Discovery and Analyses of Melanin-concentrating Hormone and a Novel Brain-gut Peptide, Neuropeptide W as the Specific Ligands for Orphan G protein-coupled Receptors

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Abstract

G protein-coupled receptors (GPCRs) with seven α-helical transmembrane domains receive exogenous and endogenous stimuli on the cell surface in a receptor-specific manner and subsequently convert them into intracellular signaling. Exogenous stimuli such as light, odor, taste, and pheromones come from the external environment, while endogenous stimuli are generated by physiologically active substances such as hormones, neurotransmitters, autacoids, and chemokines, which are produced within organisms. A huge amount of knowledge has accumulated so as to reveal that homeostasis and disease onset are tightly involved in the active or inactive state of the relevant GPCRs or in the variation in the amount of the relevant endogenous ligands. In order to improve disease conditions, many antagonists or agonists targeting the disease-relevant GPCRs have been used to treat patients. In the late 1990s, many genes with structural features similar to known GPCRs were identified mainly in bioinformatics studies. The identified genes were called orphan GPCRs, since endogenous ligands for these receptors were unidentified. In this study, the author focused on orphan GPCR genes, SLC-1, GPR7, and GPR8, and attempted to identify and characterize the endogenous ligands for these receptors.

SLC-1, an orphan GPCR, with homology to the somatostatin receptor and opioid receptor families is expressed predominantly in the brain. Its endogenous ligand has not been identified. In Chapter I, the author reports the identification of melanin-concentrating hormone (MCH) as the endogenous ligand for SLC-1 and the characterization of SLC-1 as a functional MCH receptor. An agonist peptide for rat SLC-1 was purified from the rat brain and was identified as rat MCH. Synthetic MCH peptide

activated G_i-coupled signaling in stable Chinese hamster ovary cell lines expressing human and rat SLC-1, which resulted in a decrease in intracellular cAMP levels with IC_{50} values in the subnanomolar range. These findings indicate that the author identified SLC-1 as a functional MCH receptor, which has been long sought since the first discovery of chum salmon MCH in 1983. The results enabled the studies of MCH as a regulator of the energy homeostasis, anxiety, and sleep from the viewpoint of its receptor, SLC-1. Moreover, the pairing of SLC-1 with MCH is expected to provide helpful information to create screening tools for the discovery of MCH receptor antagonists.

The structurally related orphan GPCRs, GPR7 and GPR8, present homology to the somatostatin receptor and opioid receptor families and are expressed in the central nervous system. Their endogenous ligands have not been identified. In Chapter II, the author reports the discovery and the characterization of a novel brain-gut peptide, neuropeptide W (NPW). An agonist peptide for GPR8 was purified from the porcine hypothalamus using stable Chinese hamster ovary cell lines expressing human GPR8, and a cDNA encoding its precursor protein was cloned. The cDNA encodes two forms of the peptide ligand with 23 and 30 amino acid residues, respectively, as mature peptides. The author designated the two ligands neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30) due to the novelty of their amino acid sequences. The amino acid sequence of NPW23 is 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. The enzyme-linked immunosorbent assays for NPW23 and NPW30 revealed that both peptides were localized in the brain, pituitary, and stomach. Intracerebroventricular administration of NPW23 in rats increased food intake and stimulated prolactin release. These findings indicate that NPW is the endogenous ligand for both GPR7 and GPR8 and

acts as a mediator of the central control of feeding behavior and the neuroendocrine system. This is the first description that the NPW molecular signaling is functioning in the central nervous system.

Abbreviations

BLAST:	Basic Local Alignment Search Tool
BLASTP:	Protein-protein BLAST
CHO:	Chinese hamster ovary
CNS:	Central nervous system
ELISAs:	Enzyme-linked immunosorbent assays
GPCRs:	G protein-coupled receptors
GTPγS:	Guanosine-5′-O-(3-thio)triphosphate
HPLC:	High performance liquid chromatography
MCH:	Melanin-concentrating hormone
NPW:	Neuropeptide W
NPW23:	Neuropeptide W-23
NPW30:	Neuropeptide W-30
PBS:	Phosphate-buffered saline

General Introduction

G protein-coupled receptors (GPCRs) belong to the largest family of cell surface receptors with a unique structural feature of seven α-helical transmembrane domains, which are associated with heterotrimeric G proteins (Rosenbaum et al., 2009). The number of human GPCRs is estimated to be about 800 to date (Isberg et al., 2015). GPCRs are stimulated by binding to extracellular signal transducing molecules such as hormones, neurotransmitters, autacoids, chemokines, tastes, and odors, and subsequently change their conformation to generate a signal via activation of the GPCR-interacted molecules inside the cells (Selbie and Hill, 1998). An approach to isolate the receptor genes for already-known bioactive molecules, also called endogenous ligands because they are synthetized within organisms, allowed the pairing of bioactive molecules with previously unknown receptors through the early 1990s (Masu et al., 1987; Arai et al., 1990; Honda et al., 1991; Hirata et al., 1991; Masu et al., 1991; Sasaki et al., 1991).

GPCRs have various physiological roles in the human body. Homeostasis of the human body is controlled by the fine tuning of GPCRs, and state of the GPCRs is adjusted by adequate amounts of their respective endogenous ligands such as dopamine and orexin in the central nervous system (CNS), adrenaline and angiotensin II in the cardiovascular system, histamine and glucagon-like peptide 1 in the digestive system, and gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone in the reproductive system. Thus, excess and lack of signaling via GPCRs cause diseases. In order to relieve the disease conditions by drug treatment, antagonists or agonists of disease-relevant GPCRs have been developed, and, historically, many drugs with better efficacy and safety have been used for the treatment of patients in almost all disease areas.

GPCRs have become a major drug target class so that about 30% of the drugs in the market act on GPCR molecules (Overington et al., 2006).

In the late 1990s, many genes with structural features similar to known GPCRs were isolated with the assistance of bioinformatics studies of the whole genome sequence and a variety of cDNA sequence data sets in several organisms (Wilson et al., 1998). The isolated genes were called orphan GPCRs. Since the genes were defined only by their sequences with the characteristic features of seven α-helical transmembrane domains and specific motifs of GPCRs (Wess, 1998; Fredriksson et al., 2003; Rovati et al., 2007), the presence of the endogenous ligands for the orphan GPCRs remained unknown. Besides, no one could predict which orphan GPCR gene was correctly translated as a GPCR protein and which orphan GPCR protein could be paired with unidentified endogenous ligands.

The presence of orphan GPCR genes in mammalian genomes provided the opportunities to initiate a distinct field of GPCR research called reverse pharmacology. Studies proceed in the following order: selection of the prioritized genes from many orphan GPCR genes, identification of the endogenous ligand for the selected orphan GPCR, estimation of the pathophysiological significance of the identified endogenous ligand, screening and chemical optimization of compounds as antagonists or agonists acting on the deorphanized GPCR, assessment of the compound efficacy *in vivo* using animal models followed by safety tests, and clinical trials for evaluating efficacy and safety in humans (Libert et al., 1991; Kotarsky and Nilsson, 2004). According to the concept of reverse pharmacology, a limited number of research groups reported deorphanization of orphan GPCRs, discovery of novel endogenous peptide ligands, and pioneering work on the biological functions of the identified endogenous ligands and

deorphanized GPCRs (Meunier et al., 1995; Reinscheid et al., 1995; Hinuma et al., 1998; Sakurai et al., 1998). Reverse pharmacology has great potential to elucidate the mechanism of unknown biological phenomena, which can be explained by the pairing of newly identified endogenous ligands and their deorphanized GPCRs. Additionally, it provides opportunities to identify entirely new biological phenomena caused by the pairs. Besides, as far as pathophysiological meanings of the pairs are verified, drug discovery efforts are implemented to create antagonists or agonists acting on the deorphanized GPCR.

The author decided to use the reverse pharmacology approach to identify endogenous ligands for the orphan GPCRs followed by molecular, cellular, and *in vivo* analysis of the biological functions of the identified endogenous ligands and the deorphanized GPCRs. The limited number of success noted above prompted us to refine each experimental step needed in the course of deorphanization. There were four imperative steps for deorphanization as follows: prioritization of orphan GPCRs applied to the assay, establishment of cell lines expressing the orphan GPCR gene in sufficient amounts, establishment of the assays dealing with any types of ligand-induced GPCR signaling, and composition of the ligand bank tested in the assay.

In this study, the author aimed to obtain useful knowledge in the fields of basic science and drug discovery by deorphanization of orphan GPCRs, which was followed by characterization of the relevant molecules. In Chapter I, the author created experimental systems focused on deorphanization of the orphan GPCRs with unidentified endogenous peptide ligands and applied them to identify melanin-concentrating hormone (MCH) as the endogenous ligand for SLC-1. Characterization of SLC-1 as a functional MCH receptor was conducted. In Chapter II, the author discovered a novel brain-gut peptide,

neuropeptide W (NPW), as the endogenous ligand for GPR7 and GPR8. Studies on molecular biology, *in vitro* pharmacology, and tissue distribution of NPW were conducted. In addition, an exploratory study on the *in vivo* effects of NPW was conducted to estimate NPW biological role.

Chapter I: Discovery and Analysis of Melanin-concentrating Hormone as the Specific Ligand for the Orphan GPCR, SLC-1

1. Introduction

A DNA clone of *SLC-1* was originally isolated by Kolakowski *et al.* from a human genomic DNA library (Kolakowski et al., 1996) and was suggested to encode an orphan GPCR with higher amino acid sequence similarity to the seven transmembrane regions of the five subtypes of the somatostatin receptor and the three subtypes of the opioid receptor (about 40% amino acid identity). *SLC-1* mRNA is expressed in the forebrain and hypothalamus in humans and in the brain, heart, kidney, and ovary in rats (Kolakowski et al., 1996). Two years later, a cDNA clone of the rat orthologue of *SLC-1* was independently isolated by screening of a rat brain cDNA library with a hybridization probe whose nucleotide sequence was derived from PCR using a set of degenerate primers of each of the third and seventh transmembrane of known GPCRs (Lakaye et al., 1998). Comparison of the deduced amino acid sequences between the two revealed that the human *SLC-1* DNA could not encode the full human SLC-1 protein due to the insertion of an intron sequence into the 5′-region of the human *SLC-1* DNA.

In Chapter I, the author reports the purification and characterization of MCH. An agonist peptide for rat SLC-1 was purified from the rat brain and was identified as rat MCH peptide. The functional characterization of MCH for human and rat SLC-1 indicated that MCH is the endogenous ligand for SLC-1.

2. Materials and Methods

2.1. Establishment of Chinese hamster ovary (CHO) cell lines expressing rat SLC-1

A cDNA encoding rat SLC-1 (Lakaye et al., 1998) was isolated by PCR from the reverse-transcribed product of poly (A) RNA of the rat whole brain (CLONTECH). Two PCR primers were designed to clone a rat cDNA with a *Sal*I cleavage site and a *Spe*I cleavage site that were located at the 5`- and 3`-ends, respectively. The two primer sequences were as follows: 5`-GTCGACATGGATCTGCAAACCTCGTTGCTGTG-3` (forward) and 5`-ACTAGTTCAGGTGCCTTTGCTTTCTGTCCTCT-3` (reverse). The *Sal*I- and *Spe*I-digested fragment of the rat cDNA was ligated into a unique site of the pAKKO-111H expression vector (Hinuma et al., 1994), which equipped the SRα promoter and *dhfr* gene as the selection marker. CHO *dhfr*$^{-}$ cells were transfected with the expression vector by calcium phosphate method using a CellPhect transfection kit (Amersham Pharmacia Biotech). CHO cell lines stably expressing the rat SLC-1 were selected under conditions wherein the growth medium lacked nucleotides.

2.2. Cloning of human SLC-1 cDNA

A cDNA encoding human SLC-1 was isolated from a cDNA library constructed from a human embryonic brain extract (Gibco BRL) using a gene-trapper method according to the supplier's protocol (GeneTrapper cDNA Positive Selection System, Gibco BRL). The gene-trapper method employed two oligonucleotides whose sequences were identical to the nucleotide sequences found in a human genomic *SLC-1* DNA clone (Kolakowski et al., 1996): 5`-CAACAGCTGCCTCAACCC-3` and 5`-CCTGGTGATCTGCCTCCT-3`. The former oligonucleotide was biotinylated and was employed for hybridization, and the

latter was used to synthesize double-stranded DNA. An isolated cDNA was sequenced by an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) using a DyeDeoxy Terminator Cycle Sequence Kit (PE Applied Biosystems).

2.3. Establishment of CHO cell lines expressing human SLC-1

CHO cell lines expressing human SLC-1 were prepared by using two different expression vectors. A human *SLC-1* nucleotide starting at the 1st ATG codon and ending at the stop codon in the cloned human *SLC-1* cDNA was named human *SLC-1 (L)*, while a human *SLC-1* nucleotide starting at the 3rd ATG codon and ending at the stop codon in the cloned human *SLC-1* cDNA was named human *SLC-1 (S)*. The human *SLC-1 (L)* DNA with a *Sal*I cleavage site at the 5`-end and a *Spe*I cleavage site at the 3`-ends was obtained by PCR from the cloned human *SLC-1* cDNA as a template using two primers: 5`-AGTCGACATGTCAGTGGGAGCCATGAAGAAGAAGGG-3` (forward) and 5`-AACTAGTTCAGGTGCCTTTGCTTTCTGTCCTCT-3` (reverse). In the same way, the human *SLC-1 (S)* DNA with a *Sal*I cleavage site at the 5`-end and a *Spe*I cleavage site at the 3`-ends was obtained by PCR from the cloned human *SLC-1* cDNA as a template using two primers: 5`-GTCGACATGGACCTGGAAGCCTCGCTGCTGC-3` (forward) and 5`-ACTAGTTCAGGTGCCTTTGCTTTCTGTCCTC-3` (reverse). Each *Sal*I- and *Spe*I-digested fragment obtained from the PCR-amplified human *SLC-1 (L)* DNA and human *SLC-1 (S)* DNA was ligated into a unique site of the pAKKO-111H expression vector. CHO *dhfr*$^{-}$ cells were transfected with the expression vector by calcium phosphate method using a CellPhect transfection kit (Amersham Pharmacia Biotech). CHO cell lines stably expressing the human SLC-1 (L) and the human SLC-1 (S) were selected under conditions wherein the growth medium lacked nucleotides.

2.4. Phylogenetic analysis of the GPCRs structurally similar to rat SLC-1 protein

One algorithm in Basic Local Alignment Search Tool (BLAST) programs, a protein-protein BLAST (BLASTP) search was conducted using the amino acid sequence of rat SLC-1 as a query against the database Non-redundant protein sequences (nr) of National Center for Biotechnology Information (NCBI). Amino acid sequences of human GPCRs were chosen from the search result in order of similarity to the rat SLC-1 by an index of Max score. Twelve GPCRs selected by the similarity were as follows: melanin-concentrating hormone receptor 1 (NP_005288), somatostatin receptor type 1 (NP_001040), somatostatin receptor type 2 (NP_001041), somatostatin receptor type 3 (NP_001042), somatostatin receptor type 4 (NP_001043), somatostatin receptor type 5 (NP_001044), mu-type opioid receptor isoform MOR-1 (NP_000905), delta-type opioid receptor (NP_000902), kappa-type opioid receptor isoform 1 (NP_000903), nociceptin receptor (NP_001186948), neuropeptides B/W receptor type 1 (NP_005276), and neuropeptides B/W receptor type 2 (NP_005277). When the author started the study in 1998, three GPCRs, melanin-concentrating hormone receptor 1, neuropeptides B/W receptor type 1, and neuropeptides B/W receptor type 2 were orphan GPCRs with unknown endogenous ligands, and were called SLC-1, GPR7, and GPR8, respectively. A phylogenetic tree was constructed among the 12 GPCRs and the rat SLC-1 by using the neighbor-joining method.

2.5. Northern blot analysis of *SLC-1* in human tissues

Northern blot experiment was conducted essentially as described (Ogi et al., 1990) with following minor modifications. The cloned human *SLC-1* cDNA was used as a template

to synthesize DNA probes that were labeled with [α-^{32}P]dCTP (Dupont/NEN) by a multi prime DNA labeling kit (Amersham Pharmacia Biotech). Human Multiple Tissue Northern Blots membranes (CLONTECH) were hybridized with the radiolabeled probes in hybridization buffer (2 × SSC, 0.1 % SDS, 10 × Denhardt's Solution, 50 % formamide) for an overnight incubation at 42°C. The Human Multiple Tissue Northern Blots were premade membranes: poly (A) RNA samples purified from a variety of human tissues were electrophoresed, and then these separated RNA molecules in the gel were transferred to nylon membranes. After the hybridization, the membrane filters were finally washed in 0.1 % SSC with 0.1 % SDS at 50°C. Signals of the radiolabeled probes that bound to human *SLC-1* mRNA were detected by a Bioimageanalyzer-BAS2000 (Fuji Film).

2.6. Assay for inhibition of forskolin-induced intracellular accumulation of cAMP

Inhibitory activities of test samples for cAMP accumulation were measured. Cell lines of CHO expressing rat SLC-1 (CHO-rat SLC-1) were plated on 24-well plates at 5 × 10^4 cells/well and were cultured for 2 days. The cells were washed three times with 0.5 ml of assay buffer (Hanks' buffered salt solution supplemented with 0.2 mM 3-isobutyl-1-methylxantine (Wako Pure Chemical), 0.05% bovine serum albumin, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) and were cultured in the assay buffer for 30 min. After washing the cells three times with 0.5 ml of the assay buffer, a test sample in 0.5 ml of the assay buffer supplemented with 1 μM forskolin (Wako Pure Chemical) was added to the cells in each well, and the cells were incubated at 37°C for 24 min. cAMP synthesis in the cells was terminated by addition of 0.1 ml of 20% perchloric acid, and intracellular cAMP was extracted with 20% perchloric acid on ice for 1 h. The amount of extracted cAMP was measured using an enzyme-linked

immunoassay kit (Amersham Pharmacia Biotech). The same procedure was used for assessing the agonist activity of MCH on the cell lines of CHO expressing human SLC-1 (L) (CHO-human SLC-1 (L)) and CHO expressing human SLC-1 (S) (CHO-human SLC-1 (S)).

2.7. Purification of MCH as an endogenous ligand for SLC-1 from the rat brain

The brain tissues of 70 male Wistar rats (8 weeks old) were dissected and were boiled in 800 ml of pure water for 10 min, immediately after decapitation. After cooling with ice, acetic acid was added to make a 1.0 M solution, and the tissue was homogenized using a Polytron homogenizer. The homogenate was centrifuged, and a double volume of acetone was added to supernatant. The precipitates were re-extracted with 800 ml of 1.0 M acetic acid and were processed in a similar fashion. The acetone-treated extract was centrifuged to remove the pellets. The supernatant was concentrated to remove the acetone and was then extracted with diethyl ether to remove lipid. The solution was applied to an YMCgel ODS-AM 120-S50 column (30.0 × 240 mm; YMC), and the column was eluted with 60% acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid. The eluate was lyophilized, and the lyophilized powder dissolved in 1.0 M acetic acid was charged on a SP-Sephadex C-25 column (25.0 × 80 mm; Amersham Pharmacia Biotech). The column was then eluted with 1.0 M acetic acid, 2.0 M pyridine, and 2.0 M pyridine-acetic acid (pH 5), successively. The 2.0 M pyridine-acetic acid fraction was lyophilized. An agonist peptide for rat SLC-1 was purified from the lyophilized materials by successive high performance liquid chromatography (HPLC) using a TSKgel ODS-80T_S column (21.5 × 300 mm; 10–60% CH_3CN gradient in 0.1% trifluoroacetic acid for 80 min at 5.0 ml/min; Tosoh), a CM-2SW column (4.6 × 150 mm; 10–500 mM ammonium formate ($HCOONH_4$) (pH 5.25)

gradient in 10% CH_3CN for 60 min at 1.0 ml/min; Tosoh), a diphenyl column of Vydac 219-TP54 (4.6 × 250 mm; 27.5–42.5% CH_3CN gradient in 0.1% trifluoroacetic acid for 90 min at 1.0 ml/min; Separation Group), and a Develosil ODS-UG-3 column (2.0 × 150 mm; 27.5–42.5% CH_3CN gradient in 0.1% trifluoroacetic acid for 120 min at 0.2 ml/min; Nomura Kagaku) combined with the assay for inhibition of forskolin-induced intracellular accumulation of cAMP in CHO-rat SLC-1 cells. The activity was recovered as a single peak at 36.8% of CH_3CN in the final HPLC.

2.8. Structural analysis of the purified peptide

The N-terminal amino acid sequence of the purified peptide was determined using two protein sequencers, Beckman LF3400 (Beckman Instruments, Inc.) and Procise 491cLC (Applied Biosystems, Inc.). The sequencing reaction by the Beckman LF3400 converts Cys to dehydroalanine, and the resulting phenylthiohydantoin (PTH) derivative is detected as PTH-dehydroalanine. The Procise 491cLC analysis provides amino terminal amino acid sequence information based on a conventional method of the Edman degradation. The mass spectrum of the purified peptide was measured using a JEOL HX-110 (Jeol Ltd., Tokyo, Japan) equipped with a Cs gun for the LSIMS mode.

2.9. Peptide

Human melanin-concentrating hormone, rat somatostatin-14, rat somatostatin-28, and rat cortistatin-29 were purchased from Peninsula Laboratories Ltd.

3. Results

3.1. Structural and gene expression features of SLC-1

SLC-1 protein showed sequence similarity to other human GPCRs with known or unknown endogenous ligands. Twelve GPCRs selected by a BLASTP search against a non-redundant protein database in order of similarity to the rat SLC-1 were as follows: human orthologue of the rat SLC-1, five subtypes of the somatostatin receptor, three subtypes of the opioid receptor, one opioid-like receptor, and two structurally related orphan GPCRs, GPR7 and GPR8 (O'Dowd et al., 1995). The author attempted to clarify an evolutionary relationship among the selected twelve human GPCRs above and the rat SLC-1 by the neighbor-joining method. The phylogenetic tree demonstrated that SLC-1 presented homology to the somatostatin receptor and opioid receptor families (Fig. 1-1).

The gene expression pattern of *SLC-1* in human tissues was examined by northern blot. Human *SLC-1* was expressed predominantly in the brain and moderately in the ovary and spinal cord (Fig. 1-2). Gene expression of human *SLC-1* was not detected in nearly all peripheral tissues, except the ovary.

3.2. Technologies specializing in the identification of the endogenous peptide ligands for orphan GPCRs

The author decided to focus on research of the orphan GPCRs with the likelihood of presenting bioactive peptides as their endogenous ligands. This strategy required a set of technologies that could be matched with an efficient detection of peptide agonist-induced activation of the orphan GPCRs in cell- or membrane-based assays. Expression levels of the transfected-orphan GPCR genes in the assay cell lines were some of the critical factors to ensure the robustness of the assay results. The cell lines were established by screening

based on the expression levels of the orphan GPCR gene, which were measured quantitatively (Fig. 1-3A). The next challenge was about the test samples to be examined in the assays. To increase the probability of detecting the agonist activities of peptide substances, the test samples were prepared from a wide variety of animal organs through a peptide-selective extraction and fractionation by reverse phase HPLC (Fig. 1-3B). The remaining challenge was what assay should be used in the experiments, since no one knew which subtype of α subunit of G protein was actually coupled with the orphan GPCR of my interests. Multiple assays were applied to detect a variety of intracellular signals that were generated in the assay cell lines: an assay for G_s-mediated intracellular cAMP synthesis, an assay for G_i-mediated inhibition of forskolin-induced intracellular accumulation of cAMP, and an assay for G_q-mediated arachidonic acid metabolite release. In addition to these assay systems, [^{35}S] guanosine-5′-O-(3-thio)triphosphate (GTPγS) binding to an activated form of G protein subunit α in the membranes of the cell lines was determined (Fig. 1-3C).

3.3. Isolation and identification of MCH as the endogenous ligand for rat SLC-1

The author purified an agonist peptide for rat SLC-1 from the rat brain using CHO-rat SLC-1 cells and demonstrated that the structure of the agonist peptide was identical to that of rat MCH. In the assays of intracellular signaling evoked by peptide fractions prepared from a variety of tissue extracts, the fractions from the rat whole brain, porcine whole brain, and porcine hypothalamus stimulated the inhibition of forskolin-induced intracellular accumulation of cAMP in the CHO-rat SLC-1 cells. Their activities were sensitive to incubation with proteinase (data not shown). Since the rat whole brain fraction presented the highest agonist activity per tissue weight among all extracts, extracts of the

rat brain were subjected to purification of an agonist substance for rat SLC-1 by successive chromatography. Its activity was assessed using the cAMP accumulation inhibition assay (Fig.1-4 A and B). The purification process yielded approximately 950 pmol of the purified agonist peptide for rat SLC-1 from 140 g of the rat brain (Fig. 1-5). The purified peptide was subjected to N-terminal amino acid sequence analysis using two protein sequencers, and the analysis afforded the following sequence, DFDMLRCMLGRVYRPC, corresponding to the 1st to 16th residues of rat MCH (Table 1-1). The Cys residues at the 7th and 16th positions were identified as PTH-dehydroalanine. Mass spectrum analysis using LSIMS of the active substance provided the protonated molecular ion peak at m/z 2387.2 (Fig. 1-6), which represented the whole sequence of rat MCH with 19 amino acid residues. Consequently, the amino acid sequence of the purified agonist peptide was determined to be DFDMLRCMLGRVYRPCWQV, and the agonist peptide for rat SLC-1 was identified as rat MCH.

3.4. Comparison of SLC-1 amino acid sequences among humans, rats, and mice

The author compared SLC-1 amino acid sequences of humans, rats, and mice. The longest open reading frame of human (Fig. 1-7), rat, and mouse *SLC-1* nucleotide sequences encode SLC-1 proteins composed of 422, 353, and 353 amino acid residues, respectively. The deduced amino acid sequences of human, rat, and mouse SLC-1 demonstrated structural features common to the rhodopsin family of GPCRs (Fig. 1-8). The respective three SLC-1 proteins possess seven membrane-spanning domains with potentially extracellular and cytoplasmic terminus, the E/DRY motif on TM3 (Rovati et al., 2007), and the NPXXY motif at the cytoplasmic end of TM7 (Fritze et al., 2003). The sequence alignment indicated that the 353-amino acid sequence of human SLC-1 starting with

Met^{70} and ending with Thr^{422} was nearly identical to the whole sequences of the rat and mouse SLC-1. The human SLC-1 protein of 353 amino acid residues was renamed human SLC-1 (S), while the human SLC-1 protein of 422 amino acid residues was renamed human SLC-1 (L). The human SLC-1 (S), the rat SLC-1, and the mouse SLC-1 shared 95.8% amino acid identity. The difference in amino acid length between the human SLC-1 (L) and the other SLC-1 results from its N-terminal region with an addition of 69 amino acids, which includes two Met residues, potential translation start sites, at positions 1 and 6.

3.5. Inhibition of intracellular cAMP accumulation by MCH in CHO cells expressing SLC-1

The author examined the functional activities of MCH on three different CHO cells expressing the human SLC-1 (S), the human SLC-1 (L), and the rat SLC-1, respectively. Amino acid sequences of human MCH and rat MCH are entirely identical to each other, and, thus, the synthetic human MCH peptide was used to assess the inhibition of intracellular cAMP accumulation. The MCH peptide inhibited cAMP accumulation in a dose-dependent manner in cell lines of CHO expressing human SLC-1 (L) (CHO-human SLC-1 (L)), CHO expressing human SLC-1 (S) (CHO-human SLC-1 (S)), and CHO expressing rat SLC-1 (CHO-rat SLC-1), but not in the CHO-mock cells that were established by the transfection of the pAKKO-111H vector without any inserts (Fig. 1-9A). MCH inhibited cAMP accumulation in both the CHO-human SLC-1 (S) and the CHO-rat SLC-1 cells with IC_{50} values of 0.08 nM and 0.1 nM, respectively. On the other hand, MCH only weakly inhibited the accumulation of cAMP in the CHO-human SLC-1 (L) cells with an IC_{50} value of 5 nM.

The author then assessed the potential of agonist peptides for the somatostatin receptor family, which showed homology with the rat and human SLC-1 based on my search for GPCRs with known endogenous ligands to inhibit intracellular cAMP accumulation in cell lines expressing SLC-1. The peptides, somatostatin-14, somatostatin-28, and cortistatin-29, are endogenous ligands for the five subtypes of the somatostatin receptor. The three peptides at the concentration of 1 μM did not inhibit cAMP accumulation in the CHO-rat SLC-1 cells (Fig. 1-9B).

4. Discussion

In this study, the author adopted a strategy to identify the endogenous ligands for orphan GPCRs with significant structural similarities to the GPCRs whose endogenous ligands had been known as peptides, but not as small molecular weight substances such as lipids, phospholipids, amines, nucleotides, amino-acid derivatives, prostanoids, or leukotrienes. In line with this strategy, experimental systems to detect agonist activities to GPCRs were developed so that subtle changes induced by the peptide-focused natural substances could be monitored accurately in the cell- or membrane-based GPCR-mediated signaling assays (Fig. 1-3C). These experimental systems were used to identify an endogenous peptide ligand for SLC-1, an orphan GPCR showing high amino acid sequence similarities to the somatostatin receptor and opioid receptor families (Fig. 1-1). The author purified rat MCH as the agonist peptide for SLC-1 from the rat brain using the CHO-rat SLC-1 cells (Fig. 1-5). This finding indicates the creation of an experimental platform for deorphanization of orphan GPCRs, which enabled the identification of MCH as the endogenous ligand for SLC-1.

The author demonstrated that MCH specifically inhibited forskolin-induced intracellular cAMP accumulation in a dose-dependent manner in CHO cells expressing the human SLC-1 (S), the human SLC-1 (L), and the rat SLC-1, with IC_{50} values of 0.08 nM, 5 nM, and 0.1 nM, respectively (Fig. 1-9A). MCH showed high agonist potency to the CHO cells expressing the human SLC-1 (S) and the rat SLC-1, but not to the CHO cells expressing the human SLC-1 (L). Human SLC-1 (L) is supposed to be a protein of 422 amino acid residues, which is translated from the 1st AUG start codon of the human *SLC-1* mRNA, while human SLC-1 (S) is supposed to be a protein of 353 amino acid residues, which is translated from the 3rd AUG start codon of the mRNA (Fig. 1-7). The

human SLC-1 (S) resembles the rat SLC-1 with 95.8 % amino acid identity (Fig. 1-8). The weak responsiveness of the putative human SLC-1 (L) to the MCH stimulation in the CHO cells can be explained that the extra N-terminal portion confers conformational changes of the human SLC-1 (L), which leads to a lower affinity for MCH or an inefficient G protein activation inside the cells. The difference in the responsiveness to the MCH stimulation between the human SLC-1 (L) and the human SLC-1 (S) does not necessarily mean that an authentic human SLC-1 protein is not produced from the human *SLC-1* mRNA *in vivo*, because the protein translation machinery might differ between an artificial protein expression system using CHO cells and *in vivo*. Based on the experimental results in this study, it is postulated that the authentic human SLC-1 protein with an efficient signal transduction can be successfully produced in the tissues such as the brain where the human *SLC-1* mRNA is predominantly expressed *in vivo* (Fig. 1-2). The gene expression profile of human *SLC-1* coincides with the experimental results about the tissue distribution of MCH peptide, which demonstrated that the immunoreactive MCH is mainly localized in the brain (Takahashi et al., 1995). These results support the idea that MCH primarily functions in the brain as a neurotransmitter via the activation of SLC-1. These findings indicate that *SLC-1* gene encodes a functional MCH receptor that signals through inhibition of intracellular cAMP synthesis.

The author identified SLC-1 as a MCH receptor in 1999 (Shimomura et al., 1999). The molecular nature of a functional MCH receptor had been unknown until 1999 when five research groups, including us, independently reported that MCH is the endogenous ligand for SLC-1 (Chambers et al., 1999; Saito et al., 1999; Shimomura et al., 1999; Bächner et al., 1999; Lembo et al., 1999). The following is the brief description of advancements in the research of MCH from the discovery to the studies of biological functions until 1998.

MCH was first discovered from piscine pituitaries as a fish skin-color regulating hormone, which stimulates the perinuclear aggregation of melanosomes within teleost melanocytes in 1983 (Kawauchi et al., 1983). Since the existence of the MCH-like immunoreactivity in the rat brain was reported in 1986 (Zamir et al., 1986; Naito et al., 1986), much attention has been paid on the studies of its molecular structure and biological functions in mammalian organisms. In addition to the structural analysis of rat MCH peptide isolated from the hypothalamus (Vaughan et al., 1989), molecular cloning of human, rat, and mouse *MCH* precursor genes revealed that each mature peptide is a 19-amino acid cyclic peptide with entirely identical amino acid sequences (Nahon et al., 1989; Presse et al., 1990; Breton et al., 1994). In the rat brain, MCH-positive perikarya were found exclusively in the lateral hypothalamus area and zona incerta, which are involved in the regulation of feeding behavior. MCH fibers are widely distributed through the brain (Bittencourt et al., 1992), suggesting that MCH may act as a neurotransmitter involved in a wide range of physiological functions in the CNS. Qu *et al*. reported in 1996 that *Pmch* mRNA expression levels in the hypothalamus positively correlated with food intake in the ob/ob mice and fasting mice models compared to control mice, and that MCH injection into the lateral ventricles of rats resulted in increased food consumption (Qu et al., 1996). An experiment using genetically modified mice with null mutation of the *Pmch* gene that was reported in 1998 suggested a role of MCH as an orexigenic peptide acting in the hypothalamus, which was drawn by the facts that the mice showed phenotypes of leanness with hypophagia and an increased metabolic rate (Shimada et al., 1998). As a considerable number of useful knowledge about MCH biology had accumulated from the identification of MCH from chum salmon in 1983 through the year of 1998, discovery of a MCH receptor became an urgent challenge to deepen the understanding of the molecular

mechanism at the site of action of MCH. Thus, the discovery of SLC-1 as a MCH receptor impacted on the research of MCH biology together with the other four groups in 1999.

Furthermore, the identification and understanding of SLC-1 advanced our knowledge on MCH. In 2001, the second MCH receptor presenting an amino acid identity of 36% with SLC-1 was reported by six groups, including us (Hill et al., 2001; Mori et al., 2001; Sailer et al., 2001; An et al., 2001; Wang et al., 2001; Rodriguez et al., 2001). The second MCH receptor exists in humans, monkeys, and dogs, but not in rodents. As a consequence of the full characterization of the endogenous ligand for SLC-1 and the second MCH receptor, the International Union of Basic and Clinical Pharmacology officially named SLC-1 and the second MCH receptor, melanin-concentrating hormone receptor 1 (MCHR1) and melanin-concentrating hormone receptor 2 (MCHR2), respectively (Foord et al., 2005). Studies using the mice with null mutation of the *Mchr1* that was formerly called *Slc-1* clarified the role of MCH in energy homeostasis as the *Mchr1* KO mice were resistant to diet-induced obesity with higher metabolic rates (Marsh et al., 2002; Chen et al., 2002). *In vitro* MCHR1 pharmacology was developed to carry out pharmacological evaluation of small molecule compounds as MCHR1 antagonists. In the recombinant cells expressing MCHR1, MCH stimulates G_i- and G_q-coupled signaling pathways (Hawes et al., 2000). Besides, MCH derivatives with high affinity binding to MCHR1 and less non-specific binding were newly synthesized to establish reliable receptor binding assays (Audinot et al., 2001; Takekawa et al., 2002; Gao et al., 2004). A non-peptide MCHR1 antagonist, T-226296, effective in a rat model of food intake was reported as a candidate compound for anti-obesity treatment by a group in our research laboratories in 2002 (Takekawa et al., 2002). Since then, different companies published a number of studies and patents concerning the medicinal chemistry of MCHR1 antagonists (Johansson,

2011). Additionally, several compounds have entered clinical trials with the indication of obesity treatment (MacNeil, 2013). In addition to MCH biological role in energy homeostasis, the distribution pattern of MCH fibers throughout the brain triggered the studies on other physiological functions in the CNS such as anxiety (Smith et al., 2006), depression (Saito et al., 2001), and sleep (Verret et al., 2003). Small molecule MCHR1 antagonists and genetically modified mice with mutations in the *Mchr1* gene have been utilized to unravel the involvement of MCH in the regulation of anxiety (Borowsky et al., 2002; Chaki et al., 2005), depression (Borowsky et al., 2002; Chaki et al., 2005), and sleep (Adamantidis et al., 2008; Willie et al., 2008).

In conclusion, the author identified MCH as the endogenous ligand for SLC-1. The discovery of SLC-1 as a functional MCH receptor will provide new insights into the physiological roles of MCH and its receptor, SLC-1, and will enable the development of MCH receptor modulators for the treatment of CNS diseases.

5. Tables

Table 1-1

N-terminal amino acid sequence analysis of an agonist peptide for rat SLC-1

	Detected amino acid (pmol)	
Cycle	Procise 491cLC analysis	LF 3400 analysis
1	Asp (1.95)	Asp (20.7)
2	Phe (2.18)	Phe (12.7)
3	Asp (2.05)	Asp (17.2)
4	Met (2.16)	Met (13.5)
5	Leu (1.92)	Leu (10.6)
6	Arg (1.38)	Arg (11.7)
7	not detected	PTH-dehydroalanine
8	Met (1.67)	Met (13.1)
9	Leu (1.55)	Leu (9.0)
10	Gly (1.05)	Gly (8.2)
11	Arg (1.09)	Arg (4.9)
12	Val (1.34)	Val (10.1)
13	Tyr (1.25)	Tyr (8.9)
14	Arg (0.91)	not detected
15	Pro (0.57)	Pro (7.9)
16	not detected	PTH-dehydroalanine
17	Trp (0.35)	not detected
18	Gln (0.28)	not detected
19	Val (0.05)	not detected

The N-terminal amino acid sequence of the agonist peptide was determined by two independent experiments named Procise 491cLC analysis and LF 3400 analysis. The Procise 491cLC analysis provided N-terminal amino acid sequence information based on a conventional method of the Edman degradation. Cysteine residue in the peptide was detected as a stable derivative of PTH-dehydroalanine in the LF 3400 analysis.

6. Figures

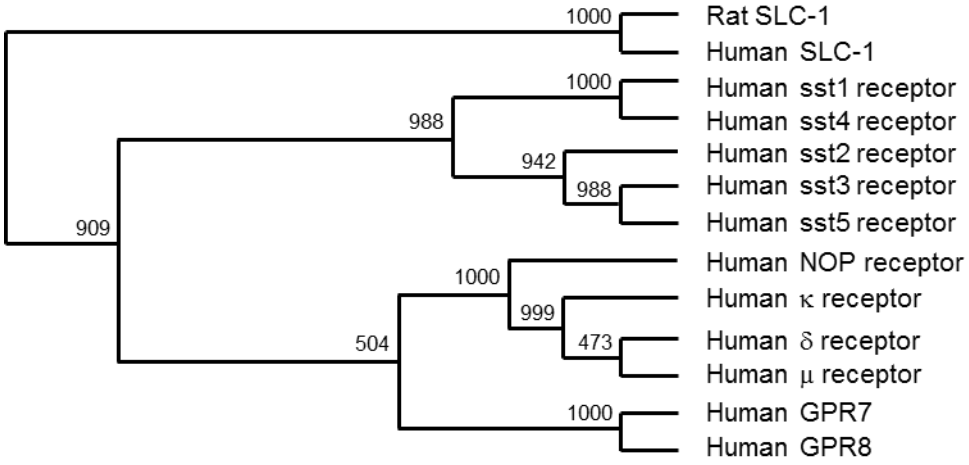

Fig. 1-1. A phylogenetic tree of GPCRs with high similarity to SLC-1 protein. Selected GPCRs presenting amino acid sequence similarity to rat SLC-1 were analyzed by the neighbor-joining method. Bootstrap values are placed on each node. Three GPCRs, GPR7, GPR8, and SLC-1, were recognized as orphan GPCRs, and the others were GPCRs with known endogenous peptide ligands when the study in Chapter I started. Sst1 receptor, somatostatin receptor 1; sst4 receptor, somatostatin receptor 4; sst2 receptor, somatostatin receptor 2; sst3 receptor, somatostatin receptor 3; sst5 receptor, somatostatin receptor 5; NOP receptor, nociceptin receptor; κ receptor, kappa-type opioid receptor isoform 1; δ receptor, delta-type opioid receptor; μ receptor, mu-type opioid receptor isoform MOR-1.

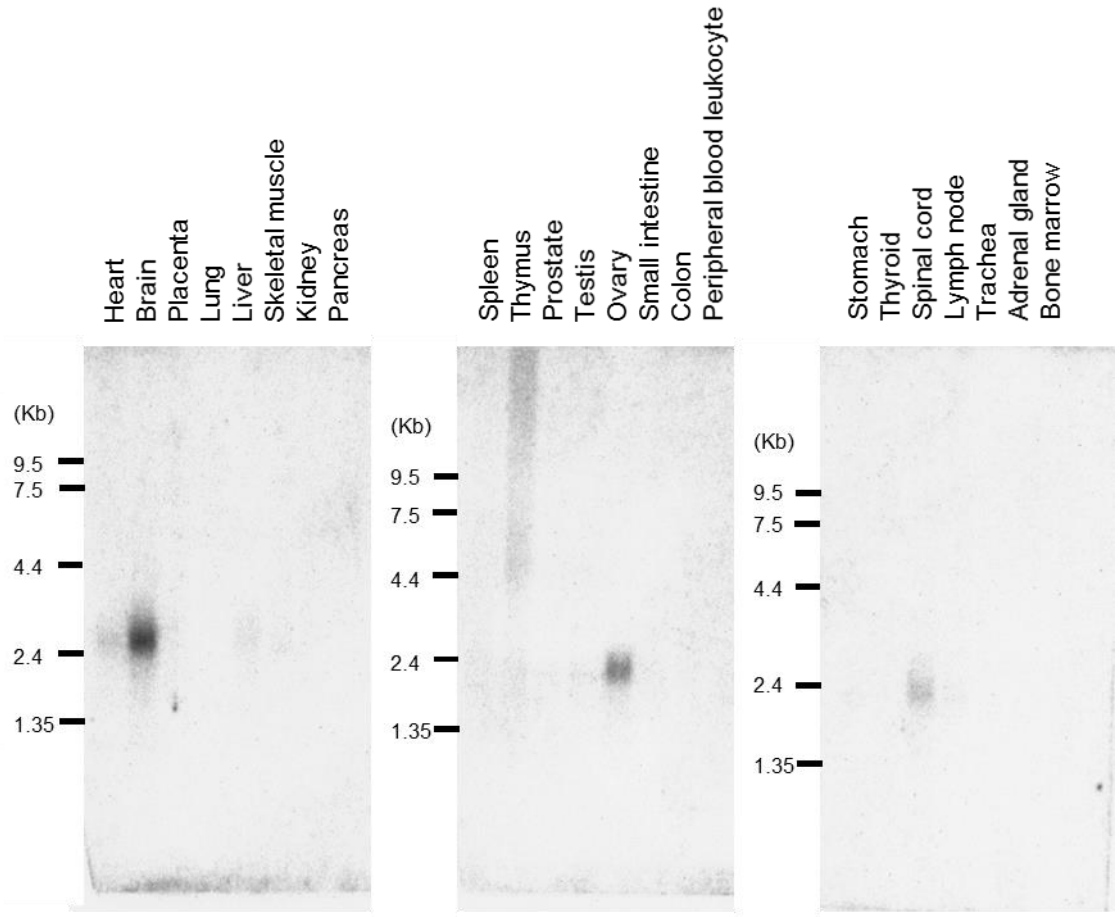

Fig. 1-2. Gene expression profile of human *SLC-1*. *SLC-1* mRNA expression levels in various human tissues were analyzed by northern blot. Premade membranes (Human Multiple Tissue Northern Blot, CLONTECH) ready to be hybridized were used. Upper lane indicates name of each tissue from which poly (A) RNA sample was purified for creation of the premade membranes.

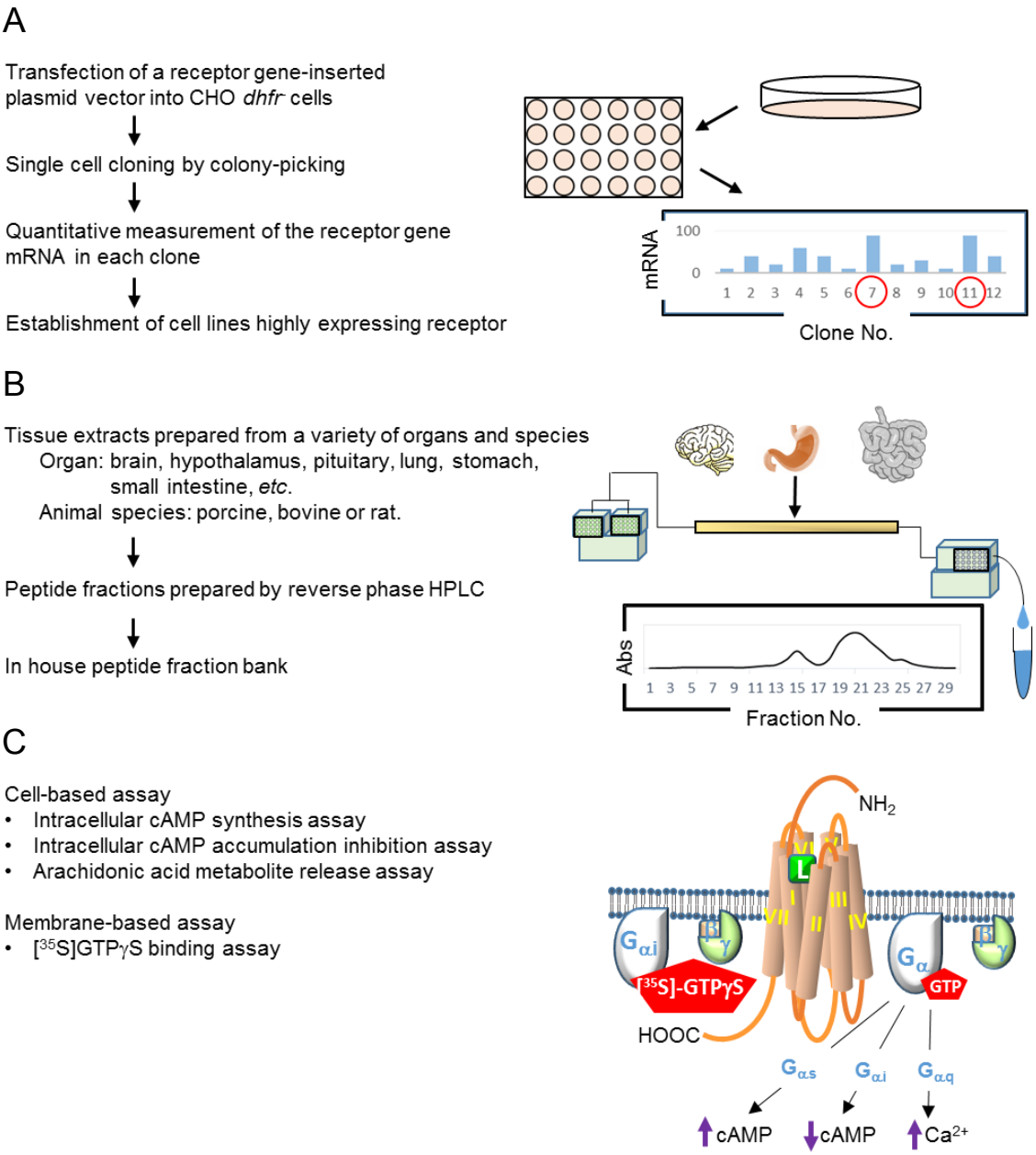

Fig. 1-3. Essential steps to be refined for successful deorphanization. *A*, establishment of orphan GPCR gene-transfected cell lines. The cell lines expressing orphan GPCR gene at the highest level were selected by a quantitative measurement of its mRNA. *B*, peptide bank. Test samples focused on peptide substances were obtained from extracts of fresh animal tissues followed by reverse phase HPLC. *C*, assay package. Multiple assays were used to detect agonist activities of ligand (L) for the orphan GPCR gene-transfected cell lines.

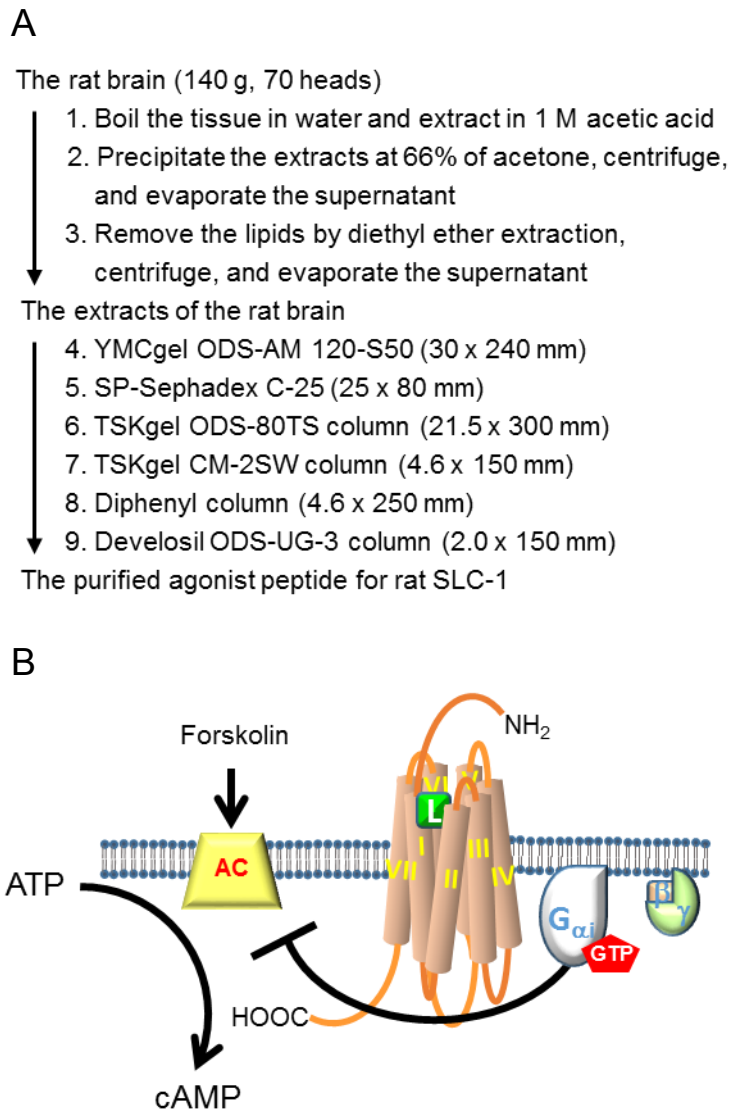

Fig. 1-4. Isolation of an agonist peptide for rat SLC-1. *A*, purification procedure of an agonist peptide for rat SLC-1. The purification procedure comprised two parts of process, extraction from the rat brain tissues (step 1 to 3) and a combination of HPLCs (step 4 to 9). Agonist activity for rat SLC-1 was monitored with an assay of intracellular cAMP accumulation inhibition. *B*, mechanistic basis of the assay. Forskolin activates a membrane protein, adenylate cyclases (AC), which catalyzes the changes of ATP to cAMP. Ligand (L) as an agonist for rat SLC-1, which is able to suppress the AC activity through $G_{\alpha i}$ protein activation results in inhibition of cAMP accumulation in CHO cells expressing rat SLC-1.

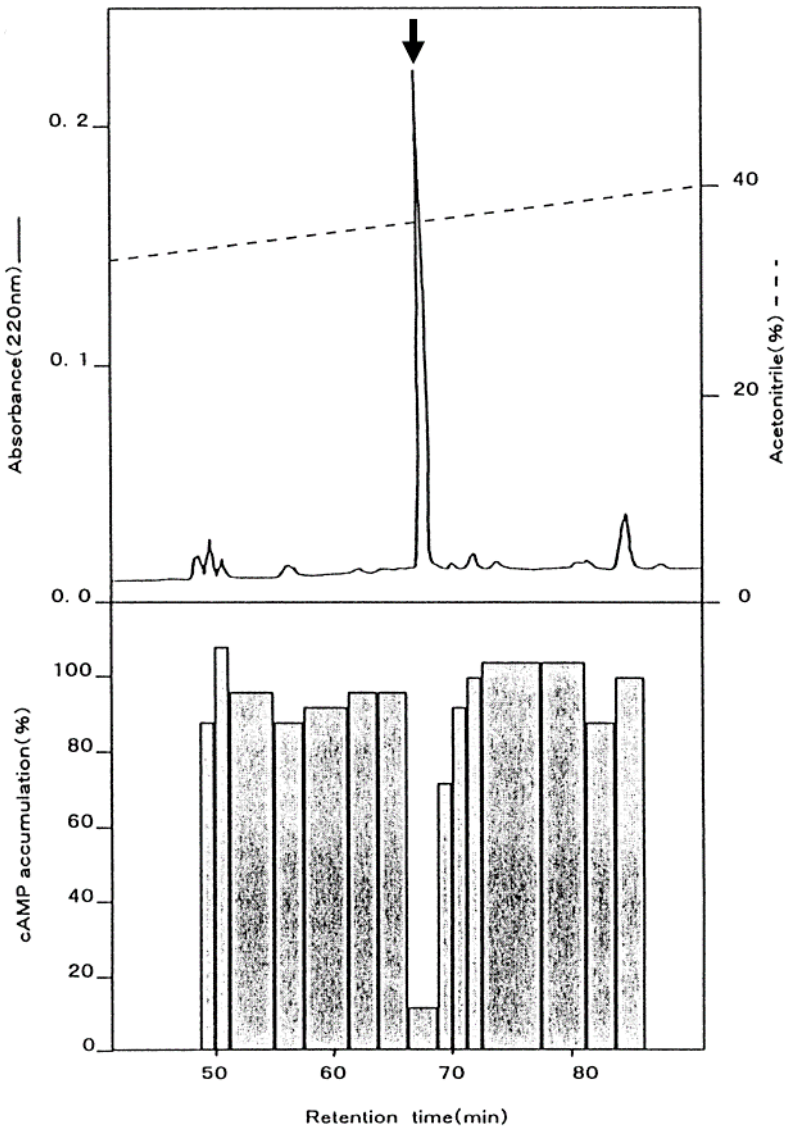

Fig. 1-5. HPLC profile of the final purification step using the Develosil ODS-UG-3 column. Elution profile of the final purification step (*upper panel*) and agonist activities of the eluate (*lower panel*) are presented. The eluate was manually fractionated, and the agonist activity of the eluate was measured with an assay of intracellular cAMP accumulation inhibition in CHO-rat SLC-1 cells. The *arrow* marks the purified agonist peptide.

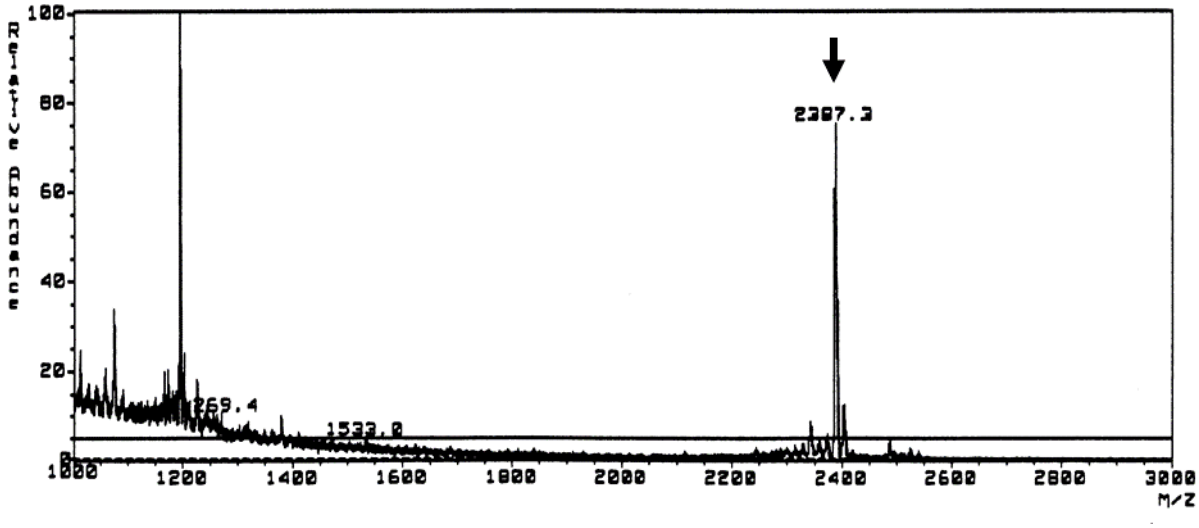

Fig. 1-6. Mass spectrometry of an agonist peptide for rat SLC-1. Mass spectrum of the agonist peptide was measured using a JEOL HX-110 Mass Spectrometer equipped with a cesium gun for the LSIMS mode. The *arrow* marks a peak of the protonated agonist peptide at the m/z value of 2387.2.

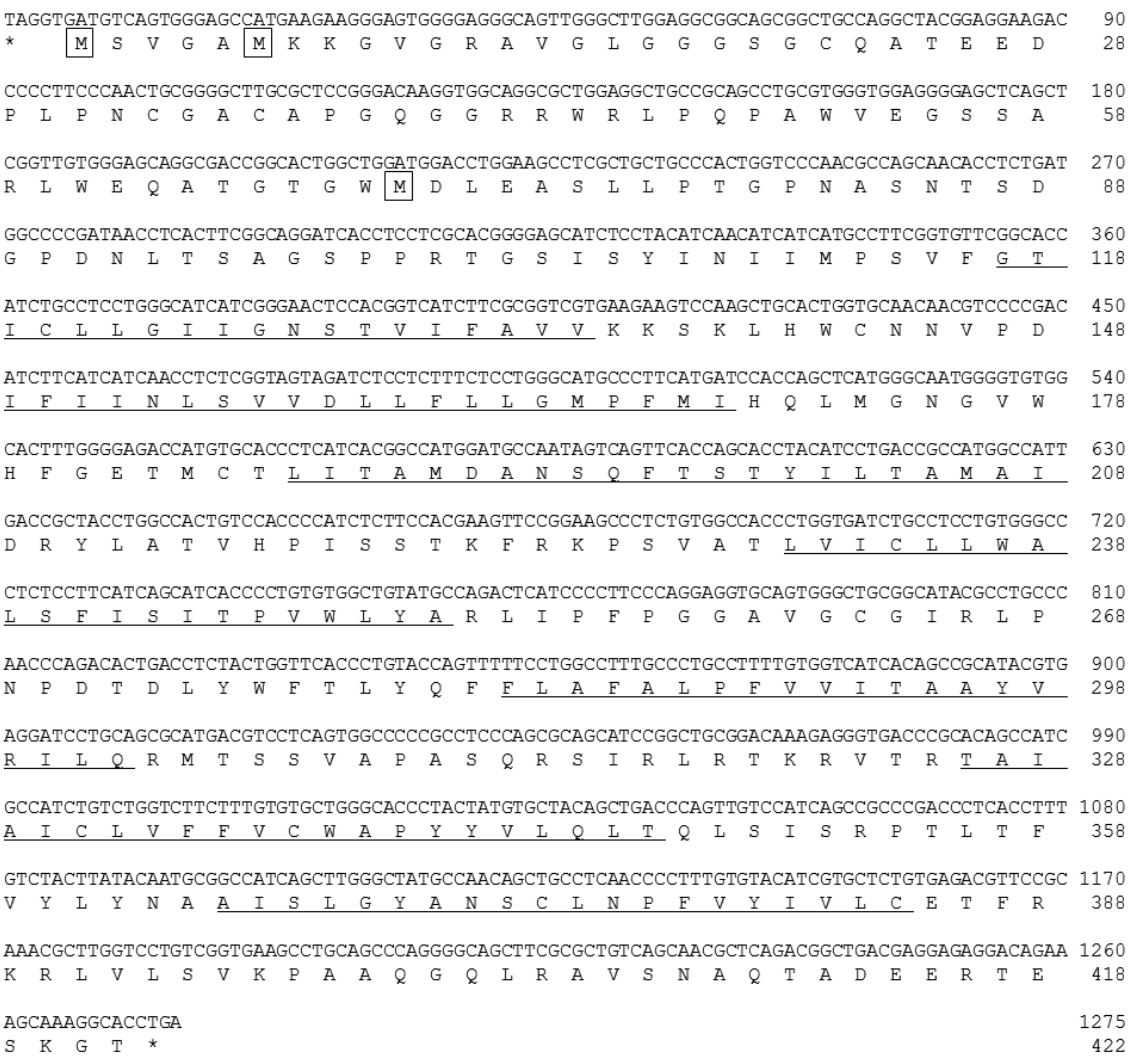

Fig. 1-7. Nucleotide sequence of human *SLC-1* cDNA and its deduced amino acid sequence. A human *SLC-1* cDNA was cloned from a cDNA library of the human embryonic brain. A deduced amino acid sequence of human SLC-1 was obtained from the nucleotide sequence with the longest open reading frame of the cDNA. Each sequence is placed with the nucleotide sequence above the amino acid sequence. Three Met residues as potential translation start site are *boxed*. Predicted transmembrane domains are *underlined*. The *asterisk* indicates a stop codon.

Fig. 1-8. Comparison of SLC-1 amino acid sequences among humans, rats, and mice. Deduced amino acid sequences of human and rat SLC-1 were obtained from the nucleotide sequences of the cloned human and rat *SLC-1* cDNA, respectively, while amino acid sequence of mouse SLC-1 was obtained from the sequence data of NP_660114 registered in NCBI. Predicted transmembrane domains are *underlined*. The E/DRY motif is *boxed in black*. The NPXXY motif is *boxed in blue*. Three Met residues as potential translation start site are *boxed in red.*

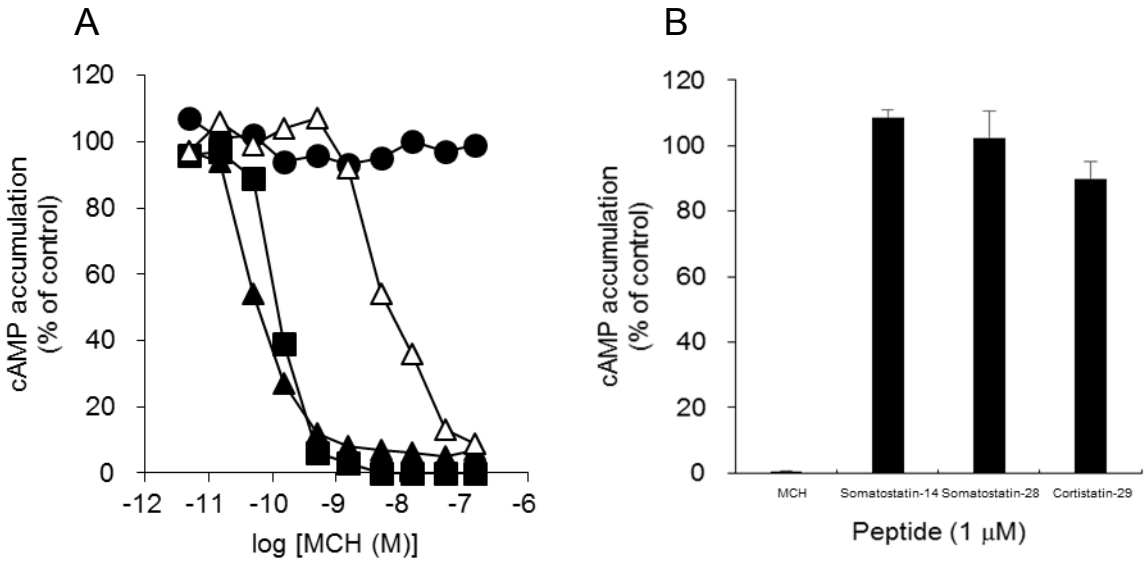

Fig. 1-9. Inhibition of forskolin-induced cAMP accumulation by MCH in CHO cells expressing SLC-1. *A*, dose-dependent inhibition of cAMP accumulation in CHO-rat SLC-1 cells, in CHO-human SLC-1 (S) cells, in CHO-human SLC-1 (L) cells, and in CHO-mock cells. Various concentrations of MCH were added to all the cells, and an assay of cAMP accumulation inhibition was conducted in the presence of forskolin at 1 μM. ■, CHO-rat SLC-1 cells; ▲, CHO-human SLC-1 (S) cells; △, CHO-human SLC-1 (L); ●, CHO-mock cells. *B*, selective activation of SLC-1 by MCH. Three natural ligands for the somatostatin receptor family, rat somatostatin-14, rat somatostatin-28, and rat cortistatin-29 at 1 μM, were added to the CHO-rat SLC-1 cells, and agonist activities of these ligands were measured with the assay of cAMP accumulation inhibition. Results shown are the means ± S.E. (n = 3).

Chapter II: Discovery and Analysis of a Novel Brain-gut Peptide, Neuropeptide W as the Specific Ligand for the Orphan GPCRs, GPR7 and GPR8

1. Introduction

GPR7 and GPR8, for which the endogenous ligands have not been identified, are structurally related orphan GPCRs. The two genes encoding GPR7 and GPR8 were originally isolated from human genomic DNA by O'Dowd *et al.* (O'Dowd et al., 1995). Human GPR7 highly resembles human GPR8 with 64% amino acid identity. Among different families of GPCRs, GPR7 and GPR8 share high similarity to the opioid and somatostatin receptor families with known endogenous ligands. In the mammalian brain, *GPR7* and *GPR8* gene expression was detected by northern blot and *in situ* hybridization analyses (O'Dowd et al., 1995). Especially, in the rat brain, *GPR7* mRNA was detected in regions of the cortex, hippocampus, and hypothalamus (Lee et al., 1999). Profiles of GPR7 and GPR8 expression mainly in the brain suggest that the endogenous ligands for the two receptors have several functions in the CNS.

In Chapter II, the author attempted to purify the agonist peptide for GPR8 and report the purification, cloning, and characterization of neuropeptide W (NPW). 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. The functional and binding characterization of NPW for GPR7 and GPR8 indicated that NPW is the endogenous ligand for both of these receptors. In order to ensure the existence of NPW at peptide levels, tissue distribution of NPW23 and

NPW30 was quantitatively examined using specific two-site enzyme-linked immunosorbent assays (ELISAs). In addition, the author describes the *in vivo* effects of NPW on feeding behavior and hormone release.

2. Materials and Methods

2.1. Establishment of CHO cell lines expressing GPR7 and GPR8

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 (O'Dowd et al., 1995). An expression vector was constructed by ligation of the receptor gene to pAKKO-111H containing *dhfr* as a selection maker (Mori et al., 1999). CHO *dhfr*$^{-}$ cells were transfected with the expression vector. Stable cell lines of CHO expressing human GPR7 (CHO-GPR7) and CHO expressing human GPR8 (CHO-GPR8) were selected under conditions wherein the growth medium lacked nucleotides.

2.2. Phylogenetic analysis of the GPCRs structurally similar to GPR7 and GPR8

A phylogenetic analysis was conducted in the same way described in Chapter I. The description of SLC-1 was changed to melanin-concentrating hormone receptor 1 due to the successful deorphanization of SLC-1 as a MCH receptor, which was demonstrated in Chapter I.

2.3. Gene expression profile of human *GPR7* and *GPR8*

Real-time PCR was used to measure copy numbers of *GPR7*, *GPR8*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA molecules, which were synthesized from poly (A) RNA samples purified from various human tissues. Relative gene expression of *GPR7* and *GPR8* were defined as follows: the copy number of each *GPR7* and *GPR8* divided by that of *GAPDH* as an internal control in each tissue sample. Human poly (A) RNA-derived cDNA samples of Multi Tissue cDNA Panels, and Marathon-Ready cDNA of each adipose and pituitary tissue were purchased from CLONTECH. Each solution of

the human cDNA samples was mixed with a TaqMan universal PCR master mix (PE Applied Biosystems) and each set of gene specific primers at final concentration of 0.2 μM and the gene specific probe at final concentration of 0.1 μM in a 25 μl of reaction mixture (Fujii et al., 2000). A calibration curve needed to convert a value of cycle threshold of the cDNA sample to a copy number of the target gene was created by adding known amounts of the *GPR7*, *GPR8* or *GAPDH* DNA fragments instead of the test cDNA samples. The primers and probe synthesized for the real-time PCR were as follows: human *GPR7*, 5`-CATGAAGACCGTCACCAACCT-3` (forward primer), 5`-CCAGCG-TGAAGAGCTCGTC-3` (reverse primer), and 5`-(FAM)-TTCATCCTCAACCTGGCC-ATCGC-(TAMRA)-3` (probe). Each set of primers and probe to measure copy number of human *GPR8* cDNA and human *GAPDH* cDNA was premixed with commercially available master mix solution (PE Applied Biosystems). The real-time PCR was carried out using an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems) under the following condition: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec.

2.4. Assay for inhibition of forskolin-induced intracellular accumulation of cAMP

Inhibitory activities of test samples for cAMP accumulation was measured as described (Shimomura et al., 1999). CHO-GPR7 or CHO-GPR8 cells were plated on 24-well plates at 5×10^4 cells/well and were 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 Pure Chemical), 0.05% bovine serum albumin, and 20 mM HEPES) and were cultured in the assay buffer for 30 min. After washing the cells three times with 0.5 ml of the assay buffer, a test sample in 0.5 ml of the assay buffer

supplemented with 1 μM forskolin (Wako Pure Chemical) was added to the cells, and the cells were incubated at 37°C for 30 min. cAMP synthesis in the cells was terminated 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).

2.5. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding assay was performed essentially as described (Takekawa et al., 2002) with minor modifications. Membrane fractions of CHO-GPR8 cells 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 was 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 60 min. The radioactivities retained on the glass filters were counted by a scintillation counter.

2.6. Purification of NPW as an endogenous ligand for GPR8 from the porcine hypothalamus

Approximately 500 g of fresh porcine hypothalamus was boiled for 10 min in pure water and was 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 was then extracted with diethyl ether. The resultant solution was applied to an YMCgel ODS-AM 120-S50 column (30.0 × 240 mm; YMC). The peptide fraction was eluted with 60% CH_3CN in 0.1% trifluoroacetic acid, and the eluate was lyophilized. The agonist peptide for human 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 $HCOONH_4$ 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.).

2.7. Cloning of porcine, human, rat, and mouse NPW precursor protein cDNAs

A BLAST search was performed using the amino acid sequence of the purified peptide as a query against the DDBJ/GenBank^{TM}/EBI Data Bank. A human genomic DNA sequence (DDBJ/GenBank^{TM}/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, a cDNA encoding porcine NPW precursor protein was obtained from a 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. Human NPW precursor protein cDNA, rat NPW precursor protein cDNA, and mouse NPW precursor protein cDNA were isolated from a human hypothalamus Marathon Ready cDNA library (CLONTECH), a rat brain Marathon Ready cDNA library (CLONTECH), and a mouse brain Marathon Ready cDNA library (CLONTECH), respectively, following the same strategies used to clone the porcine NPW precursor protein cDNA. The PCR primers for cloning the porcine, human, rat, and mouse full-length cDNAs were as follows: pig, 5`-TTCCCGACACCCCTGCGCCCAGAC-3` (forward) and 5`-GGGCTGGCGAAGGCG-GTTCCCTGC-3` (reverse); human, 5`-AGCGGTACTGAGGGGGCGGAACGA-3` (forward) and 5`-GGGTCTATGAGCGGCTCCTGGAAG-3’ (reverse); rat, 5'-GGGGC-GGGGCCATTGAGAAGC-3' (forward) and 5`-TGACCAGACAACGAGACCTGA-3` (reverse); and mouse, 5`-AAAGGCTGTAGTCGCACCAAC-3` (forward) and 5`-ACCAGAAACACGAGGCCTGAC-3` (reverse). The PCR conditions for cloning the porcine, human, rat, and mouse full-length cDNAs were as follows: pig, 96°C for 60 sec; 4 cycles of 96°C for 30 sec, 72°C for 75 sec; 4 cycles of 96°C for 30 sec, 70°C for 75 sec; 4 cycles of 96°C for 30 sec, 68°C for 75 sec; 5 cycles of 96°C for 30 sec, 64°C for 30 sec, 72°C for 45 sec; 20 cycles of 96°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec; 72°C for 10 min; human, 96°C for 60 sec; 35 cycles of 96°C for 30 sec, 64°C for 30 sec, 72°C for 120 sec; 72°C for 10 min; rat, 96°C for 60 sec; 35 cycles of 96°C for 30sec, 60°C for 30 sec, 72°C for 60 sec; 72°C for 10 min; and mouse, 96°C for 120 sec; 40 cycles of 96°C for 30 sec, 64°C for 30 sec, 72°C for 120 sec; 72°C for 10 min. The porcine, human, rat, and mouse NPW precursor protein cDNA clones were sequenced on both strands using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems).

2.8. Peptide synthesis of human, porcine, rat, and mouse NPW23 and NPW30

Human, porcine, rat, and mouse 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.

2.9. Comparison of the elution times of synthetic NPW with those of the [^{35}S]GTPγS binding activities of porcine hypothalamus fractions

Extracts of the porcine hypothalamus were fractionated by HPLC using the 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) as described above for the purification of NPW. Active fractions obtained by the 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 human 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 (4.6 × 250 mm; 25–40% CH_3CN gradient in 0.1% trifluoroacetic acid for 30 min at 1.0 ml/min).

2.10. Expression and purification of human NPW in a transient expression system using COS-7 cells

An expression vector was constructed by ligation of PCR-amplified human NPW precursor protein cDNA covering the DNA region from the 5` to 3` in-frame stop codons

to the mammalian expression vector pCR3.1 (Invitrogen). The PCR primers for cloning the human NPW precursor protein cDNA were 5`-AGCGGTACTGAGGGGGCGGA-ACGA-3` (forward) and 5`-AACTAGTCGGCCACTCCTCCTGGGTCAG-3` (reverse). COS-7 cells were transfected with the expression vector and were cultured for 2 days. The conditioned medium was concentrated using a Sep-Pak Plus C18 cartridge (Waters). The agonist peptide for human 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 using CHO-GPR8 cells. 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 a Procise 491cLC protein sequencer (Applied Biosystems, Inc.). 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).

2.11. 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 were 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.

2.12. 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 reverse phase HPLC. Specific activity of the 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 (Takekawa et al., 2002) with minor modifications. Membrane fractions of CHO-GPR7 cells and CHO-GPR8 cells suspended in 200 μl of receptor binding assay buffer (25 mM Tris-HCl (pH 7.4), 0.1% bovine serum albumin, 0.05% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (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 was then filtered onto a Whatman GF/F glass filter. After washing three times with 1.5 ml of the binding buffer, the glass filters were dried at 37°C for 60 min. 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 by a γ-counter.

2.13. Generation of monoclonal antibodies to NPW23 and NPW30

Three immunogens were prepared to obtain monoclonal antibodies recognizing the N-terminal region of NPW23 and NPW30, the C-terminal region of NPW23, and the C-terminal region of NPW30. Human [^{14}Cys]NPW-[1–13] and human [^{1}Cys]NPW-[11–23] were conjugated to porcine thyroglobulin, and rat [^{1}Cys]NPW-[16–30] was conjugated to

keyhole limpet hemocyanin, by using sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate (Pierce Chemical Co.). Female BALB/c mice (6–8 weeks old) were immunized with each immunogen, which was mixed with the Freund's complete or incomplete adjuvant just before the immunization. The spleen cells isolated from the immunized mice 3 days after intravenous injection of the immunogens were fused with mouse myeloma cells, P3X63Ag8·653, as described previously (Suzuki et al., 1989). Three monoclonal antibodies, ANPW-N (IgG1, κ) for the N-terminal region of NPW23 and NPW30, ANPW23-C (IgG1, κ) for the C-terminal region of NPW23, and ANPW30-C (IgG2b, κ) for the C-terminal region of NPW30, were screened, and IgG fractions of the three antibodies were purified from ascites fluids using an immobilized protein A column (SEIKAGAKU CORPORATION).

2.14. Construction of two-site ELISAs to quantify immunoreactive NPW23 and NPW30

The ANPW23-C and ANPW30-C were used as immobilized antibody for the two-site ELISAs of NPW23 and NPW30, respectively. The ANPW-N was conjugated to horseradish peroxidase (HRP) as described previously (Ichimori et al., 1987), and the HRP-labeled ANPW-N was used as the 2nd antibody to detect NPW23 and NPW30. The assay protocol of these two-site ELISAs was essentially the same as described by Ichimori *et al*. (Ichimori et al., 1987) with the following minor modifications. Immobilized antibody plates were prepared at 4°C by incubation with 20 μg/ml of the ANPW23-C for the NPW23 ELISA and with 20 μg/ml of the ANPW30-C for the NPW30 ELISA in 96-well ELISA plates (50 mM sodium carbonate buffer (pH 9.6), 100 μl/well) for an overnight followed by replacement of the antibody solution with 300 μl/well of Block

Ace (Snow Brand Milk Products Co., Ltd.). After the incubation with the Block Ace at least for an overnight, known amounts of the standard peptides, synthetic NPW23 and NPW30, and test samples in 100 μl of buffer C (20 mM phosphate buffer (pH 7.0), 1% bovine serum albumin, 0.4 M NaCl, and 2 mM EDTA) were added to each well of the antibody immobilized plates, and the plates were incubated at 4°C for 16 h. After washing with phosphate-buffered saline (PBS), the plates were treated with 100 μl/well of the HRP-labeled ANPW-N at a 1000-fold dilution in buffer C at 4°C for an additional 16 h. After washing with PBS, bound enzyme activity was measured using a TMB microwell peroxidase system (Kirkegaard and Perry Laboratories).

2.15. Preparation of peptide fractions from animal organs for the measurement of immunoreactive NPW23 and NPW30

Fresh animal organs were used to prepare samples for the ELISAs of NPW23 and NPW30. Each porcine organ (the whole brain, spinal cord, hypothalamus, pituitary, adrenal gland, esophagus, stomach, duodenum, small intestine, atrium, spleen, pancreas, kidney, ovary, thymus, and lung) with its total weight around 500 g was boiled for 10 min in 2 liters of pure water and was subsequently homogenized by a Polytron homogenizer in the same volume of 1.0 M acetic acid. The homogenate was centrifuged, and subsequently the supernatant was subjected to acetone precipitation at a final acetone concentration of 66%. After removal of the precipitates, the supernatant was evaporated and was extracted with diethyl ether. The resultant solution was applied to an YMCgel ODS-AM 120-S50 column (30.0 × 240 mm; YMC). Peptide fraction was eluted with 60% CH_3CN in 0.1% trifluoroacetic acid, and the eluate was lyophilized. The lyophilized material dissolved in 1.0 M acetic acid was purified by the following 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). Each fraction obtained from the second HPLC was lyophilized, and the lyophilized materials in tubes were dissolved in DMSO as assay samples for the ELISAs. Rodent organs were dissected from male Wistar rats (6–8 weeks old) and male C57BL/6 mice (6–8 weeks old): rats, the whole brain, spinal cord, pituitary, stomach, duodenum, colon, heart, spleen, pancreas, liver, kidney, testis, and lung; and mice, the whole brain, pituitary, and stomach. Each rodent organ described above was boiled for 10 min in pure water with its weight equivalent to at least 4 times weight of the organ and was subsequently homogenized by a Polytron homogenizer in the same volume of 1.0 M acetic acid. Supernatant of the initial extraction was obtained by centrifugation, and the residual tissue as a pellet was re-homogenized by the Polytron homogenizer in the same volume of 1.0 M acetic acid. Supernatant of the second extraction was obtained by the centrifugation and was mixed with that of the initial extraction followed by a semi-purification using an octadecyl-bonded silica cartridge, Sep-Pak C18 Classic Cartridge (Waters). The mixture of the extract was absorbed onto the Sep-Pak C18 Classic Cartridge followed by washing with 1.0 M acetic acid, and peptide fraction was eluted with 60% CH_3CN in 0.1% trifluoroacetic acid. The eluates were lyophilized, and the lyophilized materials were dissolved in the buffer C as assay samples for the ELISAs. Quantities of immunoreactive NPW23 and NPW30 in the assay samples were calculated as the average of duplicate assays.

2.16. 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 PBS 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.

2.17. 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, Kyoto, Japan) 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 PBS 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).

3. Results

3.1. Structural and gene expression features of GPR7 and GPR8

GPR7 and GPR8 are structurally related orphan GPCRs with an amino acid identity of 64% (O'Dowd et al., 1995). The two orphan GPCRs showed sequence similarity to other human GPCRs, human orthologue of the rat SLC-1, five subtypes of the somatostatin receptor, three subtypes of the opioid receptor, and one opioid-like receptor. A phylogenetic tree of the twelve above mentioned GPCRs and the rat SLC-1 was constructed by the neighbor-joining method. The two orphan GPCRs, GPR7 and GPR8, showed the highest homology to each other (Fig. 2-1). The closest group of GPCRs to the two orphan GPCRs was the subtypes of the opioid receptor with the opioid-like receptor.

Gene expression patterns of *GPR7* and *GPR8* in human tissues were examined by real-time PCR. Both *GPR7* and *GPR8* are moderately expressed in the brain, testis, and pituitary (Fig. 2-2). Other tissues in which either *GPR7* or *GPR8* are expressed to the same degree as that in the brain are the liver, small intestine, and spleen.

3.2. Purification and cDNA cloning of the agonist peptide for human GPR8

The author purified the agonist peptide for human GPR8 from the porcine hypothalamus using CHO-GPR8 cells, using the so-called "reverse pharmacology" technique (Libert et al., 1991; Kotarsky and Nilsson, 2004). While assessing intracellular signal changes in CHO-GPR8 cells induced by the test samples, including various tissue extracts and known bioactive substances, reverse phase HPLC fractions of the porcine hypothalamus extracts showed an inhibitory effect on cAMP accumulation induced by forskolin. Treatment of the reverse phase HPLC fractions with proteinases diminished the agonist activity (data not shown), suggesting that a peptide ligand for human GPR8 was present

in the porcine hypothalamus extracts. Because the same reverse phase HPLC fractions also stimulated [^{35}S]GTPγS binding to membranes of CHO-GPR8 cells, the porcine hypothalamus extracts were subjected to purification by successive chromatography to isolate an agonist peptide for human GPR8 (Fig. 2-3A). Its activity was assessed using the [^{35}S]GTPγS binding assay (Fig. 2-3B). The process yielded 3 pmol of the purified agonist peptide for human GPR8 from 500 g of the porcine hypothalamus (Fig. 2-4A and B). The N-terminal amino acid sequence of the purified peptide was determined to be WYKHTASPRYHTVGRAAXLL (X, not identified) using a protein sequencer.

The author then attempted to isolate the cDNA encoding this peptide to reveal its whole amino acid sequence. A cDNA was cloned from a porcine spinal cord cDNA library 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 isolated cDNA preceded an in-frame stop codon (DDBJ/GenBank^{TM}/EBI accession number AB084277), the 2nd ATG codon conforms more to the rules of Kozak (Kozak, 1996). The open reading frame starting at the 1st ATG codon encodes a porcine polypeptide of 159 amino acid residues (Fig. 2-5). The PSORT II algorithm based on the method of von Heijne (von Heijne, 1986) demonstrated that a signal peptide cleavage site of this polypeptide exists between Ala^{39} and Trp^{40} (von Heijne, 1986; Nakai and Horton, 1999). Therefore, it is predicted that two mature peptides of 23 and 30 amino acid residues will be generated from the precursor protein by signal peptide cleavage and proteolytic processing at two pairs of basic amino acid residues, Arg^{63}-Arg^{64} and Arg^{70}-Arg^{71}, respectively (Douglass et al., 1984). The mature peptide sequence of the 23-amino acid residue peptide starts at Trp^{40} and ends at Leu^{62}, whereas that of the 30-amino acid residue peptide starts at Trp^{40} and ends at Trp^{69}. The amino acid sequence of the 23-amino acid residue peptide is

identical to that of the N-terminal 23 amino acid residues in the 30-amino acid residue peptide. Since both the first and last amino acids of the 30-amino acid residue peptide are tryptophan, the 30-amino acid residue peptide was designated as neuropeptide W-30 (NPW30), and then the 23-amino acid residue peptide was designated as neuropeptide W-23 (NPW23).

3.3. Activation of human GPR8 by NPW23 and NPW30 in the porcine hypothalamus

To clarify the molecular species showing agonist activities for human GPR8, the author measured the [^{35}S]GTPγS binding activities of the porcine hypothalamus peptide fractions for human GPR8 and compared the elution times of the fractions with [^{35}S]GTPγS binding activities with those of synthetic peptides of porcine NPW23 and NPW30 under the same HPLC conditions. The synthetic porcine NPW23 and NPW30 were eluted at 24.5 min and at 26.5 min, respectively (Fig. 2-6A). The [^{35}S]GTPγS binding activities of the porcine hypothalamus peptide fractions for human GPR8 were recovered mainly in fractions 25 (24–25 min) and 27 (26–27 min) with almost the same activity (Fig. 2-6B), indicating that fractions 25 and 27 contained porcine NPW23 and NPW30, respectively. These results suggest that porcine NPW23 and NPW30 present in the porcine hypothalamus activate human GPR8.

3.4. Structures of NPW precursor protein and mature NPW peptide

The author isolated human NPW precursor protein cDNA (DDBJ/GenBank^{TM}/EBI accession number AB084276) lacking an AUG start codon from a human hypothalamus cDNA library and examined whether human NPW with agonist activity for human GPR8

is generated from this cDNA. As shown in Fig. 2-7, an open reading frame of the cDNA represents the amino acid sequences of NPW23 and NPW30, which are flanked by a putative signal peptide cleavage site and a pair of basic amino acid residues and which show amino acid sequence similarity to 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 (Peabody, 1989; Mehdi et al., 1990). To ensure that the agonist peptide for human GPR8 is synthesized from this human cDNA, the author analyzed peptides secreted from COS-7 cells that were 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 human GPR8 was purified to homogeneity (Fig. 2-8A and B). The structure of the purified peptide was 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 precursor protein and signal peptide cleavage proceeded as expected, but the proteolytic processing at a pair of basic amino acid residues failed to proceed accurately. These results indicate that the agonist peptide for human GPR8 is generated through intracellular processing of the precursor protein initiated from a non-AUG start codon in the transcript of the human NPW precursor protein cDNA.

The author subsequently isolated cDNA clones encoding rat NPW precursor protein and mouse NPW precursor protein. A cDNA encoding rat NPW precursor protein (DDBJ/EMBL/Gen-Bank™ accession number AB084278) demonstrated the existence of a precursor protein of 185 amino acid residues, which is translated from the longest open

reading frame in the cDNA nucleotide sequence (Fig. 2-9). The amino acid sequence of the rat NPW precursor protein includes a signal peptide cleavage site between Ala^{41} and Trp^{42}, which was predicted by the PSORT II algorithm (Nakai and Horton, 1999) and two pairs of basic amino acid residues, Arg^{65}-Arg^{66} and Arg^{72}-Arg^{73}. Two peptides of 23 and 30 amino acid residues are predicted to be generated from the precursor protein as mature peptides via the signal peptide cleavage between Ala^{41} and Trp^{42} followed by proteolytic processing at the amino terminal ends of the Arg^{65}-Arg^{66} and the Arg^{72}-Arg^{73}, respectively. The mature peptide sequences of rat NPW23 and rat NPW30 are suggested to be WYKHVASPRYHTVGRASGLLMGL and WYKHVASPRYHTVGRASGLLMGLRR-SPYLW, respectively. As for the human *NPW* precursor nucleotide sequence, a cDNA of the mouse NPW precursor protein lacking an AUG start codon presented two peptide sequences of 23 and 30 amino acid residues, which are flanked by a putative signal peptide cleavage site and by two pairs of Arg-Arg basic amino acid residues (Fig. 2-10). The amino acid sequences of the 23- and 30-amino acid residue peptides in the mouse NPW precursor protein are quite similar to those of the human, porcine, and rat NPW23 and NPW30 (Fig. 2-11).

The author compared the amino acid sequences of NPW precursor protein and mature NPW peptide from the humans, pigs, rats, and mice. A deduced amino acid sequence of each porcine and rat NPW precursor protein was defined unambiguously as the translated protein from the open reading frame of the cloned cDNA with normal AUG start codons. Since each cDNA encoding human and mouse NPW precursor protein lacked the AUG start codon, the author assumed that a potential non-AUG start codon was either ACG, CUG, or GUG, which existed at 123-base to 96-base upstream of the tryptophan codon for the N-terminal amino acid of the mature NPW peptide sequences due to the fact that

the AUG start codon of the porcine and rat NPW precursor protein nucleotides was placed in the same region. As a result, the human NPW precursor protein, 165 amino acids, with translation initiated from the CUG codon at the nucleotide position 187–189 and the mouse NPW precursor protein, 176 amino acids, with translation initiated from the CUG codon at the nucleotide position 121–123 were aligned with the porcine and rat NPW precursor proteins using CLUSTAL W multiple sequence alignment (Fig. 2-12). The alignment pointed out the conserved amino acid sequences and motifs: a putative signal peptide sequence with hydrophobic amino acids, leucine or proline, mature NPW peptide sequences of NPW23 and NPW30, and two pairs of Arg-Arg basic amino acid residues. Especially, the amino acid sequences of the mature NPW peptide, NPW23 and NPW30 showed quite higher sequence similarity among the four species, 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 at tryptophan and ends at leucine, and that of NPW30 starts at tryptophan and ends at tryptophan.

Thus, the author identified novel bioactive peptides, NPW23 and NPW30 that are encoded by the *NPW* precursor gene. The database search was conducted with the BLAST and the FASTA programs using the amino acid sequences of NPW23 and NPW30 as queries against as diverse as protein and DNA sequence databases available. The amino acid sequences of NPW23 and NPW30 did not show significant similarity to any known proteins registered in those databases. The database search results demonstrated that NPW was first discovered by this study aiming at the deorphanization of GPR8.

3.5. Functional activity and binding affinity of NPW for GPR7 and GPR8

The author 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. 2-13A and B; and Table 2-1). 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. 2-13 A and B; and Table 2-1). In CHO-GPR7 and CHO-GPR8 cells, no elevation of intracellular calcium induced by NPW23 and NPW30 was observed by measurement of $[Ca^{2+}]_i$ response using a calcium fluorescent dye indicator, Fluo 3-AM (data not shown), and the inhibitory effect of NPW on forskolin-induced cAMP accumulation was abolished by preincubation with pertussis toxin (Fig. 2-13C and D), indicating that human GPR7 and GPR8 both are coupled with the $G_{\alpha i}$ protein. Saturation binding analysis using ^{125}I-labeled human NPW23 showed that membrane fractions of CHO-GPR7 and CHO-GPR8 cells displayed high affinity, saturable, and specific binding (human GPR7, *Kd* = 31.8 ± 3.0 pM and *Bmax* = 2.02 ± 0.10 pmol/mg; human GPR8: *Kd* = 20.7 ± 0.6 pM and *Bmax* = 4.37 ± 0.04 pmol/mg) (Fig. 2-13E and F). ^{125}I-labeled human NPW23 bound both human GPR7 and GPR8 with nearly the same affinity. Competition binding analysis demonstrated high affinity binding of NPW23 and NPW30 to human GPR7 and GPR8 (Table 2-1). NPW binds to and activates these receptors with high affinity and potency. These results indicate that NPW is the endogenous ligand for both GPR7 and GPR8.

3.6. Tissue distribution of immunoreactive NPW23 and NPW30

The author created two-site ELISAs to assess the tissue distribution of the mature NPW peptides, NPW23 and NPW30. The two-site ELISAs were essentially constructed with

three monoclonal antibodies, the ANPW-N, the ANPW23-C, and the ANPW30-C, which recognize the N-terminal sequence of both NPW23 and NPW30, the C-terminal sequence of NPW23, and the C-terminal sequence of NPW30, respectively. The ANPW23-C and ANPW30-C were used as immobilized antibodies to trap NPW23 and NPW30, respectively, by binding to individual C-terminal regions, and the ANPW-N labeled with HRP was used as the 2nd antibody to capture the free N-terminal regions of the NPW23 and NPW30, which were already trapped by the immobilized antibodies in 96-well ELISA plates. The two ELISAs, NPW23 ELISA and NPW30 ELISA enabled the specific measurement of human, porcine, rat, and mouse NPW23 and NPW30 with a minimum detectable quantity of 0.3 fmol/well (Fig. 2-14).

The author observed a preferential expression of NPW23 and NPW30 peptides in the brain, pituitary, and stomach in a variety of animal tissues. Porcine peptide fractions prepared from sixteen different organs (the brain, spinal cord, hypothalamus, pituitary, adrenal gland, esophagus, stomach, duodenum, small intestine, atrium, spleen, pancreas, kidney, ovary, thymus, and lung) were used for the measurement of NPW23 and NPW30 by the two-site ELISAs. Significant levels of immunoreactive NPW23 (ir-NPW23) and immunoreactive NPW30 (ir-NPW30) were detected in the brain, spinal cord, hypothalamus, pituitary, adrenal gland, stomach, and duodenum (Table 2-2). Rat peptide fractions prepared from thirteen different organs (the brain, spinal cord, pituitary, stomach, duodenum, colon, heart, spleen, pancreas, liver, kidney, testis, and lung) were applied to the two-site ELISAs, and significant levels of ir-NPW23 and ir-NPW30 were detected in the brain, spinal cord, pituitary, and stomach. Mouse peptide fractions were prepared from a limited number of organs (the brain, pituitary, and stomach) where both porcine and rat NPW peptides were detected as mentioned above. Significant amounts of ir-NPW23 and

ir-NPW30 were detected in all the mouse peptide fractions. The two-site ELISAs for the extracts of porcine, rat, and mouse organs demonstrated that NPW23 and NPW30 are preferentially localized in the brain, pituitary, and stomach.

3.7. *In vivo* effects of NPW on feeding behavior and hormone release

The author investigated the central effects of NPW on feeding behavior and hormone release because rat GPR7 is expressed at relatively high levels in the brain (Lee et al., 1999). First, human NPW23 was intracerebroventricularly administered to rats, and food intake was monitored (Fig. 2-15A). Injection of 10 nmol of NPW23 significantly increased food intake, and the magnitude of food consumption during a 2-h period was about 3-fold relative to vehicle controls. Second, human NPW23 was intracerebroventricularly administered to rats, and hormone concentrations in the blood were measured (Fig. 2-15B). 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 20 min after injection and decreased to control levels after 60 min.

4. Discussion

The author purified a novel bioactive peptide, NPW, as the agonist for GPR8 using CHO cells expressing human GPR8 from the porcine hypothalamus. The discovery of NPW resulted from the attempt of the deorphanization of GPR8. The purified peptide is encoded by the porcine *NPW* precursor gene, and the precursor protein sequence predicts the existence of two mature peptides composed of 23 and 30 amino acid residues. The author demonstrated the existence of the 23- and 30-amino acid residue NPW peptides as mature peptides in the porcine hypothalamus by HPLC analysis combined with the [^{35}S]GTPγS binding assay (Fig. 2-6A and B). The porcine hypothalamus contained nearly equal amounts of NPW23 and NPW30 because both fractions 25 and 27 showed nearly the same agonist activity, indicating that NPW23 and NPW30 would exhibit their own biological activities in the mammalian body.

Uniqueness of the human NPW precursor protein cDNA sequence postulated that translation of human NPW precursor protein, unlike that of pigs and rats, is not initiated from a typical AUG start codon. The author demonstrated that NPW21 with agonist activity for human GPR8 was synthesized from the human NPW precursor protein cDNA lacking an AUG start codon in a transient expression system using COS-7 cells. In this expression system, translation of the human NPW precursor polypeptide was initiated from a non-AUG start codon, and the signal peptide cleavage subsequently occurred at an expected site, but the proteolytic processing of the polypeptide failed to create the expected C terminus of mature NPW, probably due to an incomplete set of processing enzymes in the COS-7 cells (Galanopoulou et al., 1993) 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 detected in c-Myc, fibroblast growth factor-2,

vascular epidermal growth factor, MHC, and TRPV6 (Hann et al., 1988; Prats et al., 1989; Meiron et al., 2001; Starck et al., 2012; Fecher-Trost et al., 2013). 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 (Peabody, 1989; Mehdi et al., 1990). If initiation of the human NPW precursor polypeptide translation takes place at a position that corresponds to the AUG start site in the porcine or rat *NPW* precursor mRNAs, a CUG triplet (nucleotides 187–189) is one of the candidates for the non-AUG start codon in human *NPW* precursor mRNA (Fig. 2-7). Although further research is required to determine the translation start site and the usage of the non-AUG start codon in the human *NPW* precursor mRNA, it is proposed that human NPW is encoded by the nucleotide sequence of the human NPW precursor protein cDNA with a unique translation from a non-AUG start codon and that the mature forms of human NPW are NPW23 (WYKHVASPRYHTVGRAAGLLMGL) and NPW30 (WYKHVASPRYHTVGRAAG-LLMGLRRSPYLW).

The mouse NPW precursor protein cDNA sequence lacking an AUG-start codon suggested that translation of its precursor protein is initiated from a non-AUG codon as experimentally elucidated for the translation of human NPW precursor protein. A CUG triplet (nucleotides 121–123) was supposed to be a potential non-AUG start codon if the translation initiation takes place at the same position as that in porcine or rat *NPW* precursor mRNAs (Fig. 2-10). The author has not attempted to ensure whether agonist peptides for GPR8 are synthesized from mouse *Npw* precursor mRNA using an experimental system with COS-7 cells that are transfected with the mouse NPW precursor protein cDNA. However, mature peptide forms of mouse NPW precursor protein, NPW23

and NPW30, have been detected in the mouse brain, pituitary, and stomach by quantitative measurement using the NPW23- and NPW30-specific two-site ELISAs (Table 2-2). Thus, it is proposed that mouse NPW is encoded by the nucleotide sequence of the mouse NPW precursor protein cDNA with a unique translation from a non-AUG start codon and that the mature forms of mouse NPW are NPW23 (WYKHVASPRYHTV-GRASGLLMGL) and NPW30 (WYKHVASPRYHTVGRASGLLMGLRRSPYQW).

The amino acid sequence features of human, porcine, rat, and mouse NPW precursor proteins indicate a possible biosynthetic pathway mechanism for the mature NPW peptides. NPW23 and NPW30 are generated from their precursor proteins by a post-translational modification, including the translocation of the NPW precursor proteins presenting signal peptide sequences across the ER membrane and processing at the two sites of the Arg-Arg basic amino acid residues in the regulated secretory vesicles. After initiation of protein translation, a signal recognition particle that binds to the signal peptide sequence in the NPW precursor protein conveys the translation complex with the ribosomes on the ER membrane in the cytosol (Nagai et al., 2003; Akopian et al., 2013). The polypeptide chain elongating inside the translocon, a transmembrane protein complex with channel activity, is then released into the ER lumen after completion of signal peptide cleavage and termination of the translation. The resulting proprotein of NPW undergoes proteolytic processing at the N-terminal Arg-Arg residue site by endoprotease in the regulated secretory vesicles (Seidah and Prat, 2012). As a consequence of the signal peptide cleavage and the processing at the two pairs of the basic amino acid residues, the NPW precursor proteins are converted to the mature peptide forms, NPW23 and NPW30.

Human NPW23 and NPW30 inhibited forskolin-induced cAMP accumulation in both

CHO-GPR7 and CHO-GPR8 cells, with subnanomolar or nanomolar IC_{50} values. In competition binding studies, human NPW23 and NPW30 showed high binding affinity for 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 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 2-1). 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 2-1). 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 the activation of and binding to its receptors.

Two-site ELISAs were developed to assess the tissue distribution of NPW23 and NPW30, which underlies predictions of where NPW exerts its biological functions *in vivo*. The two-site ELISAs constructed by using three different monoclonal antibodies enabled the specific and sensitive quantification of NPW23 and NPW30 in the tissue extracts from pigs, rats, and mice. The author measured the amounts of ir-NPW23 and ir-NPW30 in a variety of organs from pigs, rats, and mice and demonstrated that NPW23 and NPW30 are commonly localized in the brain, pituitary, and stomach of these three animals (Table 2-2). Although significant amounts of ir-NPW23 and ir-NPW30 were detected in the

spinal cord of pigs and rats, the existence of NPW in the mouse spinal cord could not be ensured due to technical difficulties in the resection of a sufficient amount of the mouse spinal cord tissues for the two-site ELISAs. Clarification of whether the mouse spinal cord produces NPW will help to obtain definitive information on the tissue distribution pattern of NPW. In spite of the detection of immunoreactive NPW (ir-NPW) in the porcine duodenum, it is believed that the duodenum cannot be an organ that produces NPW. The significant amounts of NPW in the extract from the porcine duodenum in those assays might result from contamination of the lower part of NPW-producing porcine stomach tissues adjacent to the duodenum tissues when the duodenum tissues used as assay samples were cut at a public slaughterhouse. Besides that, the author obviously showed that no ir-NPW was detected in the tissues of rat duodenum, which were carefully removed from the adjacent organs such as the stomach and small intestine in our animal research facilities. These results indicate that NPW23 and NPW30 are categorized into brain-gut peptides.

The data on the tissue distribution of NPW peptides or the gene expression profile of its receptors, GPR7 and GPR8, allowed us to unravel unknown biological functions of NPW. The existence of NPW peptides as well as *GPR7* and *GPR8* gene expression in the brain naturally predicted that NPW acts as a neurotransmitter in the CNS. The localization of NPW peptides in the stomach and pituitary that have a function of endocrine activity raised the possibility that the NPW stored in these organs is being released into the bloodstream and subsequently reaches to the target tissues where the NPW activates GPR7 and GPR8. Further histological studies using techniques of immunohistochemistry and *in situ* hybridization for NPW and its receptors will enable us to obtain information on the cellular distribution of NPW and its receptors, which might narrow down focus

when attempting to elucidate the biological functions of NPW.

An exploratory study on the *in vivo* effects of NPW was conducted to determine the physiological and pathophysiological roles of NPW. The author demonstrated that NPW was functioning as a neurotransmitter, modulating feeding behavior and neuroendocrine release. In rats, intracerebroventricular administration of NPW23 resulted in an acute increase in food intake. The orexigenic effect could be accounted for by activation of GPR7-expressing neurons localized in the hypothalamic areas, which are considered to regulate feeding behavior and energy homeostasis (Spiegelman and Flier, 2001). Stimulation of prolactin release was observed upon the intracerebroventricular administration of NPW23. Although GPR7 is expressed in the pituitary gland, where prolactin is secreted in response to physiological stimuli (Wynick et al., 1998; Yang et al., 2000), 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 behavior 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 behavior and prolactin release, the broad expression patterns of GPR7 and GPR8 in the mammalian brain raise the possibility that NPW influences many physiological processes in the CNS.

Some GPCRs have more than one endogenous ligand, which activates and binds to receptors of the same family (Inoue et al., 1989). These 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 *NPW* precursor gene. In the

search for an endogenous ligand for GPR7 or GPR8, it was not possible to identify 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.

Since the discovery and molecular characterization of NPW reported in 2002, much attention has been paid on the understanding of the biological functions of NPW and its receptors. The followings are summary of the advancements in the elucidation of the NPW's biological functions. Regulation of feeding behavior and energy homeostasis by NPW was investigated with a high priority due to the initial finding that NPW acutely increased food intake in the light phase (Shimomura et al., 2002; Levine et al., 2005). Mondal *et al.* reported that the intracerebroventricular administration of NPW acutely decreased food intake in the dark phase, and the continuous intracerebroventricular infusion of NPW suppressed food intake and body weight gain in rats (Mondal et al., 2003). In the single intracerebroventricular administration of NPW experiment, rise in body temperature and heat production were also observed. An experiment using genetically modified mice with null mutation of GPR7 confirmed NPW function as a regulator of feeding behavior and metabolism. Rodents present an orthologue of human GPR7, but not an orthologue of human GPR8. Ishii *et al.* demonstrated that male *Gpr*7$^{-/-}$ mice developed adult-onset obesity along with hyperphagia (Ishii et al., 2003). Studies on the molecular mechanism involved in NPW anorectic activity in the brain (Date et al., 2010) and cellular level distribution of NPW-positive neurons (Dun et al., 2003; Takenoya et al., 2008; Takenoya et al., 2010; Motoike et al., 2015) have accumulated to reinforce the evidence for a role of NPW as an anorectic peptide. A function of NPW in neuroendocrine regulation has been intensively studied. In addition to the prolactin-releasing activity, intracerebroventricular administration of NPW induced elevated

plasma corticosterone or ACTH levels, which were mediated by corticotrophin-releasing hormone (Baker et al., 2003; Taylor et al., 2005). These reports suggest that NPW acts as a mediator of stress via activation of the hypothalamus-pituitary-adrenal axis. NPW is also involved in pain regulation in the spinal cord so that spinally applied NPW produced anti-hyperalgesia action in a model of inflammatory pain in rats (Yamamoto et al., 2005). In the stomach, NPW is expressed in gastric antral G cells as observed by immunoelectron microscopy and is colocalized with a gastric hormone, gastrin (Mondal et al., 2006). Although the determination of NPW immunoreactivity within G cells strongly suggests that NPW in the stomach may function in an endocrine manner, biological roles of the gastric NPW remain to be clarified. Apparent expression of GPR7 and substantial projections of NPW fibers in the extended amygdala have drawn attention to the involvement of NPW and its receptor in the control of emotion. Phenotypic analyses of *Gpr7*$^{-/-}$ mice in terms of social behavior suggested the link between NPW and the regulation of fear and anxiety (Sakurai, 2013).

Neuropeptide B (NPB), a structurally different peptide from NPW, was simultaneously reported as another endogenous ligand for GPR7 and GPR8 by a group in our research laboratories in 2002 (Fujii et al., 2002). One year later, two other groups independently reported the discovery and analyses of NPW and NPB (Brezillon et al., 2003; Tanaka et al., 2003). As a consequence of full deorphanization of both GPR7 and GPR8, the International Union of Basic and Clinical Pharmacology officially named GPR7 and GPR8, neuropeptides B/W receptor 1 (NPBWR1) and neuropeptides B/W receptor 2 (NPBWR2), respectively (http://www.iuphar-db.org/nciupharPublications.jsp).

In conclusion, the author identified NPW, a novel brain-gut peptide that is the endogenous ligand for both GPR7 and GPR8 and that affects the central control of feeding

behavior and the release of prolactin in rats. This study will provide new insights into the physiological roles of NPW and its receptors, GPR7 and GPR8.

5. Tables

Table 2-1
Functional activity and binding affinity of human NPW for GPR7 and GPR8

	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

IC_{50} values for functional activity (nM) were determined by the inhibition of cAMP accumulation assay in the presence of forskolin (1 μM). IC_{50} values for binding affinity (nM) 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).

Table 2-2

Quantification of NPW in animal tissues by NPW23- and NPW30-specific two-site ELISAs

	ir-NPW in porcine tissues* (fmole/ 1g wet tissue)		ir-NPW in rat tissues† (fmole/ 1g wet tissue)		ir-NPW in mouse tissues† (fmole/ 1g wet tissue)	
	NPW23	NPW30	NPW23	NPW30	NPW23	NPW30
Whole brain	91	111	13	44	52	2
Spinal cord	72	123	1	9	NA	NA
Hypothalamus	282	230	NA	NA	NA	NA
Pituitary	1230	423	32	676	6645	2
Adrenal gland	2	51	NA	NA	NA	NA
Esophagus	ND‡	ND	NA	NA	NA	NA
Stomach	55	776	3	88	1	1
Duodenum	31	361	ND	ND	NA	NA
Small intestine	ND	ND	NA	NA	NA	NA
Colon	NA§	NA	1	ND	NA	NA
Atrium	ND	ND	NA	NA	NA	NA
Heart	NA	NA	ND	ND	NA	NA
Spleen	ND	ND	ND	ND	NA	NA
Pancreas	ND	ND	ND	ND	NA	NA
Liver	NA	NA	ND	ND	NA	NA
Kidney	ND	ND	ND	ND	NA	NA
Ovary	ND	ND	NA	NA	NA	NA
Testis	NA	NA	ND	ND	NA	NA
Thymus	ND	ND	NA	NA	NA	NA
Lung	1	3	ND	ND	NA	NA

*ir-NPW in porcine tissues: each result is a total of the mean of duplicate assays for each fraction where the extract of one organ was separated into 40 fractions by reverse phase HPLC.

†ir-NPW in rat tissues and ir-NPW in mouse tissues: each result is the mean of duplicate assays for the peptide fraction prepared using octadecyl-bonded silica cartliges.

‡ND: not detectable. §NA: not available.

Peptide fractions were obtained from extracts of animal tissues followed by reverse phase HPLC for porcine tissues, and by batch absorption and elution using octadecyl-bonded silica cartridges for rat and mouse tissues. The peptide fractions as assay samples were subjected to the ELISAs to measure immunoreactive NPW (ir-NPW) of 23 and 30 amino acid residues.

6. Figures

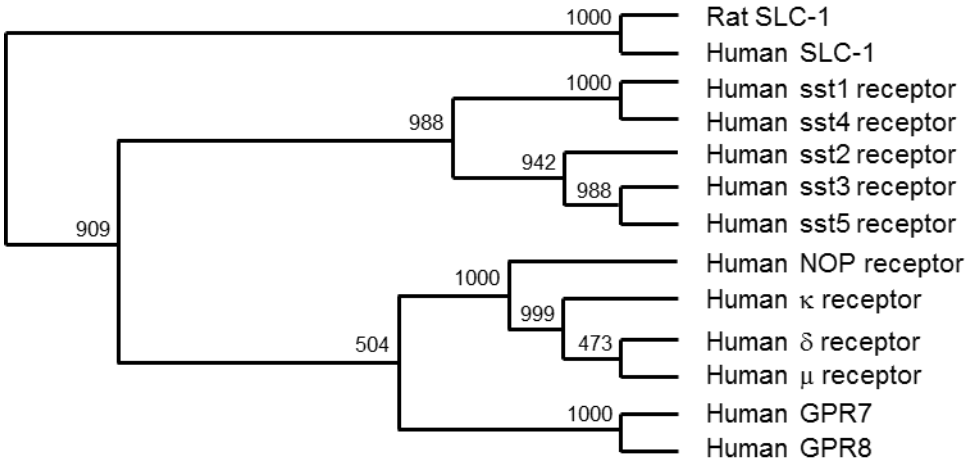

Fig. 2-1. A phylogenetic tree of GPCRs with higher similarity to human GPR7 and GPR8. Selected GPCRs presenting amino acid sequence similarity to SLC-1 were analyzed by the neighbor-joining method. Bootstrap values are placed on each node. Two GPCRs, GPR7 and GPR8, were recognized as orphan GPCRs, and the others were GPCRs with known endogenous ligands when the study in Chapter II started. SLC-1, melanin-concentrating hormone receptor 1; sst1 receptor, somatostatin receptor 1; sst4 receptor, somatostatin receptor 4; sst2 receptor, somatostatin receptor 2; sst3 receptor, somatostatin receptor 3; sst5, somatostatin receptor 5; NOP receptor, nociceptin receptor; κ receptor, kappa-type opioid receptor isoform 1; δ receptor, delta-type opioid receptor; μ receptor, mu-type opioid receptor isoform MOR-1.

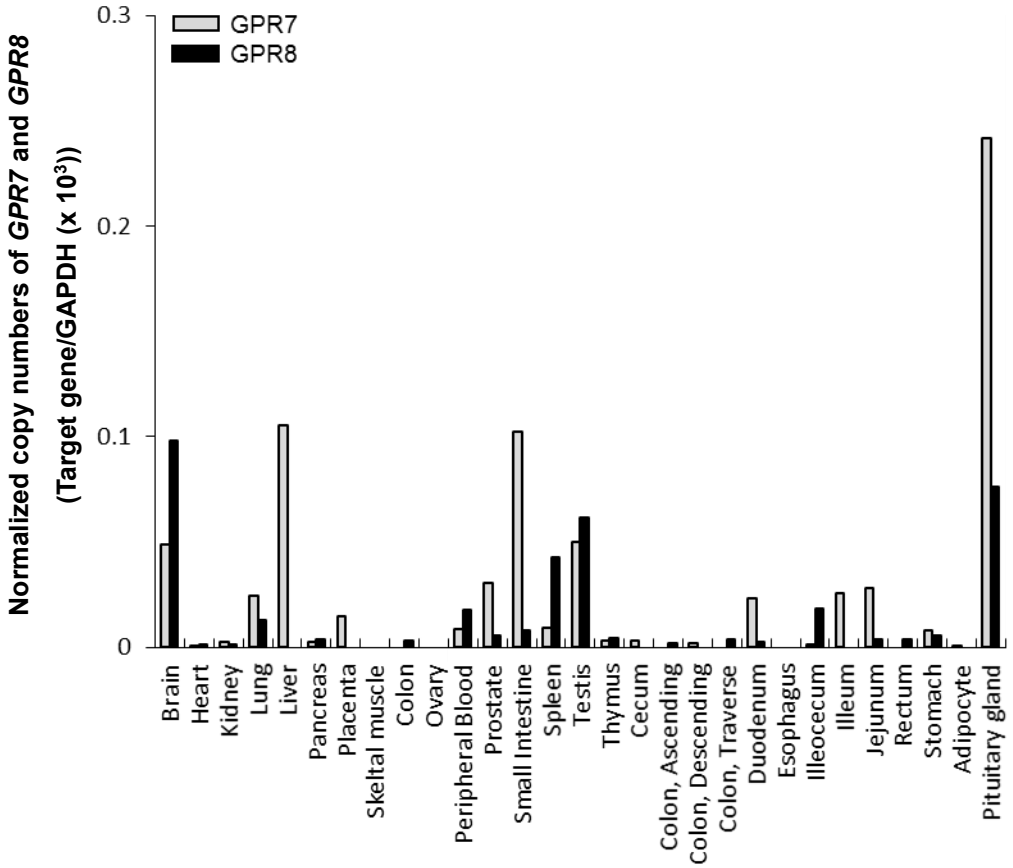

Fig. 2-2. Gene expression profile of human *GPR7* and *GPR8*. Copy numbers of *GPR7* and *GPR8* mRNA in human tissues were measured by real-time PCR. The gene expression in the respective tissues was represented as normalized copy number that was calculated as follows: each copy number of *GPR7* and *GPR8* mRNA was divided by copy number of *GAPDH* mRNA in the same tissue.

A

The porcine hypothalamus (500 g, 30 heads)

1. Boil the tissue in water and extract in 1 M acetic acid
2. Precipitate the extracts at 66% of acetone, centrifuge, and evaporate the supernatant
3. Remove the lipids by diethyl ether extraction, centrifuge, and evaporate the supernatant

The extracts of the porcine hypothalamus

4. YMCgel ODS-AM 120-S50 column (30 x 200 mm)
5. TSKgel ODS-80TM column (21.5 x 240 mm)
6. TSKgel SP-5PW column (21 x 150 mm)
7. Develosil CN-UG-5 column (4.6 x 250 mm)
8. Wakosil-II 3C18HG column (2.0 x 150 mm)

The purified agonist peptide for GPR8

B

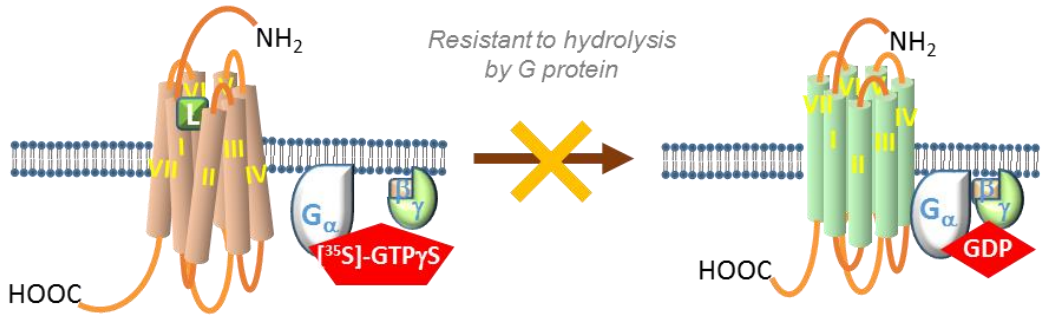

Fig. 2-3. Isolation of an agonist peptide for human GPR8. *A*, purification procedure of an agonist peptide for human GPR8. The purification procedure comprised two parts of process, extraction from the porcine hypothalamus (step 1 to 3) and a combination of HPLCs (step 4 to 8). Agonist activity for human GPR8 was monitored with an assay of [^{35}S]GTPγS binding. *B*, mechanistic basis of the assay. Once ligand (L) as an agonist for human GPR8 binds to the receptor, heterotrimeric G proteins associated with the receptor are activated via the changes from GDP- to GTP-bound form, which induces intracellular signaling by activating downstream molecules in cells expressing human GPR8. The activated state returns to inactivated state through hydrolysis of the GTP to GDP, which is catalyzed by α subunit of the G protein. A GTP analogue, [^{35}S]GTPγS with highly resistant to the GTPase activity of the α subunit remains to bind to the G protein for a longer period of time so that an agonist binding to human GPR8 can be detected as an increase in radioactivity of [^{35}S]GTPγS in the membrane fractions of the cells expressing human GPR8.

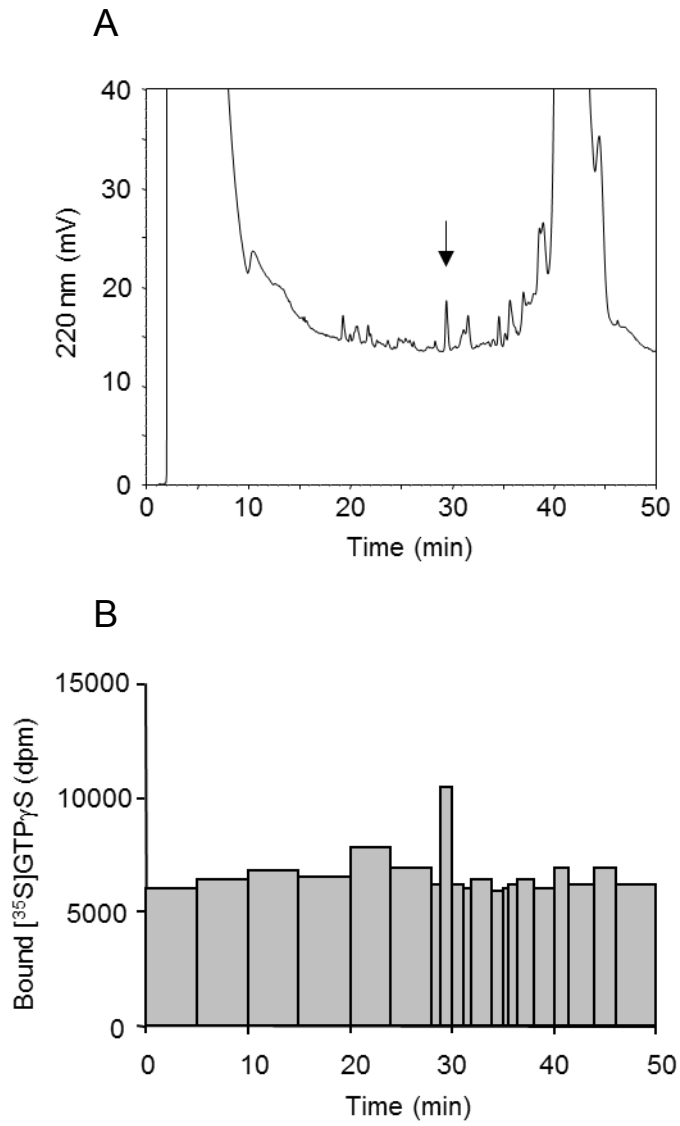

Fig. 2-4. Identification of an endogenous ligand for human GPR8 from the extracts of the porcine hypothalamus. *A*, HPLC profile of the final purification step using the Wakosil-II 3C18HG column. The *arrow* marks the purified material. *B*, detection of [^{35}S]GTPγS binding activity to membrane fractions of CHO-GPR8 cells. The eluate was manually collected, and the activity was recovered as a single peak with an elution time of 29.4 min.

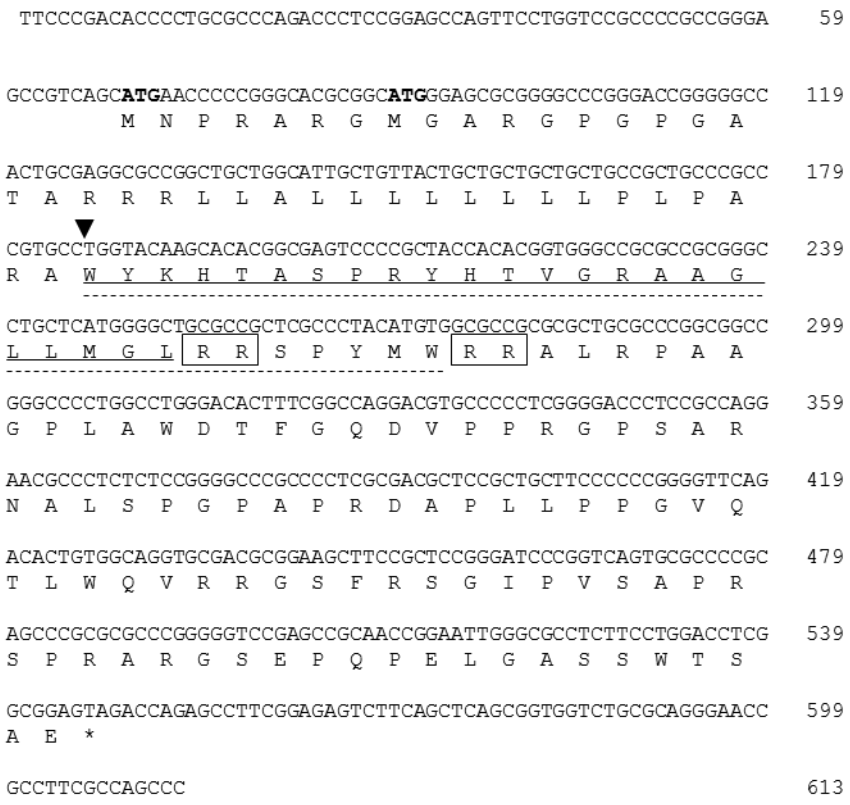

Fig. 2-5. Porcine NPW precursor protein cDNA and mature peptide sequences. A cDNA clone encoding an agonist peptide for human GPR8 was isolated from a cDNA library of the porcine spinal cord. A deduced amino acid sequence of porcine NPW precursor protein was obtained from the longest open reading frame of the cDNA. Each sequence is placed with the nucleotide sequence above the amino acid sequence. Two Met codons as potential translation start site are indicated by *boldface letters*. The *asterisk* indicates a stop codon. The *arrowhead* indicates a putative signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. Porcine NPW23 and NPW30 are *underlined* with a *solid line* and with a *broken line*, respectively.

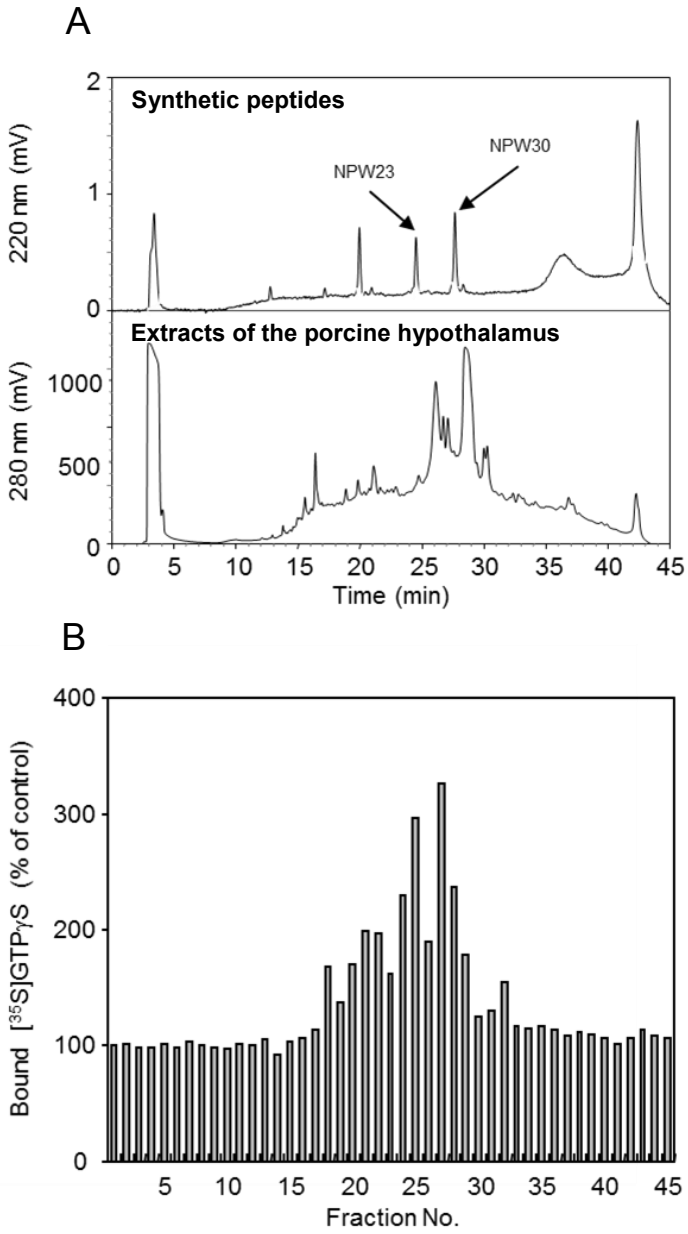

Fig. 2-6. Activation of human GPR8 by porcine NPW23 and NPW30. *A*, HPLC profiles of synthetic porcine NPW peptides and extracts of the porcine hypothalamus. Two *arrows* mark the elution peaks of the synthetic porcine NPW23 and NPW30 (*upper panel*). The extracts of the porcine hypothalamus were fractionated under the same HPLC conditions (*lower panel*). *B*, detection of agonist activities in the fractionated extracts of the porcine hypothalamus for human GPR8. The agonist activity of each fraction prepared from extracts of the porcine hypothalamus was measured by [^{35}S]GTPγS binding assay.

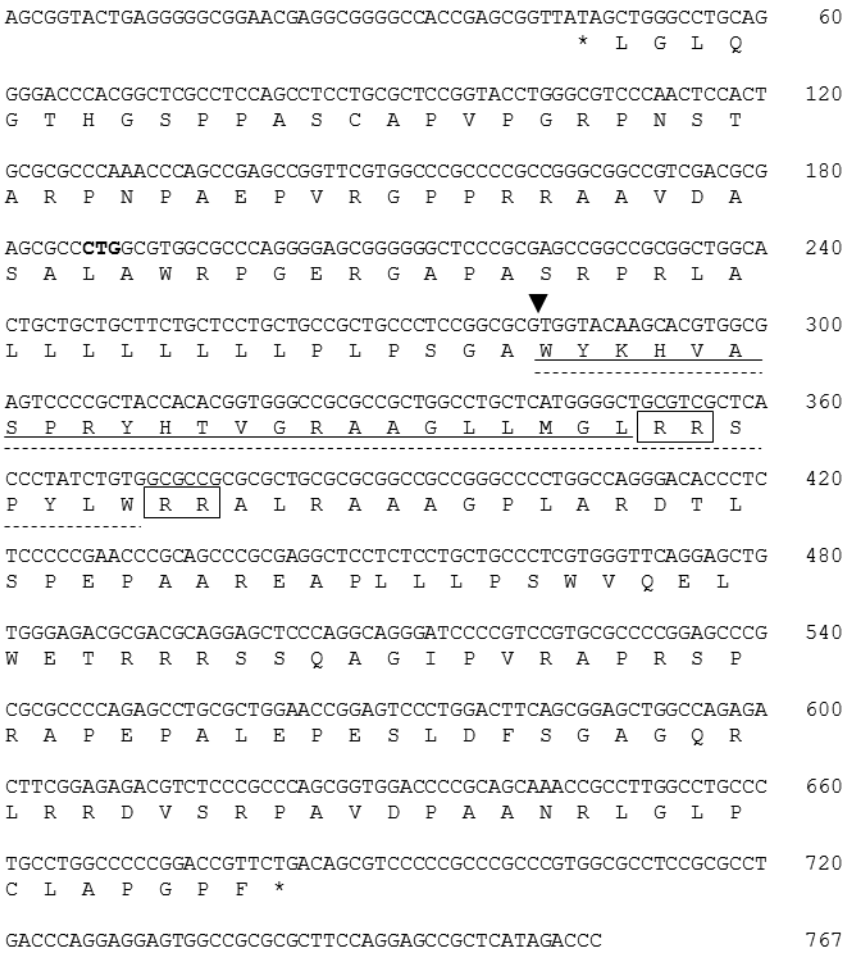

Fig. 2-7. Human NPW precursor protein cDNA and mature peptide sequences. A cDNA clone encoding human NPW precursor protein was isolated from a cDNA library of the human hypothalamus. An amino acid sequence was obtained from an open reading frame of the cDNA. Each sequence is placed with the nucleotide sequence above the amino acid sequence. A putative non-AUG start codon is indicated by *boldface letters*. The *asterisk* indicates a stop codon. The *arrowhead* indicates a putative signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. Human NPW23 and NPW30 are *underlined* with a *solid line* and with a *broken line*, respectively.

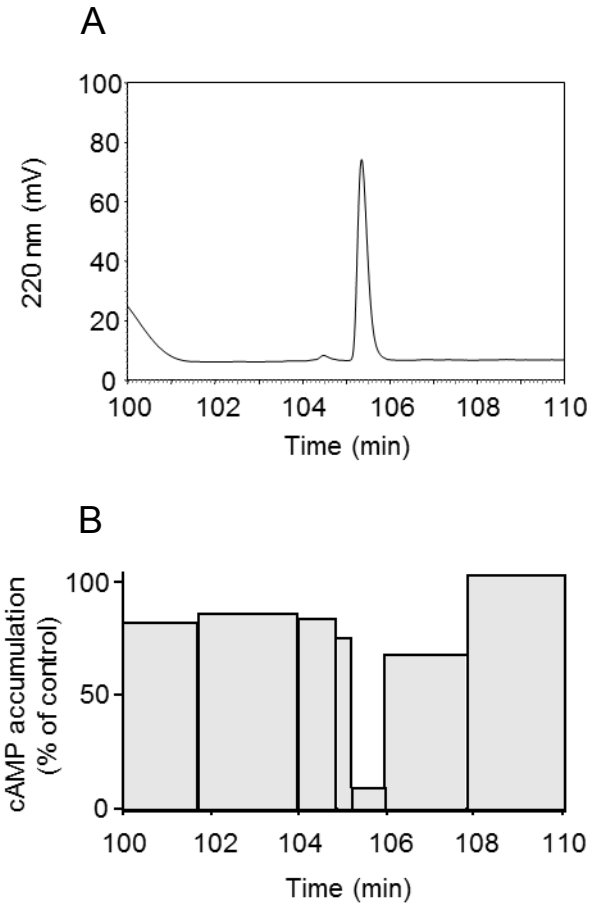

Fig. 2-8. Expression of NPW peptide produced from human NPW precursor protein cDNA with non-AUG start codon. *A*, purification of an agonist peptide for human GPR8 from the supernatant of COS-7 cells that were transfected with a human NPW precursor protein expression vector. Shown is the HPLC elution profile of the final purification step using the 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 assay of cAMP accumulation inhibition 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.

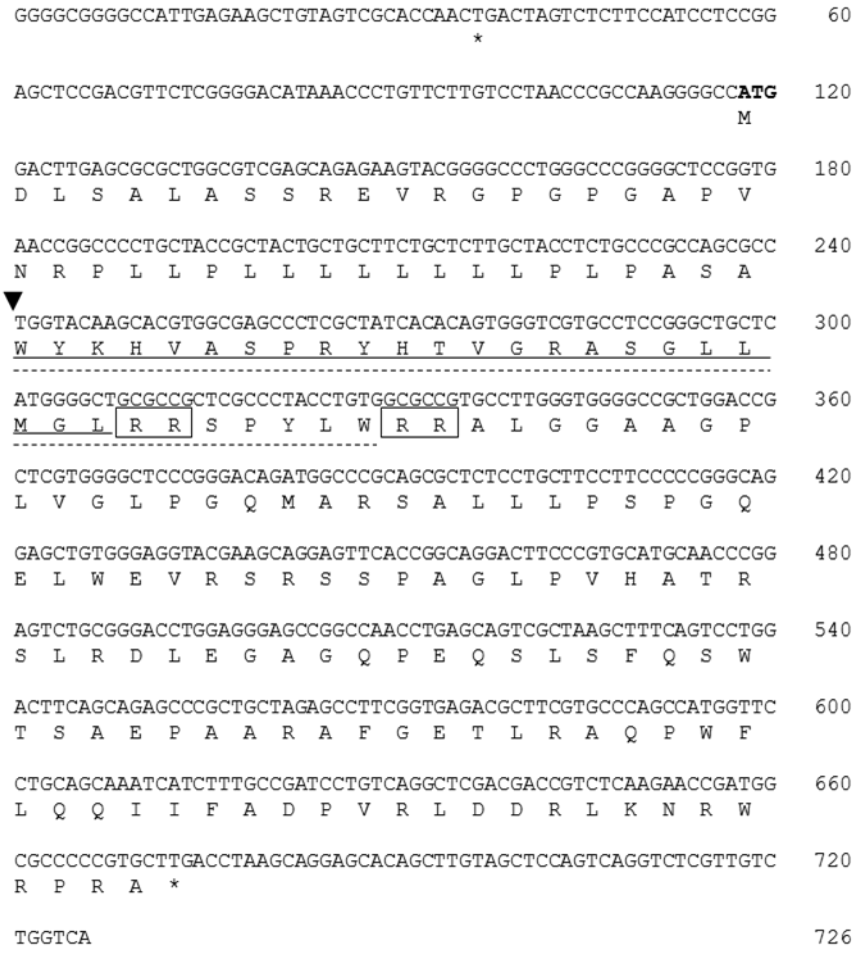

Fig. 2-9. Rat NPW precursor protein cDNA and mature peptide sequences. A cDNA clone encoding rat NPW precursor protein was isolated from a cDNA library of the rat brain. A deduced amino acid sequence of rat NPW precursor protein was obtained from the longest open reading frame of the cDNA. Each sequence is placed with the nucleotide sequence above the amino acid sequence. One Met codon as potential translation start site is indicated by *boldface letters*. The *asterisk* indicates a stop codon. The *arrowhead* indicates a putative signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. Rat NPW23 and NPW30 are *underlined* with a *solid line* and with a *broken line*, respectively.

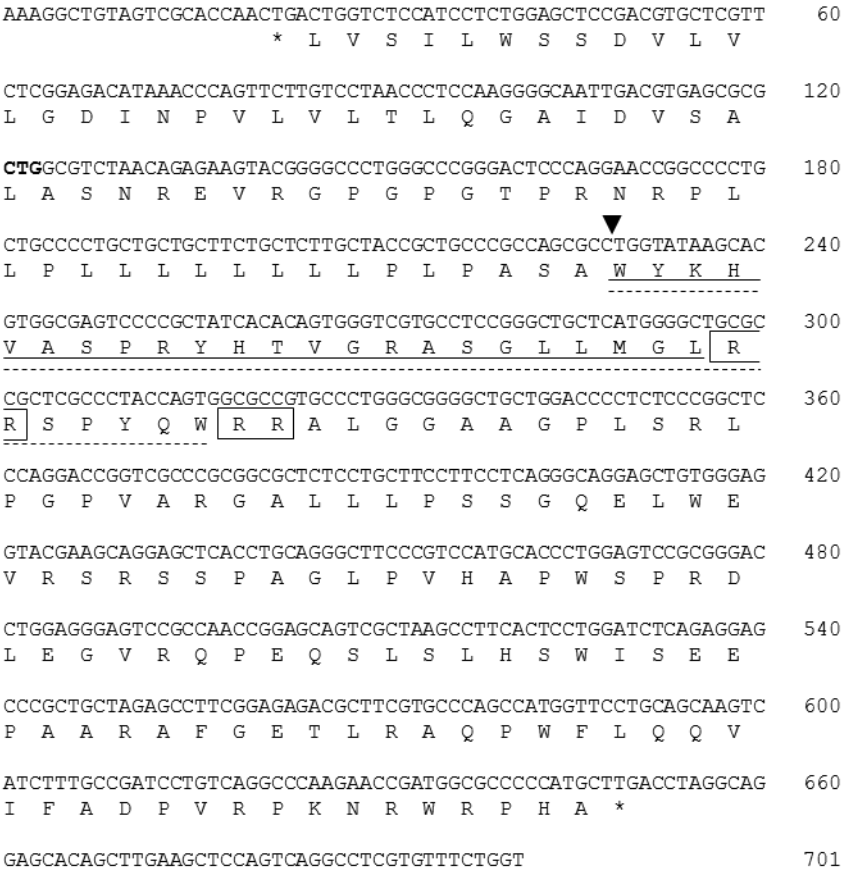

Fig. 2-10. Mouse NPW precursor protein cDNA and mature peptide sequences. A cDNA clone encoding mouse NPW precursor protein was isolated from a cDNA library of the mouse brain. An amino acid sequence was obtained from an open reading frame of the cDNA. Each sequence is placed with the nucleotide sequence above the amino acid sequence. A putative non-AUG start codon is indicated by *boldface letters*. The *asterisk* indicates a stop codon. The *arrowhead* indicates a putative signal peptide cleavage site. A pair of basic amino acid residues is *boxed*. Mouse NPW23 and NPW30 are *underlined* with a *solid line* and with a *broken line*, respectively.

Fig. 2-11. Comparison of mature NPW23 and NPW30 amino acid sequences among humans, pigs, rats, and mice. Human, porcine, rat, and mouse NPW23 and NPW30 sequences were aligned with each other. The amino acid residues identical among the four species are *shaded in black*.

Fig. 2-12. Comparison of NPW precursor proteins among humans, pigs, rats, and mice. Deduced NPW precursor proteins were aligned with each other using CLUSTAL W multiple sequence alignment. Human and mouse NPW precursor proteins were presumed to undergo translation from non-AUG start codons, while porcine and rat NPW precursor proteins were predicted to be translated from AUG start codons. Among them, the *asterisk* indicates an identical amino acid in the precursor proteins, and the amino acid residues are written in *red or blue*. A putative signal peptide sequence with hydrophobic amino acid cluster preceding the mature NPW peptides is *boxed in blue*. The NPW30 sequence is *boxed in black*. Two pairs of basic amino acid residues, Arg-Arg, are written in *blue*.

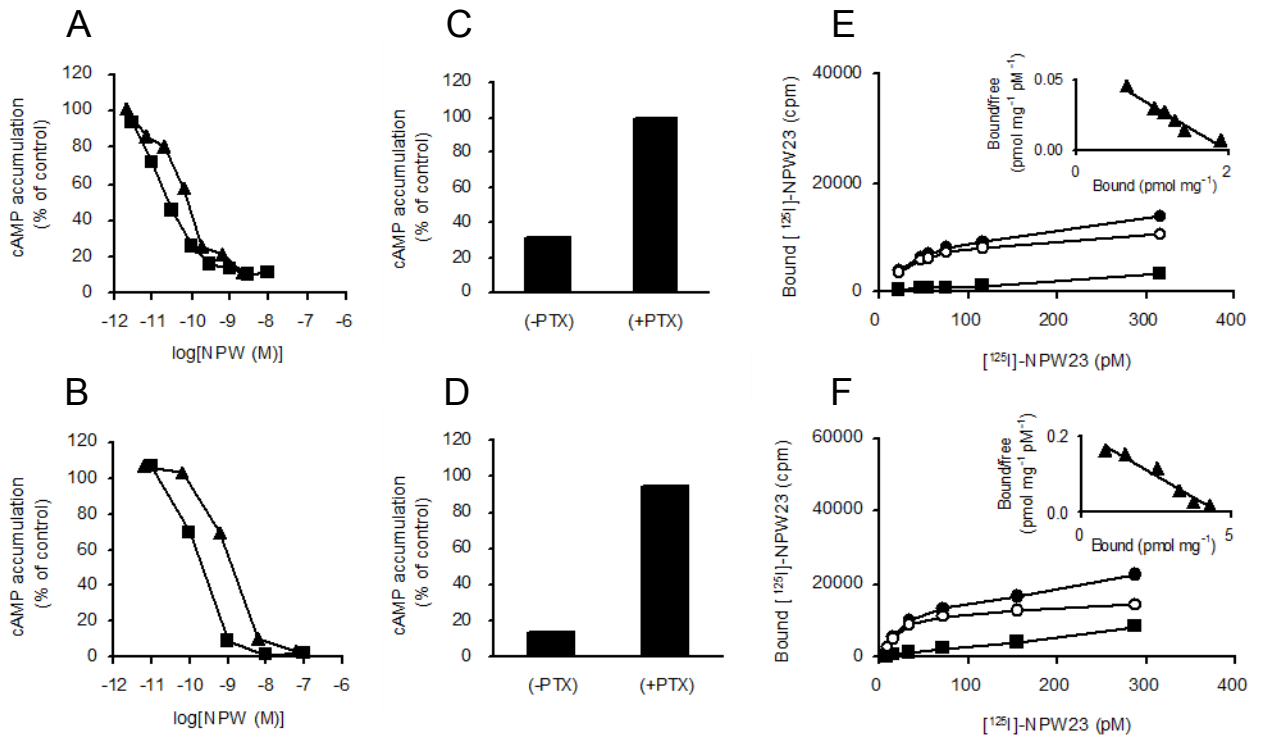

Fig. 2-13. Functional and binding characterization of NPW. *A* and *B*, dose-dependent inhibition of cAMP accumulation by NPW in CHO-GPR7 and CHO-GPR8 cells, respectively. Various concentrations of NPW23 or NPW30 were added to CHO-GPR7 and CHO-GPR8 cells, and cAMP accumulation inhibition assay was conducted 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.

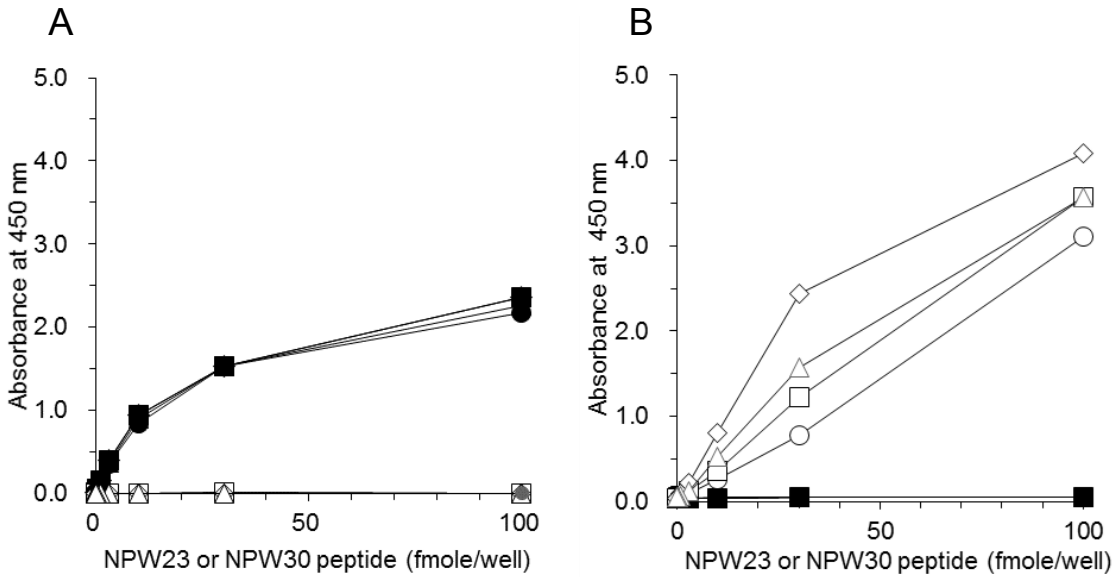

Fig. 2-14. Two different ELISAs for quantification of NPW23 and NPW30. *A* and *B*, standard curve of each NPW peptide in NPW23 ELISA and NPW30 ELISA, respectively. Various concentrations of synthetic NPW23 and NPW30 of human, porcine, rat, and mouse sequences were subjected to the ELISAs, and the NPW23 and NPW30 peptides captured on the ELISA assay plates were detected. ●, human NPW23; ▲, porcine NPW23; ■, rat NPW23; ⧫, mouse NPW23; ○, human NPW30; △, porcine NPW30; □, rat NPW30; ◊, mouse NPW30.

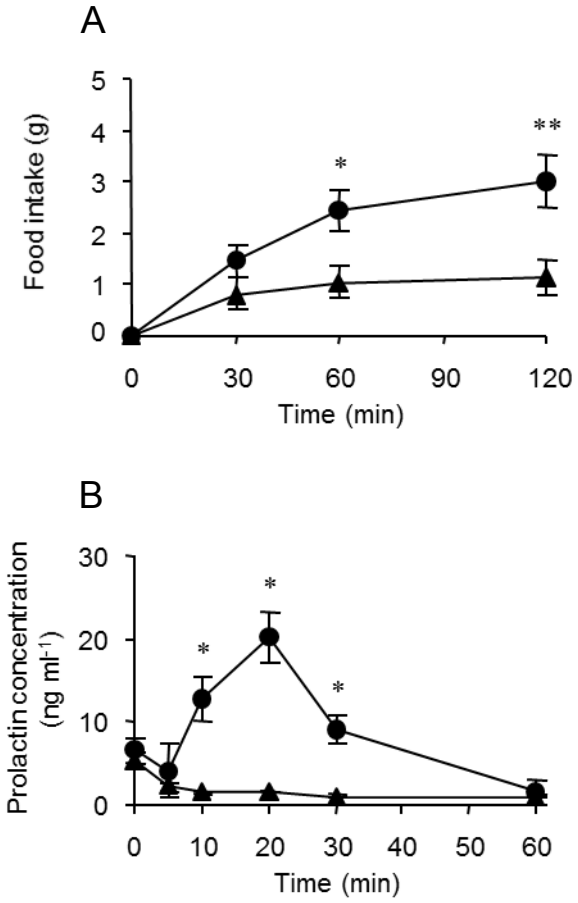

Fig. 2-15. *In vivo* effects of NPW in rats. *A*, increase of food intake by intracerebroventricular administration of human NPW23 in rats. After injection at 15:00, food intake was measured. Cumulative food consumption was plotted over a 2-h period. ●, 10 nmol of NPW23; ▲, vehicle. Results shown are 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. *, $p < 0.05$.

General Conclusion

The results obtained in this study were acquired by focusing on the analysis of the molecular nature of two pairs of deorphanized GPCRs and their endogenous ligands. The discovery of SLC-1 as a functional MCH receptor enables the understanding of a variety of MCH functions in the CNS as collective responses of the neural networks containing SLC-1 positive neurons. In addition, the pairing of SLC-1 with MCH is expected to accelerate research and development of the MCH receptor antagonists with better pharmacological profile for the treatment of obesity, anxiety, or sleep disorders. The discovery of NPW, a novel brain-gut peptide as the endogenous ligand for GPR7 and GPR8 paved the way for the field of NPW biology. The selective localization of NPW peptide and preferential gene expression of its receptors, GPR7 and GPR8, in the brain triggered studies on biological roles of NPW in the CNS. It is expected that the significance of the pathophysiological roles of NPW will be elucidated by taking advantage of the initial knowledge and basic technologies on NPW, which were provided by the efforts in this study.

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References

Adamantidis, A., Salvert, D., Goutagny, R., Lakaye, B., Gervasoni, D., Grisar, T., Luppi, P.H., Fort, P., 2008. Sleep architecture of the melanin-concentrating hormone receptor 1-knockout mice. Eur J Neurosci. 27, 1793-1800.

Akopian, D., Shen, K., Zhang, X., Shan, S.O., 2013. Signal recognition particle: an essential protein-targeting machine. Annu Rev Biochem. 82, 693-721.

An, S., Cutler, G., Zhao, J.J., Huang, S. G., Tian, H., Li, W., Liang, L., Rich, M., Bakleh, A., Du, J., Chen, J.L., Dai, K., 2001. Identification and characterization of a melanin-concentrating hormone receptor. Proc Natl Acad Sci U S A. 98, 7576-7581.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S., 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature. 348, 730-732.

Audinot, V., Lahaye, C., Supl, T., Beauverger, P., Rodriguez, M., Galizzi, J.P., Fauchère, J.L., Boutin, J.A., 2001. [^{125}I]-S36057: a new and highly potent radioligand for the melanin-concentrating hormone receptor. Br J Pharmacol. 133, 371-378.

Bächner, D., Kreienkamp, H., Weise, C., Buck, F., Richter, D., 1999. Identification of melanin concentrating hormone (MCH) as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1). FEBS Lett. 457, 522-524.

Baker, J.R., Cardinal, K., Bober, C., Taylor, M.M., Samson, W.K., 2003. Neuropeptide W acts in brain to control prolactin, corticosterone, and growth hormone release. Endocrinology. 144, 2816-2821.

Bittencourt, J.C., Presse, F., Arias, C., Peto, C., Vaughan, J., Nahon, J.L., Vale, W., Sawchenko, P.E., 1992. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol. 319, 218-245.

Borowsky, B., Durkin, M.M., Ogozalek, K., Marzabadi, M.R., DeLeon, J., Lagu, B., Heurich, R., Lichtblau, H., Shaposhnik, Z., Daniewska, I., Blackburn, T.P., Branchek, T.A., Gerald, C., Vaysse, P.J., Forray, C., 2002. Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. Nat Med. 8, 825-830.

Breton, C., Presse, F., Hervieu, G., Nahon, J.L., 1994. Structure and regulation of the mouse melanin-concentrating hormone mRNA and gene. Mol Cell Neurosci. 4, 271-284.

Brezillon, S., Lannoy, V., Franssen, J.D., Le Poul, E., Dupriez, V., Lucchetti, J., Detheux, M., Parmentier, M., 2003. Identification of natural ligands for the orphan G protein-

coupled receptors GPR7 and GPR8. J Biol Chem. 278, 776-783.

Chaki, S., Funakoshi, T., Hirota-Okuno, S., Nishiguchi, M., Shimazaki, T., Iijima, M., Grottick, A.J., Kanuma, K., Omodera, K., Sekiguchi, Y., Okuyama, S., Tran, T.A., Semple, G., Thomsen, W., 2005. Anxiolytic- and antidepressant-like profile of ATC0065 and ATC0175: nonpeptidic and orally active melanin-concentrating hormone receptor 1 antagonists. J Pharmacol Exp Ther. 313, 831-839.

Chambers, J., Ames, R.S., Bergsma, D., Muir, A., Fitzgerald, L.R., Hervieu, G., Dytko, G.M., Foley, J.J., Martin, J., Liu, W.S., Park, J., Ellis, C., Ganguly, S., Konchar, S., Cluderay, J., Leslie, R., Wilson, S., Sarau, H.M., 1999. Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. Nature. 400, 261-265.

Chen, Y., Hu, C., Hsu, C.K., Zhang, Q., Bi, C., Asnicar, M., Hsiung, H.M., Fox, N., Slieker, L.J., Yang, D.D., Heiman, M.L., Shi, Y., 2002. Targeted disruption of the melanin-concentrating hormone receptor-1 results in hyperphagia and resistance to diet-induced obesity. Endocrinology. 143, 2469-2477.

Date, Y., Mondal, M.S., Kageyama, H., Ghamari-Langroudi, M., Takenoya, F., Yamaguchi, H., Shimomura, Y., Mori, M., Murakami, N., Shioda, S., Cone, R.D., Nakazato, M., 2010. Neuropeptide W: an anorectic peptide regulated by leptin and metabolic state. Endocrinology. 151, 2200-2210.

Douglass, J., Civelli, O., Herbert, E., 1984. Polyprotein gene expression: generation of diversity of neuroendocrine peptides. Annu Rev Biochem. 53, 665-715.

Dun, S.L., Brailoiu, G.C., Yang, J., Chang, J.K., Dun, N.J., 2003. Neuropeptide W-immunoreactivity in the hypothalamus and pituitary of the rat. Neurosci Lett. 349, 71-74.

Fecher-Trost, C., Wissenbach, U., Beck, A., Schalkowsky, P., Stoerger, C., Doerr, J., Dembek, A., Simon-Thomas, M., Weber, A., Wollenberg, P., Ruppert, T., Middendorff, R., Maurer, H.H., Flockerzi, V., 2013. The in vivo TRPV6 protein starts at a non-AUG triplet, decoded as methionine, upstream of canonical initiation at AUG. J Biol Chem. 288, 16629-16644.

Foord, S.M., Bonner, T.I., Neubig, R.R., Rosser, E.M., Pin, J.P., Davenport, A.P., Spedding, M., Harmar, A.J., 2005. International Union of Pharmacology. XLVI. G protein-coupled receptor list. Pharmacol Rev. 57, 279-288.

Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Habata, Y., Hinuma, S., Onda, H., Nishimura, O., Fujino, M., 2000. Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. J Biol Chem. 275, 29528-29532.

Fujii, R., Yoshida, H., Fukusumi, S., Habata, Y., Hosoya, M., Kawamata, Y., Yano, T., Hinuma, S., Kitada, C., Asami, T., Mori, M., Fujisawa, Y., Fujino, M., 2002. Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. J Biol Chem. 277, 34010-34016.

Fredriksson, R., Lagerström, M.C., Lundin, L.G., Schiöth, H.B., 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 63, 1256-1272.

Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K.P., Ernst, O.P., 2003. Role of the conserved NPxxY(x) 5,6F motif in the rhodopsin ground state and during activation. Proc Natl Acad Sci U S A. 100, 2290-2295.

Galanopoulou, A.S., Kent, G., Rabbani, S.N., Seidah, N.G., Patel, Y.C., 1993. Heterologous processing of prosomatostatin in constitutive and regulated secretory pathways. Putative role of the endoproteases furin, PC1, and PC2. J Biol Chem. 268, 6041-6049.

Gao, X., Hsu, C.K., Heinz, L.J., Morin, J., Shi, Y., Shukla, N.K., Smiley, D.L., Xu, J., Zhong, B., Slieker, L.J., 2004. Europium-labeled melanin-concentrating hormone analogues: ligands for measuring binding to melanin-concentrating hormone receptors 1 and 2. Anal Biochem. 328, 187-195.

Hann, S.R., King, M.W., Bentley, D.L., Anderson, C.W., Eisenman, R.N., 1988. A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell. 52, 185-195.

Hawes, B.E., Kil, E., Green, B., O'Neill, K., Fried, S., Graziano, M.P., 2000. The melanin-concentrating hormone receptor couples to multiple G proteins to activate diverse intracellular signaling pathways. Endocrinology. 141, 4524-4532.

Hill, J., Duckworth, M., Murdock, P., Rennie, G., Sabido-David, C., Ames, R.S., Szekeres, P., Wilson, S., Bergsma, D.J., Gloger, I.S., Levy, D.S., Chambers, J.K., Muir, A.I., 2001. Molecular cloning and functional characterization of MCH2, a novel human MCH receptor. J Biol Chem. 276, 20125-20129.

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., Fujino, M., 1998. A prolactin-releasing peptide in the brain. Nature. 393, 272-276.

Hinuma, S., Hosoya, M., Ogi, K., Tanaka, H., Nagai, Y., Onda, H., 1994. Molecular cloning and functional expression of a human thyrotropin-releasing hormone (TRH) receptor gene. Biochim Biophys Acta. 1219, 251-259.

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., Narumiya,

S., 1991. Cloning and expression of cDNA for a human thromboxane A2 receptor. Nature. 349, 617-620.

Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., Shimizu, T., 1991. Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. Nature. 349, 342-346.

Ichimori, Y., Suzuki, N., Kitada, C., Tsukamoto, K., 1987. Monoclonal antibodies to human interferon-gamma. II: Antibodies with neutralizing activity. Hybridoma. 6, 173-181.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., Masaki, T., 1989. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A. 86, 2863-2867.

Isberg, V., de Graaf, C., Bortolato, A., Cherezov, V., Katritch, V., Marshall, F.H., Mordalski, S., Pin, J.P., Stevens, R.C., Vriend, G., Gloriam, D.E., 2015. Generic GPCR residue numbers - aligning topology maps while minding the gaps. Trends Pharmacol Sci. 36, 22-31.

Ishii, M., Fei, H., Friedman, J.M., 2003. Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. Proc Natl Acad Sci U S A. 100, 10540-10545.

Johansson, A., 2011. Recent progress in the discovery of melanin-concentrating hormone 1-receptor antagonists. Expert Opin Ther Pat. 21, 905-925.

Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M., Baker, B.I., 1983. Characterization of melanin-concentrating hormone in chum salmon pituitaries. Nature. 305, 321-323.

Kolakowski, L.F. Jr., Jung, B.P., Nguyen, T., Johnson, M.P., Lynch, K.R., Cheng, R., Heng, H.H., George, S.R., O'Dowd, B.F., 1996. Characterization of a human gene related to genes encoding somatostatin receptors. FEBS Lett. 398, 253-258.

Kotarsky, K., Nilsson, N.E., 2004. Reverse pharmacology and the de-orphanization of 7TM receptors. Drug Discov Today Technol. 1, 99-104.

Kozak, M., 1996. Interpreting cDNA sequences: some insights from studies on translation. Mamm Genome. 7, 563-574.

Lakaye, B., Minet, A., Zorzi, W., Grisar, T., 1998. Cloning of the rat brain cDNA encoding for the SLC-1 G protein-coupled receptor reveals the presence of an intron in the gene. Biochim Biophys Acta. 1401, 216-220.

Lee, D.K., Nguyen, T., Porter, C.A., Cheng, R., George, S.R., O'Dowd, B.F., 1999. Two related G protein-coupled receptors: the distribution of GPR7 in rat brain and the

absence of GPR8 in rodents. Brain Res Mol Brain Res. 71, 96-103.

Lembo, P.M., Grazzini, E., Cao, J., Hubatsch, D.A., Pelletier, M., Hoffert, C., St-Onge, S., Pou, C., Labrecque, J., Groblewski, T., O'Donnell, D., Payza, K., Ahmad, S., Walker, P., 1999. The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. Nat Cell Biol. 1, 267-271.

Levine, A.S., Winsky-Sommerer, R., Huitron-Resendiz, S., Grace, M.K., de Lecea, L., 2005. Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. Am J Physiol Regul Integr Comp Physiol. 288, 1727-1732.

Libert, F., Vassart, G., Parmentier, M., 1991. Current developments in G-protein-coupled receptors. Curr Opin Cell Biol. 3, 218-223.

MacNeil, D.J., 2013. The role of melanin-concentrating hormone and its receptors in energy homeostasis. Front Endocrinol (Lausanne). 4: 49.

Marsh, D.J., Weingarth, D.T., Novi, D.E., Chen, H.Y., Trumbauer, M.E., Chen, A.S., Guan, X.M., Jiang, M.M., Feng, Y., Camacho, R.E., Shen, Z., Frazier, E.G., Yu, H., Metzger, J.M., Kuca, S.J., Shearman, L.P., Gopal-Truter, S., MacNeil, D.J., Strack, A.M., MacIntyre, D.E., Van der Ploeg, L.H., Qian, S., 2002. Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. Proc Natl Acad Sci U S A. 99, 3240-3245.

Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., Nakanishi, S., 1987. cDNA cloning of bovine substance K receptor through oocyte expression system. Nature. 329, 836-838.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., Nakanishi, S., 1991. Sequence and expression of a metabotropic glutamate receptor. Nature. 349, 760-765.

Mehdi, H., Ono, E., Gupta, K.C., 1990. Initiation of translation at CUG, GUG, and ACG codons in mammalian cells. Gene. 91, 173-178.

Meiron, M., Anunu, R., Scheinman, E.J., Hashmueli, S., Levi, B.Z., 2001. New isoforms of VEGF are translated from alternative initiation CUG codons located in its 5'UTR. Biochem Biophys Res Commun. 282, 1053-1060.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., Costentin, J., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature. 377, 532-535.

Mihalic, J.T., Fan, P., Chen, X., Chen, X., Fu, Y., Motani, A., Liang, L., Lindstrom, M., Tang, L., Chen, J.L., Jaen, J., Dai, K., Li, L., 2012. Discovery of a novel melanin concentrating hormone receptor 1 (MCHR1) antagonist with reduced hERG inhibition. Bioorg Med Chem Lett. 22, 3781-3785.

Mondal, M.S., Yamaguchi, H., Date, Y., Shimbara, T., Toshinai, K., Shimomura, Y., Mori, M., Nakazato, M., 2003. A role for neuropeptide W in the regulation of feeding behavior. Endocrinology. 144, 4729-4733.

Mondal, M.S., Yamaguchi, H., Date, Y., Toshinai, K., Kawagoe, T., Tsuruta, T., Kageyama, H., Kawamura, Y., Shioda, S., Shimomura, Y., Mori, M., Nakazato, M., 2006. Neuropeptide W is present in antral G cells of rat, mouse, and human stomach. J Endocrinol. 188, 49-57.

Motoike, T., Skach, A.G., Godwin, J.K., Sinton, C.M., Yamazaki, M., Abe, M., Natsume, R., Sakimura, K., Yanagisawa, M., 2015. Transient expression of neuropeptide W in postnatal mouse hypothalamus--a putative regulator of energy homeostasis. Neuroscience. 301, 323-337.

Mori, M., Harada, M., Terao, Y., Sugo, T., Watanabe, T., Shimomura, Y., Abe, M., Shintani, Y., Onda, H., Nishimura, O., Fujino, M., 2001. Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor. Biochem Biophys Res Commun. 283, 1013-1018.

Mori, M., Sugo, T., Abe, M., Shimomura, Y., Kurihara, M., Kitada, C., Kikuchi, K., Shintani, Y., Kurokawa, T., Onda, H., Nishimura, O., Fujino, M., 1999. Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). Biochem Biophys Res Commun. 265, 123-129.

Nagai, K., Oubridge, C., Kuglstatter, A., Menichelli, E., Isel, C., Jovine, L., 2003. Structure, function and evolution of the signal recognition particle. EMBO J. 22, 3479-3485.

Nahon, J.L., Presse, F., Bittencourt, J.C., Sawchenko, P.E., Vale, W., 1989. The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. Endocrinology. 125, 2056-2065.

Naito, N., Kawazoe, I., Nakai, Y., Kawauchi, H., Hirano, T., 1986. Coexistence of immunoreactivity for melanin-concentrating hormone and alpha-melanocyte-stimulating hormone in the hypothalamus of the rat. Neurosci Lett. 70, 81-85.

Nakai, K., Horton, P., 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci. 24, 34-36.

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., George, S.R., 1995. The cloning and chromosomal mapping of two novel human opioid-somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. Genomics. 28, 84-91.

Ogi, K., Kimura, C., Onda, H., Arimura, A., Fujino, M., 1990. Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). Biochem Biophys Res Commun. 173, 1271-1279.

Overington, J.P., Al-Lazikani, B., Hopkins, A.L., 2006. How many drug targets are there? Nat Rev Drug Discov. 5, 993-996.

Peabody, D.S., 1989. Translation initiation at non-AUG triplets in mammalian cells. J Biol Chem. 264, 5031-5035.

Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lélias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., Caput, D., 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Natl Acad Sci U S A. 86, 1836-1840.

Presse, F., Nahon, J.L., Fischer, W.H., Vale, W., 1990. Structure of the human melanin concentrating hormone mRNA. Mol Endocrinol. 4, 632-637.

Qu, D., Ludwig, D.S., Gammeltoft, S., Piper, M., Pelleymounter, M.A., Cullen, M.J., Mathes, W.F., Przypek, R., Kanarek, R., Maratos-Flier, E., 1996. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature. 380, 243-247.

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., Civelli, O., 1995. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science. 270, 792-794.

Rodriguez, M., Beauverger, P., Naime, I., Rique, H., Ouvry, C., Souchaud, S., Dromaint, S., Nagel, N., Suply, T., Audinot, V., Boutin, J.A., Galizzi, J.P., 2001. Cloning and molecular characterization of the novel human melanin-concentrating hormone receptor MCH2. Mol Pharmacol. 60, 632-639.

Rosenbaum, D.M., Rasmussen, S.G., Kobilka, B.K., 2009. The structure and function of G-protein-coupled receptors. Nature. 459, 356-363.

Rovati, G.E., Capra, V., Neubig, R.R., 2007. The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. Mol Pharmacol. 71, 959-964.

Sailer, A.W., Sano, H., Zeng, Z., McDonald, T.P., Pan, J., Pong, S.S., Feighner, S.D., Tan, C.P., Fukami, T., Iwaasa, H., Hreniuk, D.L., Morin, N.R., Sadowski, S.J., Ito, M., Ito, M., Bansal, A., Ky, B., Figueroa, D.J., Jiang, Q., Austin, C.P., MacNeil, D.J., Ishihara, A., Ihara, M., Kanatani, A., Van der Ploeg, L.H., Howard, A.D., Liu, Q., 2001. Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R. Proc Natl Acad Sci U S A. 98, 7564-7569.

Saito, Y., Cheng, M., Leslie, F.M., Civelli, O., 2001. Expression of the melanin-

concentrating hormone (MCH) receptor mRNA in the rat brain. J Comp Neurol. 435, 26-40.

Saito, Y., Nothacker, H.P., Wang, Z., Lin, S.H., Leslie, F., Civelli, O., 1999. Molecular characterization of the melanin-concentrating-hormone receptor. Nature. 400, 265-269.

Sakurai, T., 2013. NPBWR1 and NPBWR2: Implications in Energy Homeostasis, Pain, and Emotion. Front Endocrinol (Lausanne). 4:23.

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., Yanagisawa, M., 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 20, 573-585.

Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J.J., Hasegawa, M., Matsuda, Y., Inagami, T., 1991. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. Nature. 351, 230-233.

Seidah, N.G., Prat, A., 2012. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov. 11, 367-383.

Selbie, L.A., Hill, S.J., 1998. G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. Trends Pharmacol Sci. 19, 87-93.

Shimada, M., Tritos, N.A., Lowell, B.B., Flier, J.S., Maratos-Flier, E., 1998. Mice lacking melanin-concentrating hormone are hypophagic and lean. Nature. 396, 670-674.

Shimomura, Y., Harada, M., Goto, M., Sugo, T., Matsumoto, Y., Abe, M., Watanabe, T., Asami, T., Kitada, C., Mori, M., Onda, H., Fujino, M., 2002. Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. J Biol Chem. 277, 35826-35832.

Shimomura, Y., Mori, M., Sugo, T., Ishibashi, Y., Abe, M., Kurokawa, T., Onda, H., Nishimura, O., Sumino, Y., Fujino, M., 1999. Isolation and identification of melanin-concentrating hormone as the endogenous ligand of the SLC-1 receptor. Biochem Biophys Res Commun. 261, 622-626.

Smith, D.G., Davis, R.J., Rorick-Kehn, L., Morin, M., Witkin, J.M., McKinzie, D.L., Nomikos, G.G., Gehlert, D.R., 2006. Melanin-concentrating hormone-1 receptor modulates neuroendocrine, behavioral, and corticolimbic neurochemical stress responses in mice. Neuropsychopharmacology. 31, 1135-1145.

Spiegelman, B.M., Flier, J.S., 2001. Obesity and the regulation of energy balance. Cell. 104, 531-543.

Starck, S.R., Jiang, V., Pavon-Eternod, M., Prasad, S., McCarthy, B., Pan, T., Shastri, N., 2012. Leucine-tRNA initiates at CUG start codons for protein synthesis and presentation by MHC class I. Science. 336, 1719-1723.

Suzuki, N., Matsumoto, H., Kitada, C., Masaki, T., Fujino, M., 1989. A sensitive sandwich-enzyme immunoassay for human endothelin. J Immunol Methods. 118, 245-250.

Takahashi, K., Suzuki, H., Totsune, K., Murakami, O., Satoh, F., Sone, M., Sasano, H., Mouri, T., Shibahara, S., 1995. Melanin-concentrating hormone in human and rat. Neuroendocrinology. 61, 493-498.

Takekawa, S., Asami, A., Ishihara, Y., Terauchi, J., Kato, K., Shimomura, Y., Mori, M., Murakoshi, H., Kato, K., Suzuki, N., Nishimura, O., Fujino, M., 2002. T-226296: a novel, orally active and selective melanin-concentrating hormone receptor antagonist. Eur J Pharmacol. 438, 129-135.

Takenoya, F., Kitamura, S., Kageyama, H., Nonaka, N., Seki, M., Itabashi, K., Date, Y., Nakazato, M., Shioda, S., 2008. Neuronal interactions between neuropeptide W- and orexin- or melanin-concentrating hormone-containing neurons in the rat hypothalamus. Regul Pept. 145, 159-164.

Takenoya, F., Yagi, M., Kageyama, H., Shiba, K., Endo, K., Nonaka, N., Date, Y., Nakazato, M., Shioda, S., 2010. Distribution of neuropeptide W in the rat brain. Neuropeptides. 44, 99-106.

Tanaka, H., Yoshida, T., Miyamoto, N., Motoike, T., Kurosu, H., Shibata, K., Yamanaka, A., Williams, S.C., Richardson, J.A., Tsujino, N., Garry, M.G., Lerner, M.R., King, D.S., O'Dowd, B.F., Sakurai, T., Yanagisawa, M., 2003. Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8. Proc Natl Acad Sci U S A. 100, 6251-6256.

Taylor, M.M., Yuill, E.A., Baker, J.R., Ferri, C.C., Ferguson, A.V., Samson, W.K., 2005. Actions of neuropeptide W in paraventricular hypothalamus: implications for the control of stress hormone secretion. Am J Physiol Regul Integr Comp Physiol. 288, 270-275.

Vaughan, J.M., Fischer, W.H., Hoeger, C., Rivier, J., Vale, W., 1989. Characterization of melanin-concentrating hormone from rat hypothalamus. Endocrinology. 125, 1660-1665.

Verret, L., Goutagny, R., Fort, P., Cagnon, L., Salvert, D., Léger, L., Boissard, R., Salin, P., Peyron, C., Luppi, P.H., 2003. A role of melanin-concentrating hormone producing neurons in the central regulation of paradoxical sleep. BMC Neurosci. 4:19.

von Heijne, G., 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14, 4683-4690.

Wang, S., Behan, J., O'Neill, K., Weig, B., Fried, S., Laz, T., Bayne, M., Gustafson, E., Hawes, B.E., 2001. Identification and pharmacological characterization of a novel human melanin-concentrating hormone receptor, mch-r2. J Biol Chem. 276, 34664-34670.

Wess, J., 1998. Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol Ther. 80, 231-264.

Willie, J.T., Sinton, C.M., Maratos-Flier, E., Yanagisawa, M., 2008. Abnormal response of melanin-concentrating hormone deficient mice to fasting: hyperactivity and rapid eye movement sleep suppression. Neuroscience. 156, 819-829.

Wilson, S., Bergsma, D.J., Chambers, J.K., Muir, A.I., Fantom, K.G., Ellis. C., Murdock, P.R., Herrity, N.C., Stadel, J.M., 1998. Orphan G-protein-coupled receptors: the next generation of drug targets? Br J Pharmacol. 125, 1387-1392.

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., Pachnis, V., 1998. Galanin regulates prolactin release and lactotroph proliferation. Proc Natl Acad Sci U S A. 95, 12671-12676.

Yang, S.P., Lee, Y., Voogt, J.L., 2000. Involvement of endogenous opioidergic neurons in modulation of prolactin secretion in response to mating in the female rat. Neuroendocrinology. 72, 20-28.

Yamamoto, T., Saito, O., Shono, K., Tanabe, S., 2005. Anti-hyperalgesic effects of intrathecally administered neuropeptide W-23, and neuropeptide B, in tests of inflammatory pain in rats. Brain Res. 1045, 97-106.

Zamir, N., Skofitsch, G., Jacobowitz, D.M., 1986. Distribution of immunoreactive melanin-concentrating hormone in the central nervous system of the rat. Brain Res. 373, 240-245.