in 52-h fasted rats receiving leptin at 12-h intervals (10 μg/kg, icv) or saline (n = 8 in each group). *, P < 0.0001.
 vascular and neural mechanisms that regulate energy intake and expenditure and that may underlie the effects of leptin on feeding behavior. It is known that the hypothalamus contains a substantial number of neuropeptide-containing neurons involved in the regulation of energy and water balance. These neurons are distributed throughout distinct hypothalamic areas and are involved in a variety of metabolic functions, including feeding, energy metabolism, and reproduction. The hypothalamic neurons that secrete the anorexigenic hormone leptin are located in the arcuate nucleus (ARC) and provide an important link between peripheral signals and the control of energy homeostasis.

In this study, the authors examined the expression of the anorexigenic hormone leptin in the hypothalamic neuron population receiving visual afferent input from the suprachiasmatic nucleus (SCN). They found that the number of leptin-expressing neurons in the ARC was significantly decreased in response to food deprivation, suggesting that these neurons are involved in the regulation of food intake and energy balance. The findings of this study support the hypothesis that leptin is a key player in the neural mechanisms that control energy homeostasis and may provide new insights into the cellular and molecular mechanisms underlying the effects of leptin on feeding behavior.
Furthermore, hypothalamic NPW expression increased in both ob/ob and db/db mice, who lack leptin signaling. The increase in NPW mRNA level seen in ob/ob mice was abrogated by leptin replacement, whereas NPW expressions in AICAR-treated ob/ob mice and ob/ob mice that were pair-fed with leptin-replaced ob/ob mice remained elevated. Glucose homeostasis in ob/ob mice was restored by AICAR treatment (Supplemental Fig. 3), which activates 5’AMP-activated protein kinase, an important mediator of muscle contraction-induced glucose transport (27). In addition, there were no significant differences in body weight between the leptin-replaced and pair-fed ob/ob mice (Supplemental Fig. 4). These results suggest that conditions associated with an absence of leptin, such as hyperglycemia or hyperphagia, did not affect NPW expression, whereas leptin deficiency increased NPW expression. Diet-induced obese animals have elevated serum leptin and impaired physiological responses to exogenously injected leptin, which together indicates the presence of leptin-resistance (28, 29). We showed that NPW expression was increased in the brains of diet-induced obese rats whose body weight gain significantly exceeded those of control group. Leptin levels of these obese rats elevated compared with those of control rats (control group, 3.90 ± 0.50 ng/ml; high-fat diet group, 21.7 ± 3.1 ng/ml; *P < 0.0001) (Mutoh, E., K. Senba, S. Akieda-Asai, A. Miyashita, and Y. Date, published data). Thus, NPW may play a role in maintaining energy homeostasis when leptin signaling was disrupted.

The ob-Rb regulates gene transcription via activation of the JAK-STAT pathway. The ob-Rb-STAT3 signaling pathway is known to be central to the regulation of food intake and energy expenditure. The ob-Rb is rapidly phosphorylated by JAK2 in response to leptin stimulation then induces tyrosine phosphorylation of STAT3 (30). Phospho-STAT3 dimerizes and translocates to the nucleus, eventually altering gene transcription (31, 32). As shown in this study, leptin increased phospho-STAT3 in most NPW neurons of the VMH, PVN, ARC, or LH. Although we failed to identify STAT-binding sites within leptin-response elements of the rat NPW gene promoter, our data suggest that NPW expression is regulated by the JAK-STAT pathway. Here, we also demonstrated that leptin significantly increased SOCS3 mRNA expression in the VMH and increased SOCS3-immunoreactivity in NPW neurons. SOCS3, one of the proteins induced by leptin-mediated STAT signaling, binds JAKs, which attenuates the ability of JAKs to phosphorylate the STATs (33, 34). Thus, SOCS3 is thought to inhibit leptin activation of the JAK-STAT pathway. Our immunocytochemical data showed that phospho-STAT3 and SOCS3 were
increased in NPW-immunoreactive neurons 20 and 60 min after leptin treatment, respectively. These findings indicate that the induction of STAT3 phosphorylation in NPW neurons by leptin signaling may be immediately suppressed by SOCS3. To further clarify the physiologic importance of leptin-mediated NPW regulation, the JAK-STAT signaling in NPW neurons should be examined after leptin administration in vivo. However, NPW is only detectable after colchicine treatment, and one report suggests that colchicine itself can affect STAT3 signaling (35). As shown here, NPW was overexpressed in both ob/ob and db/db mice and returned to the control level in ob/ob mice with leptin treatment. In addition, we found that SOCS3 mRNA expression in ob/ob mice decreased compared with that of control mice (data not shown). Our data also showed that NPW mRNA of db/db mice who lack the ob-Rb increased more than that of ob/ob mice. As previously mentioned, leptin administrations to rats increased SOCS3 mRNA and inclined to decrease NPW mRNA. Taken together, there would be a possibility that impaired leptin signaling in the NPW neurons may reduce the SOCS3-mediated inhibition of STAT3, increasing NPW gene expression.

To investigate the mechanism of NPW-induced feeding reduction, we performed quantitative RT-PCR and electrophysiologic studies. NPW significantly increased POMC and decreased AGRP, but did not affect CART or NPY, mRNA expression. Additionally, electrophysiologic studies showed that NPW decreases action potential firing activity of NPY/AGRP neurons and sIPSCs of POMC neurons. The decreased inhibitory synaptic input to POMC neurons, presumably arriving from neighboring NPY neurons, should lead to increased firing activity (2). Immunoelectron microscopic studies demonstrated that POMC-immunoreactive perikaryon often received synapses from NPW-containing axon terminals. Furthermore, NPW-containing axon terminals also made synapses with NPY/AGRP neurons of the rat ARC. These findings suggest that NPW may regulate energy metabolism through the MC4-R system. Therefore, we investigated the functional relationship between NPW and MC4-R pathway in feeding behavior. Intracerebroventricular injection of 40 μg/kg of NPW led to significantly decreased feeding in rats, but administration of either AGRP or SHU9119, inhibitors of POMC-MC4-R signaling, before NPW abrogated the NPW-induced reductions in feeding. Although we could not confirm the expression of GPR7, the NPW receptor, in the POMC or NPY/AGRP-expressing neurons, the histologic and electrophysiologic studies make a compelling argument that NPY modulates feeding at least partly through the POMC-MC4-R pathway. To confirm this hypothesis, further studies using MC4-R deficient animals are needed. Additionally, future experiments should be tried to clarify which population of NPW-expressing neurons is important for the regulation of the POMC-MC4-R pathway. To confirm this hypothesis, further studies using MC4-R deficient animals are needed. Moreover, considering that leptin can directly or indirectly regulate TRH, a potent anorectic neuropeptide, which is located in the parvocellular area of the PVN, we also should evaluate the functional and anatomical relationship between NPW and TRH or other hypothalamic substances. The extensive distribution of NPW or GPR7 in the hypothalamus suggests possibilities that NPW may contribute to several kinds of neural circuit involved in energy metabolism. To fully understand the mechanism of NPW-induced feeding reduction or energy expenditure, development of NPW deficient animals or detailed evaluation of GPR7−/− animals would be crucial.

Classically, anorectic neuropeptides are up-regulated and orexigenic neuropeptides are down-regulated by leptin. As an anorectic neuropeptide, it was surprising that NPW expression is not up-regulated by leptin administration and up-regulated in ob/ob mice or diet-induced obese rats. However, we hypothesize that such kinds of systems as to compensate leptin function would be necessary to restore an imbalance of energy homeostasis which occurs.
under conditions of disrupted leptin signaling including leptin resistance. Although the contribution of NPW to the regulation of metabolism may be minor under normal conditions, it may play an important compensatory role under some circumstances. Further studies are needed to resolve the molecular mechanisms by which NPW affects feeding behavior. Such studies may identify new regulatory mechanisms in the leptin signaling pathway and potentially lead to development of novel therapeutic agents for the treatment of leptin resistance.

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