Melanin-concentrating hormone (MCH) is a neuropeptide highly expressed in the brain that regulates several physiological functions mediated by receptors in the G protein-coupled receptor family. Recently an orphan receptor, SLC-1, has been identified as an MCH receptor (MCH-R1). Herein we identify and characterize a novel receptor for human MCH (MCH-R2). The receptor is composed of 340 amino acids encoded by a 1023-base pair cDNA and is 35% homologous to SLC-1. 125I-MCH specifically bound to Chinese hamster ovary cells stably expressing MCH-R2. MCH stimulated dose-dependent increases in intracellular free Ca\(^{2+}\) and inositol phosphate production in these cells but did not affect cAMP production. The pharmacological profile for mammalian MCH, [Phe\(^{13},\)Tyr\(^{19}\)]MCH, and salmon MCH at MCH-R2 differed compared with MCH-R1 as assessed by intracellular signaling and radioligand binding assays. The EC\(_{50}\) in signaling assays and the IC\(_{50}\) in radioligand binding assays of salmon MCH was an order of magnitude higher than mammalian MCH at MCH-R2. By comparison, the EC\(_{50}\) and IC\(_{50}\) values of salmon MCH and mammalian MCH at MCH-R1 were relatively similar. Blot hybridization revealed exclusive expression of MCH-R2 mRNA in several distinct brain regions, particularly in the cortical area, suggesting the involvement of MCH-R2 in the central regulation of MCH-mediated functions.

The most active area of research on MCH has focused on the role of MCH in the regulation of feeding behavior and energy balance. Several studies provide evidence for a role for MCH in regulation of body weight.1) Mice that lack MCH are hypophagic and exhibit an increased metabolic rate, resulting in decreased body weight and body fat content (5). 2) Intracerebroventricular injection of MCH increases feeding in rats (6–8). 3) MCH, like several other appetite-regulating neuropeptides, is regulated by leptin in the hypothalamus (9, 10).

Other physiological roles of MCH include regulation of the hypothalamic-pituitary adrenal gland axis (11), modulation of water and electrolyte fluxes in the gut (12), stimulation of oxytocin secretion from isolated rat neurohypophysis (13), regulation of sensory processing (14), and modulation of monoaminergic activity in the medial preoptic area (15). In addition, several lines of evidence also suggest a role for MCH in modification of memory retention (16), modulation of other hormones regulating food intake (17) and sexual behavior (18) and involvement in seizure (19). MCH is also localized in neurons functionally involved in circuits of the extrapyramidal motor systems from striatal centers to the thalamus and cerebral cortex and to the midbrain and spinal cord (20). The complex central nervous system expression pattern of MCH together with the many diverse functions mediated by MCH suggest the existence of more than one MCH receptor.

The molecular identities of MCH receptors were unknown until recent reports of the first MCH receptor (MCH-R1) (21–25). MCH-R1 is composed of seven transmembrane domains typical of G protein-coupled receptors and binds to MCH with high affinity. MCH-R1 is coupled to G\(_i\), G\(_o\), and G\(_q\)-type G proteins and mediates decreases in intracellular cAMP and increases in intracellular Ca\(^{2+}\) levels, inositol phosphate production, and mitogen-activate protein kinase activity (26). In this report, we describe a novel MCH receptor identified through bioinformatic and molecular cloning approaches. The receptor is similar (35% identical) to the previously published MCH receptor, is able to bind 125I-labeled MCH, and can be activated by MCH leading to intracellular signaling. In addition, the expression of the receptor is localized to distinct regions of the brain.

### MATERIALS AND METHODS

Human Human Marathon-Ready cDNAs and the rapid amplification of cDNA ends (RACE) kit were from CLONTECH (Palo Alto, CA). Oligonucleotides were custom-synthesized by Life Technologies, Inc. The 293-EBNA cell line (293 cells) was obtained from Invitrogen. MCH and other peptides were purchased from Sigma or BACHEM. 125I-MCH was from PerkinElmer Life Sciences.

**Isolation of cDNA for MCH-R2**—The amino acid sequences of known G protein-coupled receptors were used to conduct a BLAST search of the public data base GenBank™. The search identified a BAC clone (accession number AAQ492353) with a sequence of 429 bp. The fragment...
contained an ~120-bp coding sequence belonging to the transmembrane regions 2 and 3 of a putative G protein-coupled receptor, and the rest was a portion of an intron. A BLAST analysis revealed high homology of the 120-bp coding sequence to the recently published MCH receptor (MCH-R1) (21–25). RACE was used to obtain the full-length cDNA for this putative G protein-coupled receptor (MCH-R2). The ORF is indicated by the long bar with the nucleotide sequence shown below (GenBank™ accession number AF399937). B, amino acid sequence alignment of MCH-R2 with those of MCH-R1 (27). Residues that match exactly are shaded black.

**Fig. 1.** Molecular identification of MCH-R2. A, cloning of cDNA containing the ORF of MCH-R2. The short bar indicates the starting 120-bp sequence (GenBank™ accession number AQ492353) for RACE. The arrows below denote multiple RACE reactions to extend the sequence toward the 5' and 3' directions. Primers oligoJ634 and oligoJ636 were used in PCR to obtain the full-length ORF of MCH-R2. The ORF is indicated by the long bar with the nucleotide sequence shown below (GenBank™ accession number AF399937). B, amino acid sequence alignment of MCH-R2 with those of MCH-R1 (27). Residues that match exactly are shaded black.

To clone the full length of MCH-R2 cDNA, a new PCR primer pair (oligoJ634 and oligoJ636) was designed to amplify in a PCR by using CLONTECH Marathon-Ready cDNA as template (see Fig. 1A). The PCR product containing the full-length cDNA was cloned into expression vector pCR3.1 (Invitrogen) to form an expression construct pCR3.1-MCH-R2. For each RACE, nested PCR primers specific to the MCH-R2 cDNA sequence and primers AP1 and AP2 that bind to Marathon-Ready cDNA adapter (CLONTECH) were used. Multiple RACE reactions were run to extend the sequence to the start codon of the MCH-R2 full-length cDNA. Similar conditions were used to obtain the 3'-RACE products to obtain the stop codon of the open reading frame (ORF).

Radioligand Binding Assay—CHO cells stably expressing MCH-R2 (CHO/MCH-R2) or MCH-R1 (CHO/MCH-R1) were plated in six-well culture plates and incubated for 24–48 h. Cells were washed with binding buffer (25 mM Hepes, pH 7.2, 10 mM MgCl₂, 1 mM MnCl₂, 1% bovine serum albumin). Cells were then preincubated with 300 µl of binding buffer containing unlabeled MCH (mammalian MCH, (IPhe³¹-Tyr³⁸)MCH, or salmon MCH as indicated in the figures) for 5
Cells were then treated with an additional 300 μl of binding buffer containing 125I-MCH at concentrations described in the figures for 45 min at room temperature. Cells were then placed on ice and washed four times with cold binding buffer. Cells were scraped into 12 ml 11003 75-mm test tubes and centrifuged at 3000 g for 5 min. Excess fluid was aspirated, and the radioactivity bound to the cell pellet was counted in a γ counter.

**Measurement of Intracellular Ca2⁺ Levels**—CHO/MCH-R2 and CHO/MCH-R1 cells were split into 96-well plates and incubated overnight. Subsequently the medium was removed, and 100 μl of FLIPR buffer (Hanks’ balanced salt solution, 20 mM Hepes, 2.5 mM probenecid, 0.1% bovine serum albumin) containing 4 μM Fluo-3/AM was added to the wells. Cells were incubated at 37 °C in 5% CO₂ for 1 h. Cells were then washed two times with 150 μl of FLIPR buffer. Subsequently 150 μl of FLIPR buffer was added to each well, and changes in intracellular Ca2⁺...
levels were measured after addition of 50 μl of assay buffer containing the appropriate concentration of ligands. The FLIPR instrument (Molecular Devices) was used to monitor intracellular free Ca\(^{2+}\) levels over a 3-min stimulation period. The maximum increase in fluorescence was determined and compared for each well. All data were analyzed, and EC\(_{50}\) values were determined by nonlinear regression using GraphPad Prism software.

**Measurement of Inositol Phosphate Production**—CHO/MCH-R2 and CHO/MCH-R1 cells were split into 12-well culture plates. Cells were then washed with inositol phosphate (IP) assay buffer (phosphate-buffered saline, 20 mM LiCl, 1 mM CaCl\(_2\)) and stimulated as indicated in the figures for 45 min. Cells were lysed by the addition of 1 ml of 0.4 M perchloric acid. The perchloric acid was then removed from the wells and added to 0.5 ml of 0.7 M KOH, 0.6 M KHCO\(_3\). A 1.0-ml aliquot from each sample was applied to a Dowex anion exchange column. Columns were washed twice with water (10 ml each), and IPs were eluted with 3.0 ml of 0.1M formic acid, 1.0 M ammonium formate. Samples were analyzed by liquid scintillation spectroscopy. All data were analyzed by nonlinear regression using GraphPad Prism software.

**Messenger RNA Expression Analysis of MCH-R2**—Tissue expression of MCH-R2 was examined using dot blots and Northern blots obtained from a commercial source (CLONTECH). Hybridization to blots was carried out using a PCR-generated DNA fragment encompassing 800 bp from a commercial source (CLONTECH). Hybridization to blots was carried out using a PCR-generated DNA fragment encompassing 800 bp spanning regions within the protein, a feature shared by the G protein-coupled receptor superfamily. BLAST analysis with the amino acid sequence of MCH-R2 against the GenBank\textsuperscript{TM} data base revealed moderately high homology to known receptors, i.e. the MCH-R1 receptor, somatostatin receptor types 5, 3, 2, and 1.

**RESULTS AND DISCUSSION**

The 120-bp coding sequence (Fig. 1A) was used as a starting sequence for the 5'- and 3'-RACE extensions. Multiple RACE reactions were performed to obtain the 5' translation initiation codon and the 3' translation termination codon by using human brain cDNA preparations. Based on the 5'- and 3'-end sequences, a full-length ORF was generated by PCR. The ORF is 1023 bp long (Fig. 1A) and encodes a protein of 340 amino acids (Fig. 1B). Immediately upstream of the ATG initiation codon is a short stretch of untranslated sequence with an in-frame termination codon (TAA tcc tgt gaa agt cca ega aca ATG), suggesting a correct assignment of the Met as the N terminus of the receptor.

Comparison with other G protein-coupled receptor amino acid sequences and hydrophobicity analysis of the 340-amino acid protein suggests that there are seven transmembrane-spanning regions within the protein, a feature shared by the G protein-coupled receptor superfamily. BLAST analysis with the amino acid sequence of MCH-R2 against the GenBank\textsuperscript{TM} data base revealed moderately high homology to known receptors, i.e. the MCH-R1 receptor, somatostatin receptor types 5, 3, 2,
and 1, and the κ and δ opioid receptor subtypes. The alignment analysis of the protein sequences by the J. Hein method (27) showed that the highest homology of MCH-R2 is to the human MCH-R1 receptor (35%). Fig. 1B illustrates the alignment of the MCH-R2 amino acid sequence with human MCH-R1. These analyses suggest that the protein encoded by the ORF sequence may be a novel MCH receptor. The amino acid sequence is identical to that in recent reports that also concluded it to be MCH-R2 (28, 29).

Radioligand binding assays were performed to test the ability of MCH-R2 to bind 125I-labeled MCH. MCH-R2 ORF cDNA was cloned into the expression vector pME18. A CHO cell line was generated that stably expressed MCH-R2 (CHO/MCH-R2). Subsequent studies were performed using this cell line. Specific binding of 125I-MCH to the CHO/MCH-R2 cells was detected (Fig. 2A). No specific binding was observed with wild-type CHO cells. Nonradiolabeled MCH competitively inhibited 125I-MCH (0.5 nM) binding to CHO/MCH-R2 with an IC50 of 5.0 ± 1.0 nM (Fig. 2B). This data demonstrates that MCH specifically binds to MCH-R2 with high affinity.

The ability of MCH to activate MCH-R2 and regulate intracellular signaling is shown in Fig. 3. MCH dose dependently stimulated an increase in intracellular free Ca2+ levels (Fig. 3A) and IP production (Fig. 3B) in CHO/MCH-R2 cells and in CHO cells expressing MCH-R1 (CHO/MCH-R1). The EC50 values of MCH at MCH-R2 (0.54 ± 0.05 nM in the Ca2+ assay and 3.4 ± 0.2 nM in the IP assay) were lower in both functional assays compared with the EC50 values of MCH at MCH-R1 (5.0 ± 0.6 nM in the Ca2+ assay and 27 ± 9 nM in the IP assay). MCH stimulation of CHO/MCH-R2 cells had no effect on basal (data not shown) or forskolin-stimulated (Fig. 3C) cAMP production. By contrast MCH stimulation of CHO/MCH-R1 cells inhibited forskolin-provoked increases in cAMP production (Fig. 3C). Cells expressing MCH-R2 were not responsive to stimulation by somatostatin, dynorphin A, dynorphin B, neuropeptide EI, or neuropeptide GE at concentrations up to 1 μM.
Identification and Characterization of MCH-R2

The expression of MCH-R2 mRNA was determined with Northern and dot blot hybridization with an 800-hp MCH-R2 cDNA fragment in the ORF as probe. The MCH-R2 mRNA was localized primarily to telencephalic regions of the human brain (Fig. 6A). As shown by Northern blot, there is a prominent 4.4-kilobase pair band in total brain and all of the cortical RNAs (Fig. 6A). The multiple tissue expression array shows strong signals in the amygdala, hippocampus, and nucleus accumbens as well as in the fetal brain (Fig. 6B). Previous studies in rat and human brain have defined the expression of MCH-R1. Northern blot and in situ hybridization studies have shown MCH-R1 to be expressed in brain, eye, skeletal muscle, and tongue (21, 22, 25, 30). In rat brain, MCH-R1 mRNA is broadly expressed and appears to be most abundant in neurons in the olfactory system, nucleus accumbens, amygdala, hippocampus, and hypothalamus (25). In contrast, the MCH-R2 mRNA is expressed almost exclusively in cortical regions of human brain (Fig. 6). Major areas of overlap between the two subtypes appear to be the nucleus accumbens, amygdala, and hippocampus. MCH-R2 also appears to be neuronal in nature because there is no signal in the corpus callosum RNA.

The differential expression of the two receptor subtypes in brain suggests that the two receptors serve different functions. Much of the functionality attributed to MCH revolves around homeostatic mechanisms such as food intake and energy balance as well as neuroendocrine processes. These functions and the MCH peptide appear to reside in subcortical regions like the hypothalamus in rodents, and thus the presence of MCH-R1 in many of these same regions suggests MCH-R1 is the receptor responsible for mediating homeostatic mechanisms.

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The interaction of a ligand with its receptor is a major determinant of affinity. Comparison of mammalian MCH (DFDMLRCMLGRVYRPCWQV) with salmon MCH (DTMRCMVGRVYRPCW) reveals that the primary difference between the two ligands is near the N terminus (FDML) outside of the cyclic structure formed by the disulfide bond between Cys7 and Cys16. Thus the lower affinity of salmon MCH for MCH-R2 suggests that the 4-amino acid stretch FDML as well as Leu9 and Gln18 may play a role in ligand binding of mammalian MCH with MCH-R2.

To determine whether the binding affinity of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH for MCH-R2 differed, whole cell binding experiments were performed. Binding of [125I]MCH to CHO/MCH-R2 cells was competitively inhibited by increasing concentrations of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH to stimulate IP production in CHO/MCH-R1 and CHO/MCH-R2 cells were determined in Fig. 4, C and D. In CHO/MCH-R2 cells (Fig. 4C), the IC50 for salmon MCH-stimulated IP production was much higher compared with that for mammalian MCH or [Phe13,Tyr19]MCH. IC50 values were 3.4 ± 0.2 nM for mammalian MCH, 3.9 ± 0.8 nM for [Phe13,Tyr19]MCH, and 95 ± 20 nM for salmon MCH. In contrast, mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH provoked IP production with relatively similar potencies in CHO/MCH-R1 cells (Fig. 4B). IC50 values were 34 ± 10 nM for mammalian MCH, 64 ± 15 nM for [Phe13,Tyr19]MCH, and 88 ± 10 nM for salmon MCH. The results in Fig. 4 illustrate different pharmacological profiles between the two cloned MCH receptors. The IC50 of mammalian MCH at MCH-R2 is an order of magnitude lower than that at MCH-R1 in both functional assays. The potency of salmon MCH is much less (10 times in the Ca2+ levels and IP production in CHO/MCH-R1 and CHO/MCH-R2 were assessed. As shown in Fig. 4A, the EC50 for mammalian MCH-stimulated increases in intracellular free Ca2+ levels and IP production in CHO/MCH-R1 and CHO/MCH-R2 were lower for MCH-R2 compared as Leu9 and Gln18 may play a role in ligand binding of mammalian and salmon MCH to stimulate increases in intracellular free Ca2+ levels and IP production in CHO/MCH-R1 and CHO/MCH-R2 were lower for MCH-R2 compared.

To compare the pharmacological profile of MCH-R2 to MCH-R1, the abilities of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH to stimulate increases in intracellular free Ca2+ levels and IP production in CHO/MCH-R1 and CHO/MCH-R2 were assessed. As shown in Fig. 4A, the EC50 for mammalian MCH-stimulated increases in intracellular free Ca2+ levels was 0.54 ± 0.05 nM. By comparison, the EC50 of [Phe13,Tyr19]MCH was higher (2.26 ± 0.43 nM). The EC50 for salmon MCH (4.0 ± 0.4 nM) was an order of magnitude higher than that for mammalian MCH. In contrast, in CHO/MCH-R1 cells (Fig. 4B), EC50 values for mammalian MCH and salmon MCH were quite similar (5.0 ± 0.6 nM for mammalian MCH and 7.1 ± 0.8 nM for salmon MCH). By comparison, the EC50 value of [Phe13,Tyr19]MCH at MCH-R1 in increasing intracellular free Ca2+ was higher (38.5 ± 3.2 nM). The abilities of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH to stimulate IP production in CHO/MCH-R1 and CHO/MCH-R2 cells were determined in Fig. 4, C and D. In CHO/MCH-R2 cells (Fig. 4C), the EC50 for salmon MCH-stimulated IP production was much higher compared with that for mammalian MCH or [Phe13,Tyr19]MCH. EC50 values were 3.4 ± 0.2 nM for mammalian MCH, 3.9 ± 0.8 nM for [Phe13,Tyr19]MCH, and 95 ± 20 nM for salmon MCH. In contrast, mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH provoked IP production with relatively similar potencies in CHO/MCH-R1 cells (Fig. 4B). EC50 values were 34 ± 10 nM for mammalian MCH, 64 ± 15 nM for [Phe13,Tyr19]MCH, and 88 ± 10 nM for salmon MCH. The results in Fig. 4 illustrate different pharmacological profiles between the two cloned MCH receptors. The EC50 of mammalian MCH at MCH-R2 is an order of magnitude lower than that at MCH-R1 in both functional assays. The potency of salmon MCH is much less (10 times in the Ca2+ assay and 30 times in the IP assay) than mammalian MCH at MCH-R2. In contrast, the potencies of salmon MCH and mammalian MCH at MCH-R1 are relatively similar.

To determine whether the binding affinity of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH for MCH-R2 differed, whole cell binding experiments were performed. Binding of [125I]MCH to CHO/MCH-R2 cells was competitively inhibited by increasing concentrations of mammalian MCH, [Phe13,Tyr19]MCH, and salmon MCH (Fig. 5A). The affinity of salmon MCH was markedly lower for MCH-R2 compared with that of mammalian MCH and [Phe13,Tyr19]MCH. The IC50 values were 5.0 ± 1.0 nM for mammalian MCH, 4.0 ± 2.0 nM for [Phe13,Tyr19]MCH, and 100 ± 20 nM for salmon MCH. By comparison, the affinities for the three MCH peptides for MCH-R1 appear to be relatively similar (Fig. 5B). The IC50 values were 3.0 ± 1.0 nM for mammalian MCH, 5.0 ± 1.0 nM for [Phe13,Tyr19]MCH, and 4.0 ± 1.0 nM for salmon MCH at MCH-R1. These data are consistent with the functional data in Fig. 4 and with recent reports characterizing MCH-R2 (28, 29).
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Identification and Pharmacological Characterization of a Novel Human Melanin-concentrating Hormone Receptor, MCH-R2
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