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Identification of Neuropeptide W as the Endogenous Ligand for Orphan G-protein-coupled Receptors GPR7 and GPR8*

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The structurally related orphan G-protein-coupled receptors GPR7 and GPR8 are expressed in the central nervous system, and their ligands have not been identified. Here, we report the identification of the endogenous ligand for both of these receptors. We purified the peptide ligand from porcine hypothalamus using stable Chinese hamster ovary cell lines expressing human GPR8 and cloned the cDNA encoding its precursor protein. The cDNA encodes two forms of the peptide ligand with lengths of 23 and 30 amino acid residues as mature peptides. We designated the two ligands neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30). The amino acid sequence of NPW23 is completely identical to that of the N-terminal 23 residues of NPW30. Synthetic NPW23 and NPW30 activated and bound to both GPR7 and GPR8 at similar effective doses. Intracerebroventricular administration of NPW23 in rats increased food intake and stimulated prolactin release. These findings indicate that neuropeptide W is the endogenous ligand for both GPR7 and GPR8 and acts as a mediator of the central control of feeding and the neuroendocrine system.

Searches for ligands for orphan G-protein-coupled receptors (GPCRs)[1] have discovered many novel peptides and have identified the previously unknown receptors of bioactive substances (1–9). Studies on the newly identified ligands and their receptors have given us a more precise understanding of the physiological processes involved in the endocrine, cardiovascular, reproductive, immune, inflammatory, digestive, metabolic, and central nervous systems (1–11). In addition, these studies have provided opportunities to discover innovative drugs that exert their pharmacological effects by interacting with an identified receptor as an agonist or antagonist (12).

GPR7 and GPR8, for which the ligands have not been identified, are structurally related orphan GPCRs. Two genes for GPR7 and GPR8 were originally isolated from human genomic DNA by O'Dowd *et al.* (13). Human GPR7 highly resembles human GPR8, with an amino acid identity of 64%. Among various families of GPCRs, GPR7 and GPR8 share high similarity to the opioid and somatostatin receptor families. In mammalian brain, gene expression of GPR7 and GPR8 was detected by Northern blot and *in situ* hybridization analyses (13). Especially in rat brain, GPR7 mRNA was detected in regions of the cortex, hippocampus, and hypothalamus (14). Profiles of GPR7 and GPR8 expressed mainly in brain suggest that the endogenous ligands for the two receptors have several functions in the central nervous system.

In this study, we report the purification, cloning, and characterization of neuropeptide W (NPW). We attempted to purify the agonist peptide for GPR8. The cDNA encoding the agonist peptide for GPR8 demonstrates the existence of neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30), which exhibit no meaningful similarity to any known peptides. With the functional and binding characterization of NPW for GPR7 and GPR8, we show that NPW is the endogenous ligand for both of these receptors. In addition, we describe the *in vivo* effects of NPW on feeding behavior and hormone release.

EXPERIMENTAL PROCEDURES

Establishment of GPR7- and GPR8-expressing Chinese Hamster Ovary (CHO) Cell Lines—The coding region of human GPR7 was cloned from human genomic DNA and human brain cDNA, and that of human GPR8 was cloned from human brain cDNA by PCR (13). An expression vector was constructed by ligation of the receptor gene to pAKKO-111H containing *dhfr* as a selection maker (15). CHO *dhfr*$^-$ cells were transfected with the expression vector. Stable CHO cell lines expressing human GPR7 (CHO-GPR7 cells) and human GPR8 (CHO-GPR8 cells) were selected under conditions wherein the growth medium lacked nucleotides.

cAMP Accumulation Inhibition Assay—The inhibitory activity of the test samples for cAMP accumulation was measured as described (16). CHO-GPR7 or CHO-GPR8 cells were plated on 24-well plates at 5×10^4 cells/well and cultured for 2 days. The cells were washed three times with 0.5 ml of assay buffer (Hanks' buffered salt solution (pH 7.4), 0.2 mM 3-isobutyl-1-methylxanthine (Wako Bioproducts), 0.05% bovine serum albumin, and 20 mM HEPES) and cultured in assay buffer for 30 min. After washing the cells three times with 0.5 ml of assay buffer, a test sample in 0.5 ml of assay buffer supplemented with 1 μM forskolin (Wako Bioproducts) was added to the cells, and the cells were incubated at 37 °C for 30 min. cAMP synthesis in the cells was stopped by addition of 0.1 ml of 20% perchloric acid, and intracellular cAMP was extracted on ice for 1 h. The amount of extracted cAMP was measured using an enzyme-linked immunoassay kit (Amersham Biosciences).

[^{35}S]GTPγS Binding Assay—The [^{35}S]GTPγS binding assay was performed essentially as described (12) with minor modifications. The

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB084276, AB084277, and AB084278.

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[1] The abbreviations used are: GPCRs, G-protein-coupled receptors; NPW, neuropeptide W; NPW23, neuropeptide W-23; NPW30, neuropeptide W-30; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-*O*-(3-thiophosphate); RP-HPLC, reverse-phase high pressure liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

human GPR8 membrane fractions suspended in 200 μl of [^{35}S]GTPγS assay buffer (50 mM Tris-HCl (pH 7.4), 0.1% bovine serum albumin, 5 mM $MgCl_2$, 150 mM NaCl, and 1 μM GDP) were mixed with a test sample, followed by addition of [^{35}S]GTPγS (PerkinElmer Life Sciences) at a final concentration of 0.5 nM. The mixture was incubated at 25 °C for 60 min and then filtered onto a Whatman GF/F glass filter. After washing three times with 1.5 ml of washing buffer (50 mM Tris-HCl (pH 7.4), 0.1% bovine serum albumin, 5 mM $MgCl_2$, and 1 mM EDTA), the glass filters were dried at 37 °C for 1 h. The radioactivities retained in the membranes were counted with a scintillation counter.

Purification of NPW from Porcine Hypothalamus—Approximately 500 g of fresh porcine hypothalamus was boiled for 10 min in water and homogenized using a Polytron homogenizer in 2 liters of 1.0 M acetic acid. The homogenate was centrifuged, and then the supernatant was subjected to acetone precipitation at a final acetone concentration of 66%. After removal of the precipitates, the supernatant was evaporated and extracted with diethyl ether. The resultant solution was applied to a column packed with YMCgel ODS-AM 120-S50 (YMC). The peptide fraction was eluted with 60% acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid, and the eluate was lyophilized. The agonist peptide for GPR8 was purified from the lyophilized materials by successive HPLC using a TSKgel ODS-80TM column (21.5 × 240 mm; 10–60% CH_3CN gradient in 0.1% trifluoroacetic acid for 80 min at 5.0 ml/min; Tosoh), a TSKgel SP-5PW column (20 × 150 mm; 10–2000 mM ammonium formate ($HCOONH_4$) gradient in 10% CH_3CN for 40 min at 5.0 ml/min; Tosoh), a Develosil CN-UG-5 column (4.6 × 250 mm; 21–26% CH_3CN gradient in 0.1% trifluoroacetic acid for 20 min at 1.0 ml/min; Nomura Chemical), and a Wakosil-II 3C18HG column (2.0 × 150 mm; 22.5–32.5% CH_3CN gradient in 0.1% trifluoroacetic acid for 40 min at 0.2 ml/min; Wako Bioproducts) combined with the [^{35}S]GTPγS binding assay. Finally, an active fraction with a single peak at both 220 and 280 nm was collected manually by HPLC using the Wakosil-II 3C18HG column. The amino acid sequence of the purified peptide was analyzed with a protein sequencer (Procise 491cLC, Applied Biosystems Inc.).

Cloning of Porcine, Human, and Rat Prepro-NPW cDNAs—A BLAST search was performed using the amino acid sequence of the purified peptide as a query against the DDBJ/GenBank™/EBI Data Bank. A human genomic DNA sequence (DDBJ/GenBank™/EBI accession number AC0050606) contained a partial nucleotide sequence of which the translated amino acid sequence was similar to the purified peptide. On the basis of this DNA sequence, the cDNA encoding prepro-NPW was obtained from the porcine spinal cord cDNA library constructed with a Marathon cDNA amplification kit (CLONTECH) using 5′- and 3′-rapid amplification of cDNA ends and PCR to clone full-length cDNA. The cDNA encoding human prepro-NPW was obtained from the human hypothalamus Marathon Ready cDNA library (CLONTECH), and the cDNA encoding rat prepro-NPW was obtained from the rat brain Marathon Ready cDNA library (CLONTECH) following the same strategies used to clone porcine prepro-NPW cDNA. The PCR primers for cloning the porcine, human, and rat full-length cDNAs were as follows: porcine, 5′-TTCCCGACACCCCTGCGCCCAGAC-3′ (forward) and 5′-GGGCTGGCGAAGGCGGTTCCCTGC-3′ (reverse); human, 5′-AGCGGTACTGAGGGGGCGGAACGA-3′ (forward) and 5′-GGGTCTATGAGCGGCTCCTGGAAG-3′ (reverse); and rat, 5′-GGGGCGGGGCCATTGAGAAGC-3′ (forward) and 5′-TGACCAGACAACGAGACCTGA-3′ (reverse). The porcine, human, and rat prepro-NPW cDNA clones were sequenced on both strands using an ABI PRISM 377 DNA sequencer (Applied Biosystems Inc.).

Synthesis of Human and Porcine NPW23 and NPW30—Human NPW23 and NPW30 and porcine NPW23 and NPW30 were chemically synthesized using an ABI 433A peptide synthesizer (Applied Biosystems Inc.) following Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) strategy. After deblocking all the protecting groups by treatment with mixture of trifluoroacetic acid/thioanisole/*m*-cresol/triisopropylsilane/1,2-ethanedithiol (85:5:5:2.5:2.5), the peptides were purified by reverse-phase HPLC (RP-HPLC).

Comparison of the Elution Times of Synthetic NPW with Those of the [^{35}S]GTPγS Binding Activities of Porcine Hypothalamus Fractions—The extracts of porcine hypothalamus were fractionated by HPLC using the TSKgel ODS-80TM column (10–60% CH_3CN gradient in 0.1% trifluoroacetic acid for 80 min at 5.0 ml/min) as described above for the purification of NPW. Active fractions obtained by ODS-HPLC were further fractionated by HPLC using a Develosil ODS-UG-5 column (4.6 × 250 mm; 25–40% CH_3CN gradient in 0.1% trifluoroacetic acid for 30 min at 1.0 ml/min; Nomura Chemical), and the eluates were collected every 1 min. The agonist activity of each fraction for GPR8 was measured by the [^{35}S]GTPγS binding assay. Synthetic porcine NPW23 and NPW30 were analyzed by HPLC using the Develosil ODS-UG-5 column (25–40% CH_3CN gradient in 0.1% trifluoroacetic acid for 30 min at 1.0 ml/min).

Expression and Purification of Human NPW in the Transient Expression System—We constructed an expression vector by ligation of PCR-amplified human prepro-NPW cDNA covering the DNA region from the 5′ to 3′ in-frame stop codons to mammalian expression vector pCR3.1 (Invitrogen). The PCR primers for cloning the human prepro-NPW cDNA were 5′-AGCGGTACTGAGGGGGCGGAACGA-3′ (forward) and 5′-AACTAGTCGGCCACTCCTCCTGGGTCAG-3′ (reverse). COS-7 cells were transfected with the expression vector and cultured for 2 days. The conditioned medium was concentrated using a Waters Sep-Pak Plus C_{18} cartridge. The agonist peptide for GPR8 was purified from the concentrated medium by successive HPLC using a TSKgel CM-2SW column (4.6 × 250 mm; 10–2000 mM $HCOONH_4$ gradient in 10% CH_3CN for 60 min at 1.0 ml/min; Tosoh), a TSKgel ODS-80TsQA column (4.6 × 250 mm; 25–45% CH_3CN gradient in 0.1% trifluoroacetic acid for 60 min at 1.0 ml/min; Tosoh), and a SymmetryShield RP18 column (2.1 × 150 mm; 15–35% CH_3CN gradient in 0.1% trifluoroacetic acid for 60 min at 0.2 ml/min; Waters Associates) combined with the cAMP accumulation inhibition assay. Finally, an active fraction with a single peak at 220 nm was collected manually by HPLC using the SymmetryShield RP18 column. The amino acid sequence of the purified peptide was analyzed with the Procise 491cLC protein sequencer. Electrospray ionization mass spectra of the purified peptide were recorded on an LCQ duo ion-trap mass spectrometer (ThermoFinnigan) equipped with a nanospray ion source (MDS Protana).

Pertussis Toxin Treatment of CHO-GPR7 and CHO-GPR8 Cells—CHO-GPR7 and CHO-GPR8 cells were cultured in 24-well plates for 24 h and then treated with or without pertussis toxin (List Biological Laboratories, Inc.) at a concentration of 100 ng/ml for 24 h. After washing these cells, the inhibitory effect of human NPW23 (1 nM) on cAMP accumulation induced by forskolin (1 μM) was analyzed.

Receptor Binding Assay—Human NPW23 was labeled by lactoperoxidase oxidation in the presence of Na^{125}I (PerkinElmer Life Sciences), and the monoiodinated peptide labeled at Tyr^{10} was purified by RP-HPLC. The specific activity of purified ^{125}I-labeled human NPW23 was ~9.5×10^{16} Bq/mol when a fresh batch of Na^{125}I was used for labeling. Membrane preparation and receptor binding assay were performed essentially as described (12) with minor modifications. CHO-GPR7 or CHO-GPR8 membrane fractions suspended in 200 μl of receptor binding assay buffer (25 mM Tris-HCl (pH 7.4), 0.1% bovine serum albumin, 0.05% CHAPS, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml pepstatin, 20 μg/ml leupeptin, and 4 μg/ml E-64) were mixed with ^{125}I-labeled human NPW23. The mixture was incubated at 25 °C for 75 min and then filtered onto a Whatman GF/F glass filter. After washing three times with 1.5 ml of binding buffer, the glass filters were dried at 37 °C for 1 h. Nonspecific binding was determined by the binding assay in the presence of unlabeled human NPW23 at 1 μM. The radioactivities retained on the glass filters were counted with a γ-counter.

Feeding Experiments—Male Wistar rats (8–9 weeks old) were maintained under controlled temperature (25 °C) and lighting (light on from 7:30 to 19:30). Food (standard chow pellets) and water were available *ad libitum*. A stainless-steel guide cannula (AG-8, EICOM, Kyoto, Japan) was inserted into the lateral ventricle under sodium pentobarbital anesthesia. After the rats had been housed for 1 week in individual cages, 10 μl of phosphate-buffered saline with or without human NPW23 was injected at a flow rate of 5 μl/min into the lateral ventricle through a microinjection cannula that was inserted into the guide cannula. All injections started at 15:00, and food intake was measured at 30, 60, and 120 min.

Hormone Release Experiments—Male Wistar rats (8–9 weeks old) were maintained under controlled temperature (25 °C) and lighting (light on from 7:30 to 19:30). Food (standard chow pellets) and water were available *ad libitum*. A stainless-steel guide cannula (AG-12, EICOM) was inserted into the third ventricle under sodium pentobarbital anesthesia. The cannula-implanted rats were housed in individual cages and kept for 1 week. One day before the experiments, the cannula-implanted rats were fitted with a catheter in the right jugular vein. The following day, 10 μl of phosphate-buffered saline with or without human NPW23 was injected at a flow rate of 5 μl/min into the third ventricle through a microinjection cannula that was inserted into the guide cannula. An intracerebroventricular injection was given between 13:00 and 15:00. After the intracerebroventricular injection of the peptide, blood samples were collected from the catheter at 5, 10, 20, 30, and 60 min. The concentration of prolactin in the plasma samples was then determined using a radioimmunoassay kit (Amersham Biosciences).

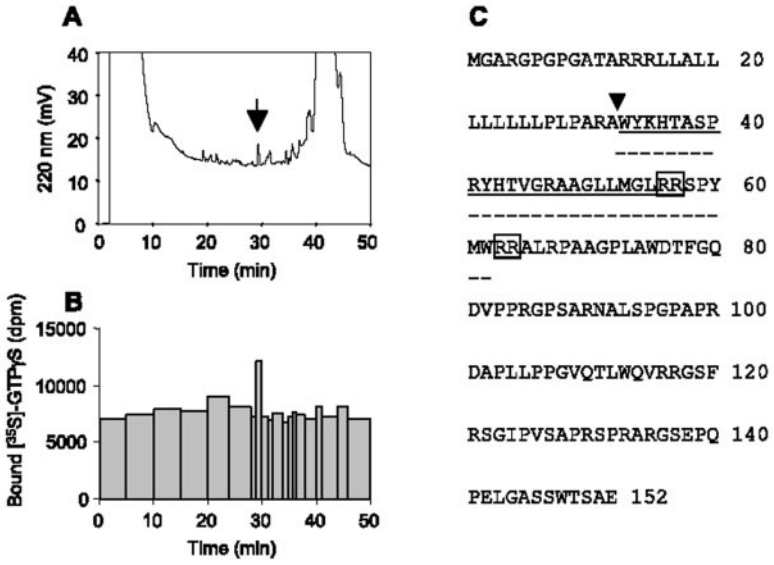

FIG. 1. **Identification of the endogenous ligand for GPR8 from extracts of porcine hypothalamus.** *A*, HPLC profile of the final purification step using the Wakosil-II 3C18HG column. The *arrow* marks the purified material. *B*, detection of [^{35}S]GTPγS binding activity for GPR8. The eluate was manually collected, and the activity was recovered as a single peak with an elution time of 29.4 min. *C*, deduced amino acid sequence of the porcine prepro-NPW polypeptide. Porcine NPW23 is *underlined* with a *solid line*, and porcine NPW30 is *underlined* with a *broken line*. The *arrowhead* indicates the signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. The DNA sequence of porcine prepro-NPW has been deposited in the DDBJ/GenBank™/EBI Data Bank (accession number AB084277).

RESULTS

Purification and cDNA Cloning of the Agonist Peptide for GPR8—We purified the agonist peptide for GPR8 from porcine hypothalamus using CHO-GPR8 cells, which is a so-called "reverse pharmacology" technique (17). In the assay of intracellular signal changes in CHO-GPR8 cells induced by the test samples, including various kinds of tissue extract and known bioactive substances, RP-HPLC fractions of porcine hypothalamus extracts showed an inhibitory effect on cAMP accumulation induced by forskolin. Treatment of the RP-HPLC fractions with proteinases that diminished the agonist activity suggested that a peptide ligand for GPR8 was present in the extracts of porcine hypothalamus. Because the same RP-HPLC fractions also stimulated [^{35}S]GTPγS binding to the membranes of CHO-GPR8 cells, the extracts of porcine hypothalamus were subjected to purification of the agonist peptide for GPR8 by successive chromatography in the [^{35}S]GTPγS binding assay. The process yielded 3 pmol of the purified agonist peptide for GPR8 from 500 g of porcine hypothalamus (Fig. 1, *A* and *B*). The N-terminal amino acid sequence of the purified peptide was determined to be WYKHTASPRYHTVGRAA*X*LL (*X*, not identified) using the protein sequencer.

We attempted to isolate the cDNA encoding this peptide to reveal its whole amino acid sequence. We cloned a cDNA from a porcine spinal cord by PCR using DNA sequence information obtained by both 5′- and 3′-rapid amplification of cDNA ends. Although two ATG codons present in the 5′-end region of the 565-bp cDNA precede an in-frame stop codon (DDBJ/GenBank™/EBI accession number AB084277), the second ATG codon conforms more to the rules of Kozak (18). The open reading frame starting at this second ATG codon encodes the porcine prepropolypeptide of 152 residues (Fig. 1*C*). The PSORT II algorithm based on the method of von Heijne (20) indicated that a signal peptide cleavage site of this polypeptide resides between Ala^{32} and Trp^{33} (19, 20). It is predicted that two mature peptides of 23 and 30 residues will be generated from the prepropolypeptide by signal peptide cleavage and proteolytic processing at two pairs of basic amino acid residues, Arg^{56}-Arg^{57} and Arg^{63}-Arg^{64}, respectively (21). The mature peptide sequence of 23 residues starts at Trp^{33} and ends at Leu^{55}, whereas that of 30 residues starts at Trp^{33} and ends at Trp^{62}. The amino acid sequence of the 23-residue peptide is identical to that of the N-terminal 23 residues in the 30-residue peptide. Because both the first and last amino acids of the 30-residue peptide are Trp, we designated the 30-residue peptide as neuropeptide W-30 and then designated the 23-residue peptide as neuropeptide W-23.

Activation of GPR8 by NPW23 and NPW30 in Porcine Hypothalamus—To clarify molecular species showing agonist activities for GPR8, we measured the [^{35}S]GTPγS binding activities of the porcine hypothalamus peptide fractions for GPR8 and compared the elution times of the [^{35}S]GTPγS binding activities with those of synthetic peptides of porcine NPW23 and NPW30 under the same HPLC conditions. The synthetic peptide of porcine NPW23 eluted at 24.5 min, and that of porcine NPW30 eluted at 26.5 min (Fig. 2*A*). The [^{35}S]GTPγS binding activities of the porcine hypothalamus peptide fractions for GPR8 were recovered mainly in fractions 25 (24–25 min) and 27 (26–27 min) with almost the same activity (Fig. 2*B*), indicating that fractions 25 and 27 contained porcine NPW23 and NPW30, respectively. These results demonstrate that porcine NPW23 and NPW30 existing in porcine hypothalamus activate GPR8.

Structures of Prepro-NPW cDNA and NPW—We isolated human prepro-NPW cDNA (DDBJ/GenBank™/EBI accession number AB084276) lacking an AUG start codon from human hypothalamus and examined whether human NPW with agonist activity for GPR8 is generated from this cDNA. As shown in Fig. 3, the open reading frame of the cDNA represents the amino acid sequences of the 23- and 30-residue peptides, which are flanked by a signal peptide cleavage site and a pair of basic amino acid residues and which resemble porcine NPW23 and NPW30, respectively. Although an AUG start codon is not present near the 5′-end of the cDNA, several potential non-AUG start codons lie within the region (22, 23). To ensure that the agonist peptide for GPR8 is synthesized from this human cDNA, we analyzed peptides secreted from COS-7 cells transfected with an expression vector containing this human cDNA. The supernatant of COS-7 cells transfected with this vector showed an inhibitory effect on forskolin-induced cAMP accumulation specifically in CHO-GPR8 cells. From 900 ml of the supernatant, 150 pmol of the agonist peptide for GPR8 was purified to homogeneity (Fig. 4, *A* and *B*). The structure of the purified peptide was determined to be WYKHVASPRYHTVGRAAGLLM (NPW21), lacking 2 amino acid residues at the C terminus compared with the predicted human NPW23 sequence. In this expression system, translation of the prepropolypeptide and signal peptide cleavage proceeded as ex-

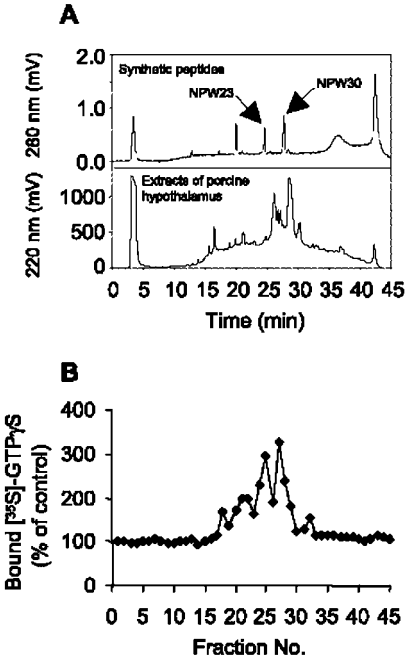

FIG. 2. **Activation of GPR8 by porcine NPW23 and NPW30.** *A*, HPLC profiles of synthetic porcine NPW and extracts of porcine hypothalamus. *Arrows* mark the elution peaks of the synthetic peptides (*upper panel*). The extracts of porcine hypothalamus were fractionated under the same HPLC conditions (*lower panel*). *B*, detection of [^{35}S]GTPγS binding activity of the fractionated extracts of porcine hypothalamus for GPR8. The agonist activity of each fraction prepared from the extracts of porcine hypothalamus was measured by the [^{35}S]GTPγS binding assay.

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AGCGGTACTGAGGGGGCGGAACGAGGCGGGGCCACCGAGCGGTTATAGCTGGGCCTGCAG   60
                                                 *  L  G  L  Q

GGGACCCACGGCTCGCCTCCAGCCTCCTGCGCTCCGGTACCTGGGCGTCCCAACTCCACT  120
G  T  H  G  S  P  P  A  S  C  A  P  V  P  G  R  P  N  S  T

GCGCGCCCAAACCCAGCCGAGCCGGTTCGTGGCCCGCCCCGCCCGGGCGGCCGTCGACGCG  180
A  R  P  N  P  A  E  P  V  R  G  P  P  R  R  A  A  V  D  A

AGCGCCCTGGCGTGGCGCCCAGGGGAGCGGGGGGCTCCCGCGAGCCGGCCGCGGCTGGCA  240
S  A  L  A  W  R  P  G  E  R  G  A  P  A  S  R  P  R  L  A

CTGCTGCTGCTTCTGCTCCTGCTGCCGCTGCCCTCCGGCGCGTGGTACAAGCACGTGGCG  300
L  L  L  L  L  L  L  P  L  P  S  G  A  W  Y  K  H  V  A
                                      ----------------

AGTCCCCGCTACCACACGGTGGGCCGCGCCGCTGGCCTGCTCATGGGGCTGCGTCCGCTCA  360
S  P  R  Y  H  T  V  G  R  A  A  G  L  L  M  G  L  R  R  S
-------------------------------------------------------------

CCCTATCTGTGGCGCCGCGCGCTGCGCGCGGCCGCCGGGCCCCTGGCCAGGGACACCCTC  420
P  Y  L  W  R  R  A  L  R  A  A  A  G  P  L  A  R  D  T  L
----------

TCCCCCGAACCCGCAGCCCGCGAGGCTCCTCTCCTGCTGCCCTCGTGGGTTCAGGAGCTG  480
S  P  E  P  A  A  R  E  A  P  L  L  L  P  S  W  V  Q  E  L

TGGGAGACGCGACGCAGGAGCTCCCAGGCAGGGATCCCGTCCGTGCGCCCCGGAGCCCG  540
W  E  T  R  R  R  S  S  Q  A  G  I  P  V  R  A  P  R  S  P

CGCGCCCCAGAGCCTGCGCTGGAACCGGAGTCCCTGGACTTCAGCGGAGCTGGCCAGAGA  600
R  A  P  E  P  A  L  E  P  E  S  L  D  F  S  G  A  G  Q  R

CTTCGGAGAGACGTCTCCGCCCAGCGGTGGACCCCGCAGCAAACCGCCTTGGCCTGCCC  660
L  R  R  D  V  S  R  P  A  V  D  P  A  A  N  R  L  G  L  P

TGCCTGGCCCCCGGACCGTTCTGACAGCGTCCCCCGCCCGCCCGTGGCGCCTCCGCGCCT  720
C  L  A  P  G  P  F  *

GACCCAGGAGGAGTGGCCGCGCGCTTCCAGGAGCCGCTCATAGACCC  767
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FIG. 3. **Human prepro-NPW cDNA and NPW sequences.** The human prepro-NPW cDNA was cloned from the human hypothalamus cDNA library. The *asterisk* indicates a stop codon. Human NPW23 is *underlined* with a *solid line*, and human NPW30 is *underlined* with a *broken line*. The *arrowhead* indicates the signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. The DNA sequence of human prepro-NPW has been deposited in the DDBJ/GenBank™/EBI Data Bank (accession number AB084276).

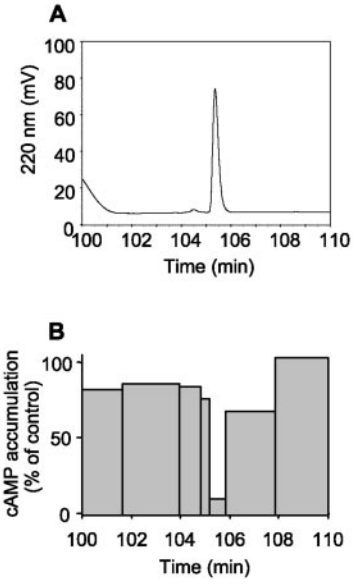

FIG. 4. **Expression and purification of human NPW.** *A*, purification of the agonist peptide for GPR8 from the supernatant of COS-7 cells transfected with the human prepro-NPW expression vector. Shown is the HPLC profile of the final purification step using a SymmetryShield RP18 column. *B*, detection of an inhibitory effect of the HPLC fractions on forskolin-induced cAMP accumulation in CHO-GPR8 cells. The eluate was manually collected, and the activity of each fraction was measured by the cAMP accumulation inhibition assay using CHO-GPR8 cells in the presence of forskolin (1 μM). The activity was recovered as a single peak with an elution time of 105.5 min.

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NPW23
Human    WYKHVASPRYHTVGRAAGLLMGL
Porcine  WYKHTASPRYHTVGRAAGLLMGL
Rat      WYKHVASPRYHTVGRASGLLMGL

NPW30
Human    WYKHVASPRYHTVGRAAGLLMGLRRSPYLW
Porcine  WYKHTASPRYHTVGRAAGLLMGLRRSPYMW
Rat      WYKHVASPRYHTVGRASGLLMGLRRSPYLW
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FIG. 5. **Sequence comparison of NPW23 and NPW30.** Human, porcine, and rat NPW23 and NPW30 sequences are aligned. Common amino acids are *boxed* in both NPW23 and NPW30.

pected, but the proteolytic processing at a pair of dibasic amino acid residues failed to proceed accurately. These results indicate that the agonist peptide for GPR8 is generated through intracellular processing of the prepropolypeptide initiated from a non-AUG start codon of the human prepro-NPW transcript.

We compared the amino acid sequences of mature NPW of different species. In addition to porcine and human prepro-NPW cDNAs, rat prepro-NPW cDNA (DDBJ/EMBL/GenBank™ accession number AB084278) was cloned from rat brain. According to the prediction of the mature peptide as used for porcine prepro-NPW, we obtained mature peptide sequences of both 23 and 30 residues from porcine, rat, and human prepro-NPW (Fig. 5). Among them, the amino acid identities of NPW23 and NPW30 are 91.3 and 90.0%, respectively. Both the N- and C-terminal amino acids of NPW are conserved such that the amino acid sequence of NPW23 starts

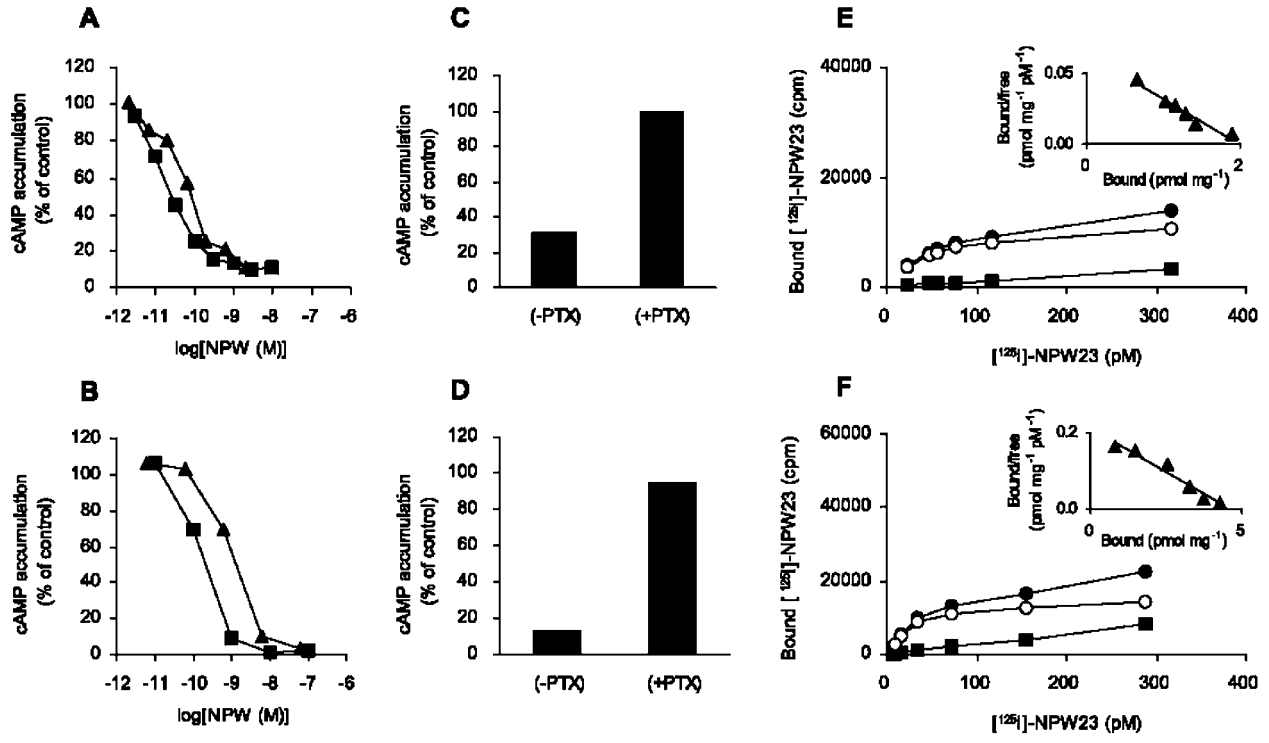

FIG. 6. **Functional and binding characterization of NPW.** *A* and *B*, dose-dependent inhibition of cAMP accumulation by NPW in CHO-GPR7 and CHO-GPR8 cells, respectively. Various concentrations of NPW23 or NPW30 were added to CHO-GPR7 and CHO-GPR8 cells, and the cAMP accumulation inhibition assay was performed in the presence of forskolin (1 μM). ■, human NPW23; ▲, human NPW30. *C* and *D*, effect of pertussis toxin on the NPW activation of CHO-GPR7 and CHO-GPR8 cells, respectively. CHO-GPR7 or CHO-GPR8 cells were treated with or without pertussis toxin (*PTX*; 100 ng/ml) for 24 h. After washing these cells, the inhibitory effect of human NPW23 (1 nM) on cAMP accumulation induced by forskolin (1 μM) was analyzed. *E* and *F*, saturation binding between ^{125}I-labeled human NPW23 and CHO-GPR7 cells and between ^{125}I-labeled human NPW23 and CHO-GPR8 cells, respectively. ●, total binding; ○, specific binding; ■, nonspecific binding. *Insets*, Scatchard analysis of the radioligand binding. Results shown are the means of triplicate experiments.

at Trp and ends at Leu, and that of NPW30 starts at Trp and ends at Trp. These results indicate that NPW23 and NPW30 are highly conserved between species.

Functional Activity and Binding Affinity of NPW for GPR7 and GPR8—We examined the functional activity and binding affinity of synthetic NPW for human GPR7 and GPR8 because human GPR8 shows higher similarity to human GPR7 than to other numerous GPCRs. Human NPW23 dose-dependently inhibited cAMP accumulation induced by forskolin in CHO-GPR7 and CHO-GPR8 cells, with IC_{50} values of 0.025 and 0.178 nM, respectively (Fig. 6, *A* and *B*; and Table I). Human NPW30 also showed an inhibitory effect on forskolin-induced cAMP accumulation in CHO-GPR7 and CHO-GPR8 cells, with IC_{50} values of 0.133 and 1.244 nM, respectively (Fig. 6, *A* and *B*; and Table I). In CHO-GPR7 and CHO-GPR8 cells, elevation of intracellular calcium induced by NPW23 and NPW30 was not observed by measurement of $[Ca^{2+}]_i$ response using a calcium fluorescent dye indicator (data not shown), and the inhibitory effect of NPW on forskolin-induced cAMP accumulation was abolished by preincubation with pertussis toxin (Fig. 6, *C* and *D*), indicating that human GPR7 and GPR8 both coupled to $G\alpha_i$. Saturation binding analyses using ^{125}I-labeled human NPW23 showed that CHO-GPR7 and CHO-GPR8 cells displayed high affinity, saturable, and specific binding (human GPR7, K_d = 31.8 ± 3.0 pM and B_{max} = 2.02 ± 0.10 pmol/mg; human GPR8: K_d = 20.7 ± 0.6 pM and B_{max} = 4.37 ± 0.04 pmol/mg) (Fig. 6, *E* and *F*). ^{125}I-Labeled human NPW23 bound both human GPR7 and GPR8 with nearly the same affinity. Competition binding analyses demonstrated high affinity binding of NPW23 and NPW30 to human GPR7 and GPR8 (Table I). NPW thus activates and binds to these receptors with high potency and affinity. These results indicate that NPW is the endogenous ligand for both GPR7 and GPR8.

In Vivo Effects of NPW on Feeding Behavior and Hormone Release—We investigated the central effects of NPW on feeding behavior and hormone release because rat GPR7 is expressed at relatively high levels in brain (14). First, human NPW23 was intracerebroventricularly administered to rats, and food intake was monitored (Fig. 7*A*). Injection of 10 nmol of NPW23 significantly increased food intake, and the magnitude of food consumption during a 2-h period was ~3-fold relative to vehicle controls. Second, human NPW23 was intracerebroventricularly administered to rats, and the hormone concentration in the blood was measured (Fig. 7*B*). Injection of 3 nmol of NPW23 significantly stimulated the release of prolactin, but not the release of other pituitary hormones such as growth hormone, adrenocorticotropic hormone, follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone. The prolactin concentration increased to maximum levels at 20 min after injection and decreased to control levels at 60 min.

DISCUSSION

We purified NPW as the agonist peptide for GPR8 using CHO-GPR8 cells from porcine hypothalamus. The purified peptide is encoded by porcine prepro-NPW cDNA, and the prepropolypeptide sequence predicts the existence of two mature peptides of 23 and 30 residues. We have demonstrated the existence of NPW peptides of 23 and 30 residues as mature peptides in porcine hypothalamus by HPLC analyses combined with the [^{35}S]GTPγS binding assay (Fig. 2, *A* and *B*). Porcine hypothalamus contains nearly equal amounts of NPW23 and NPW30 because both fractions 25 and 27 showed nearly the

TABLE I

Functional activity and binding affinity of human NPW for GPR7 and GPR8

IC_{50} values for functional activity were determined by the cAMP accumulation inhibition assay in the presence of forskolin (1 μM). IC_{50} values for binding affinity were determined by the competition binding assay using ^{125}I-labeled human NPW23 (80 pM). Results shown are the means ± S.E. (n = 3 or 5).

	Human GPR7		Human GPR8	
	cAMP	Binding	cAMP	Binding
	nM		*nM*	
NPW23	0.025 ± 0.004	0.096 ± 0.007	0.178 ± 0.007	0.210 ± 0.021
NPW30	0.133 ± 0.034	0.025 ± 0.005	1.244 ± 0.131	0.021 ± 0.002

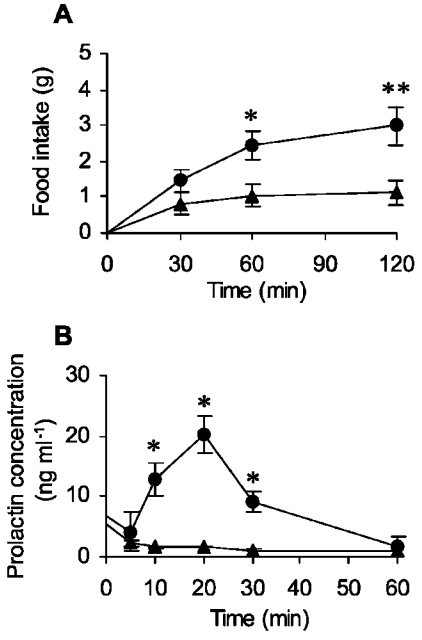

FIG. 7. ***In vivo* effects of NPW in rats.** *A*, increase of food intake by intracerebroventricular administration of human NPW23 in rats. After injection at 15:00, food intake was measured. Cumulative food consumption was plotted over a 2-h period. ●, 10 nmol of NPW23; ▲, vehicle. Results shown are the means ± S.E. (n = 10). Statistical comparisons were performed using Student's t test. *, $p < 0.05$; **, $p < 0.01$. *B*, stimulation of prolactin release by intracerebroventricular administration of human NPW23 in rats. After injection at 13:00, blood samples were collected, and the concentration of prolactin in the plasma samples was determined. ●, 3 nmol of NPW23; ▲, vehicle. Results shown are the means ± S.E. (n = 9 or 10). Statistical comparisons were performed using Student's t test. *, $p < 0.05$.

same agonist activity, indicating that NPW23 and NPW30 would exhibit their own biological activities in the mammalian body.

The prepro-NPW cDNA sequences suggested that translation of human prepro-NPW, unlike that of porcine and rat, does not initiate from a typical AUG start codon. We have demonstrated that NPW21 with agonist activity for GPR8 is synthesized from the human prepro-NPW cDNA lacking an AUG start codon in the transient expression system in COS-7 cells. In this expression system, translation of the human prepro-NPW polypeptide initiated from a non-AUG start codon, and signal peptide cleavage subsequently occurred at an expected site, but proteolytic processing of the propeptide failed to create the expected C terminus of mature NPW, probably due to an incomplete set of processing enzymes in the COS-7 cells (24) or susceptibility to proteolysis of the C terminus of mature NPW in the culture medium. Natural non-AUG start codons are very rare in eukaryotes, but have been found in c-Myc, fibroblast growth factor-2, and vascular epidermal growth factor (25–27). In almost all cases, alternate initiation takes place at upstream non-AUG codons in addition to the first in-frame AUG codons. Non-AUG triplets functioning as start codons have been experimentally determined to be ACG, CUG, and GUG (22, 23). If initiation of human prepro-NPW translation takes place at a position that corresponds to the AUG start site in porcine prepro-NPW, a CUG triplet (nucleotides 187–189) is one of the candidates for the non-AUG start codon in human prepro-NPW (Fig. 3). Although further research is required to determine the translation start site and the usage of the non-AUG start codon in human prepro-NPW, we propose that human NPW is encoded by the cDNA of human prepro-NPW with a unique translation from a non-AUG start codon and that the mature forms of human NPW are NPW23 (WYKHVASPRYHTVGRAAGLLMGL) and NPW30 (WYKHVASPRYHTVGRAAGLLMGLRRSPYLW).

Human NPW23 and NPW30 showed a high potency of inhibitory effects on forskolin-induced cAMP accumulation in both CHO-GPR7 and CHO-GPR8 cells, with subnanomolar or nanomolar IC_{50} values. In the competition binding studies, human NPW23 and NPW30 showed high affinity binding to both GPR7 and GPR8, with subnanomolar IC_{50} values. The high potency and affinity of NPW23 and NPW30 for both GPR7 and GPR8 indicate that NPW23 and NPW30 are the endogenous ligands for both GPR7 and GPR8. A slight difference between NPW23 and NPW30 was observed in that NPW23 showed a tendency to activate both GPR7 and GPR8 more efficiently compared with NPW30. In the cAMP accumulation inhibition assay, the IC_{50} values of NPW23 for GPR7 and GPR8 were 5.3- and 7.0-fold lower than those of NPW30 for the two receptors, respectively (Table I). In contrast, NPW30 showed a tendency to bind to both GPR7 and GPR8 with high affinity compared with NPW23. In the competition binding assay, the IC_{50} values of NPW30 for GPR7 and GPR8 were 3.8- and 10.0-fold lower than those of NPW23 for the two receptors, respectively (Table I). The C-terminal 7 amino acid residues in human NPW30 should result in relatively low potency and high affinity of NPW30 for its receptors compared with NPW23. Structure-activity relationship studies of NPW are required to reveal the roles of the C-terminal 7 amino acid residues in activation of its receptors and binding to its receptors.

In rats, intracerebroventricular administration of NPW resulted in an acute increase in food intake. The orexigenic effect could be accounted for activation of GPR7-expressing neurons localized in the hypothalamic areas, which are considered to regulate feeding behavior and energy homeostasis (14, 28, 29). Stimulation of prolactin release was observed upon the intracerebroventricular administration of NPW. Although it is known that GPR7 is expressed in pituitary gland, where prolactin is secreted in response to physiological stimuli (13, 30, 31), further studies will be required to determine whether NPW activates the pituitary cells directly or indirectly by other mechanisms. To define the physiological importance of NPW in complex pathways of feeding and prolactin release, development of selective antagonists for the receptors of NPW and genetically engineered mice with a deletion of NPW or its receptor genes will be useful. In addition to the effects of NPW on feeding and prolactin release, the broad expression patterns

of GPR7 and GPR8 in mammalian brain raise the possibility that NPW may influence many physiological processes.

Several GPCRs have more than two endogenous ligands, which activate and bind to receptors of the same family (32). The endogenous ligands share structural similarity or common motifs in their peptide sequences and are usually encoded by different genes. The structurally related GPCRs GPR7 and GPR8 are functional receptors for NPW23 and NPW30, which are encoded by the prepro-NPW gene. In our search for an endogenous ligand for GPR8, we were unable to isolate other endogenous ligands with structures different from that of NPW. However, these results do not exclude the possibility that other endogenous ligands may exist for GPR7 or GPR8.

In conclusion, we have identified NPW, a novel hypothalamic peptide that is the endogenous ligand for both GPR7 and GPR8 and that affects the central control of feeding and the release of prolactin in rats. This study will provide new insight into the physiological roles of NPW and its receptors, GPR7 and GPR8.

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REFERENCES

1. Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J., Jr., and Civelli, O. (1995) *Science* **270,** 792–794
2. Hinuma, S., Habata, Y., Fujii, R., Kawamata, Y., Hosoya, M., Fukusumi, S., Kitada, C., Masuo, Y., Asano, T., Matsumoto, H., Sekiguchi, M., Kurokawa, T., Nishimura, O., Onda, H., and Fujino, M. (1998) *Nature* **393,** 272–276
3. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., and Fujino, M. (2001) *Nature* **411,** 613–617
4. Feighner, S. D., Tan, C. P., McKee, K. K., Palyha, O. C., Hreniuk, D. L., Pong, S. S., Austin, C. P., Figueroa, D., MacNeil, D., Cascieri, M. A., Nargund, R., Bakshi, R., Abramovitz, M., Stocco, R., Kargman, S., O'Neill, G., Van Der Ploeg, L. H., Evans, J., Patchett, A. A., Smith, R. G., and Howard, A. D. (1999) *Science* **284,** 2184–2188
5. Howard, A. D., Wang, R., Pong, S. S., Mellin, T. N., Strack, A., Guan, X. M., Zeng, Z., Williams, D. L., Jr., Feighner, S. D., Nunes, C. N., Murphy, B., Stair, J. N., Yu, H., Jiang, Q., Clements, M. K., Tan, C. P., McKee, K. K., Hreniuk, D. L., McDonald, T. P., Lynch, K. R., Evans, J. F., Austin, C. P., Caskey, C. T., Van der Ploeg, L. H., and Liu, Q. (2000) *Nature* **406,** 70–74
6. Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. (1998) *Cell* **92,** 573–585
7. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) *Nature* **402,** 656–660
8. Saito, Y., Nothacker, H. P., Wang, Z., Lin, S. H., Leslie, F., and Civelli, O. (1999) *Nature* **400,** 265–269
9. Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., Romanic, A. M., Louden, C. S., Foley, J. J., Sauermelch, C. F., Coatney, R. W., Ao, Z., Disa, J., Holmes, S. D., Stadel, J. M., Martin, J. D., Liu, W. S., Glover, G. I., Wilson, S., McNulty, D. E., Ellis, C. E., Elshourbagy, N. A., Shabon, U., Trill, J. J., Hay, D. W., Ohlstein, E. H., Bergsma, D. J., and Douglas, S. A. (1999) *Nature* **401,** 282–286
10. Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1999) *Cell* **98,** 437–451
11. Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001) *Nature* **409,** 194–198
12. Takekawa, S., Asami, A., Ishihara, Y., Terauchi, J., Kato, K., Shimomura, Y., Mori, M., Murakoshi, H., Kato, K., Suzuki, N., Nishimura, O., and Fujino, M. (2002) *Eur. J. Pharmacol.* **438,** 129–135
13. O'Dowd, B. F., Scheideler, M. A., Nguyen, T., Cheng, R., Rasmussen, J. S., Marchese, A., Zastawny, R., Heng, H. H., Tsui, L. C., Shi, X., Asa, S., Puy, L., and George, S. R. (1995) *Genomics* **28,** 84–91
14. Lee, D. K., Nguyen, T., Porter, C. A., Cheng, R., George, S. R., and O'Dowd, B. F. (1999) *Mol. Brain Res.* **71,** 96–103
15. Mori, M., Sugo, T., Abe, M., Shimomura, Y., Kurihara, M., Kitada, C., Kikuchi, K., Shintani, Y., Kurokawa, T., Onda, H., Nishimura, O., and Fujino, M. (1999) *Biochem. Biophys. Res. Commun.* **265,** 123–129
16. Shimomura, Y., Mori, M., Sugo, T., Ishibashi, Y., Abe, M., Kurokawa, T., Onda, H., Nishimura, O., Sumino, Y., and Fujino, M. (1999) *Biochem. Biophys. Res. Commun.* **261,** 622–626
17. Civelli, O., Nothacker, H. P., Saito, Y., Wang, Z., Lin, S. H., and Reinscheid, R. K. (2001) *Trends Neurosci.* **24,** 230–237
18. Kozak, M. (1996) *Mamm. Genome* **7,** 563–574
19. Nakai, K., and Horton, P. (1999) *Trends Biochem. Sci.* **24,** 34–36
20. von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683–4690
21. Douglass, J., Civelli, O., and Herbert, E. (1984) *Annu. Rev. Biochem.* **53,** 665–715
22. Mehdi, H., Ono, E., and Gupta, K. C. (1990) *Gene* (*Amst.*) **91,** 173–178
23. Peabody, D. S. (1989) *J. Biol. Chem.* **264,** 5031–5035
24. Galanopoulou, A. S., Kent, G., Rabbani, S. N., Seidah, N. G., and Patel, Y. C. (1993) *J. Biol. Chem.* **268,** 6041–6049
25. Hann, S. R., King, M. W., Bentley, D. L., Anderson, C. W., and Eisenman, R. N. (1988) *Cell* **52,** 185–195
26. Prats, H., Kaghad, M., Prats, A. C., Klagsbrun, M., Lelias, J. M., Liauzun, P., Chalon, P., Tauber, J. P., Amalric, F., Smith, J. A., and Caput, D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1836–1840
27. Meiron, M., Anunu, R., Scheinman, E. J., Hashmueli, S., and Levi, B. Z. (2001) *Biochem. Biophys. Res. Commun.* **282,** 1053–1060
28. Beck, B. (2000) *Nutrition* **16,** 916–923
29. Spiegelman, B. M., and Flier, J. S. (2001) *Cell* **104,** 531–543
30. Wynick, D., Small, C. J., Bacon, A., Holmes, F. E., Norman, M., Ormandy, C. J., Kilic, E., Kerr, N. C., Ghatei, M., Talamantes, F., Bloom, S. R., and Pachnis, V. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12671–12676
31. Yang, S. P., Lee, Y., and Voogt, J. L. (2000) *Neuroendocrinology* **72,** 20–28
32. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2863–2867

MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

Identification of Neuropeptide W as the Endogenous Ligand for Orphan G-protein-coupled Receptors GPR7 and GPR8

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