0026-895X/01/6004-632–639$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:632–639, 2001

Vol. 60, No. 4
1159/934830
Printed in U.S.A.

ACCELERATED COMMUNICATION

Cloning and Molecular Characterization of the Novel Human Melanin-Concentrating Hormone Receptor MCH2

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Received June 15, 2001; accepted July 11, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Using a genomics-based approach for screening orphan G-protein-coupled receptors, we have identified and cloned a novel high-affinity, melanin-concentrating hormone (MCH) receptor. This receptor, named S643b, displays the greatest overall identity (32%) with the previously reported human SLC-1 receptor (MCH1) and to a lesser extent with the somatostatin receptor subtypes. The gene encoding the S643b receptor spans more than 23 kilobase pairs (kb) and was mapped, by radiation hybrid experiments, on chromosome 6q14.3-q15. Comparison of the S643b cDNA with human genomic sequence reveals that the 340-amino-acid receptor is encoded by five exons. Its tissue distribution, as determined by Northern blot and reverse transcription-polymerase chain reaction analysis, indicates that a 4-kb transcript is predominantly expressed in the brain. When expressed in Chinese hamster ovary (CHO) cells, the S643b receptor displays a strong, dose-dependent, transient elevation of intracellular calcium in response to MCH (EC_{50} = 9.5 nM). During the present study, we isolated a splice variant, designated S643a, encoding for a receptor that was not activated by MCH in a cellular calcium mobilization assay. Comparative pharmacological studies using CHO cells stably expressing either SLC-1 or S643b receptors demonstrated that similar structural features of MCH are required to stimulate intracellular Ca^{2+} mobilization at both receptors. The identification and localization of this new MCH receptor (MCH2) provides further insight into the physiological implication of MCH in modulating behavioral responses, including food intake.

Melanin-concentrating hormone (MCH) is a cyclic, 19-amino-acid peptide expressed predominantly in brain. MCH-synthesizing neurons are located in the zona inserta and lateral hypothalamus and project broadly throughout the central nervous system (Bittencourt et al., 1992). MCH seems to be a key regulator in energy balance and food intake (Qu et al., 1996; Rossi et al., 1997; Shimada et al., 1998). In addition to its orexigenic action, MCH is involved in numerous behavioral responses such as auditory stimuli (Miller et al., 1993), grooming (Sanchez et al., 1997), sexual behavior (Tsukamura et al., 2000), anxiety (Gonzalez et al., 1996), and modulation of the hypothalamo-pituitary adrenal axis during stress (Jezova et al., 1992; Ludwig et al., 1998). Like many hypothalamic peptides that regulate food intake (Leu-enkephalin, galanin, motilin, neuropeptide Y) MCH is also expressed in the gastroentero-pancreatic systems and exerts effects on metabolic axes such as insulin release (Tadayyon et al., 2000) and leptin secretion from adipocytes (Bradley et al., 2000).

Several groups have concomitantly identified the orphan G protein-coupled receptor (GPCR) SLC-1 as a receptor for the neuropeptide MCH (Bachner et al., 1999; Chambers et al., 1999; Lembo et al., 1999; Saito et al., 1999; Shimomura et al., 1999). This receptor is widely expressed throughout the brain and peripheral tissues and its distribution is consistent with all the reported biological actions of MCH in mammalian systems (Hervieu et al., 2000). Extensive pharmacological studies of the SLC-1 recombinant receptor using specific radioligands and MCH analogs (Audinot et al., 2001a,b) as well as the evaluation of the effects of these peptides upon

T.S. was the recipient of a Convention Industrielle de Formation par la Recherche between the Association Nationale de la Recherche Technique, the Institut de Recherche Servier, and the Centre National de la Recherche Scientifique.

ABBREVIATIONS: MCH, melanin-concentrating hormone; GPCR, G-protein-coupled receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pair; HTGS, high throughput genome sequences; RACE, rapid amplification of cDNA ends; SSC, standard saline citrate; CHO, Chinese hamster ovary; FLIPR, fluorometric imaging plate reader; HEK, human embryonic kidney.

food intake (Suply et al., 2001) suggest that SLC-1 receptor was involved in the central MCH regulation of feeding behavior.

SLC-1 was also shown to be expressed in insulinoma cell lines in which rat/human MCH significantly stimulated insulin release, whereas salmon MCH was less active in eliciting a response (Tadayyon et al., 2000). However, MCH-induced insulin release was found to be insensitive to pertussis-toxin. This result was surprising because in cells expressing the SLC-1 recombinant receptor, salmon MCH is equipotent to rat/human MCH in binding and functional assays, and MCH-induced calcium mobilization is at least partially inhibited by pertussis toxin (Saito et al., 2000). These discrepancies suggest the existence of as-yet-unknown MCH receptor(s).

In an effort to search for additional members of the G-protein-coupled receptor family, we performed homology searching of the HTGS database, using known GPCRs as baits. We identified a bacterial artificial chromosome clone (GenBank accession number AC027643) containing a fragment related to the SLC-1 receptor. This genomic DNA fragment was demonstrated to be a gene and was mapped on chromosome 6q14.3-q15. The full-length coding region of this new sequence was isolated from human brain; once expressed in cells, this receptor was selectively activated by nanomolar concentrations of MCH. Meanwhile, as reported elsewhere (Hill et al., 2001; Mori et al., 2001), the same receptor was independently discovered by two other groups. In the present study, we describe for the first time the chromosomal localization of this new gene together with part of its genomic organization. To gain more insight in the functional implications of this receptor, we have investigated its distribution in human tissues and compared its pharmacological profile using peptide derivatives with that of SLC-1, the initial MCH receptor, to assess the structural features of MCH required to stimulate intracellular Ca^{2+} mobilization.

Experimental Procedures

Identification and Cloning of the Human S643b Receptor cDNA. The complete human SLC-1 receptor amino acid sequence (Shimomura et al., 1999) was used to conduct a search (using the Basic Local Alignment Search Tool) of the high-throughput genome sequences (HTGS) database. A bacterial artificial chromosome clone (GenBank accession number AC027643) containing sequence homologous to the SLC-1 receptor was identified and analyzed with Fgene sofware (Salamov and Solovye, 2000) to assess the start/stop codons and the intron/exon boundaries. Specific sense and antisense primers were synthesized to PCR-amplify the open reading frame of the predicted GPCR with cDNA prepared from human brain mRNA. The sequences of the primers are as follows (the underlined nucleotides are the *Hin*dIII and *Kpn*I sites, respectively): forward, 643aH 5′-GAGCTTAAGCTTCAAAATGGATTCAGA-ATTAGTGC-3′; reverse, 643aK 5′-GGATCCGGTACCAAAGTGTGATTTCAGA-GTGTTTC-3′. PCR thermal cycling conditions used were as follows: 35 cycles of 94°C; 1 min; 55°C, 1 min; 72°C, 2 min. The resultant 920-bp PCR product, named S643a, was cloned into the pcDNA3.1 vector (Invitrogen, Cergy Pontoise, France). The recombinant plasmid, designated pS643a, was sequenced on both strands by automated sequencing. The 5′ end of the S643a cDNA fragment was further extended using CLONTECH's Human Brain ClonCapture cDNA library as a template for 5′ rapid amplification of cDNA ends (RACE). PCR reaction was performed with the forward primer pE1 5′-CGAGCTCGGATCGATATCTG-3′ based on the pEXP1 vector sequence and the reverse specific primer 643R 5′-TGTGGACCAAATCAGCCACA-3′ based on the S643a cDNA. PCR thermal cycling conditions used were as follows: 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. The resulting PCR products were then subcloned into the pT-Adv vector (CLONTECH, Palo Alto, CA) and sequenced on both strands by automated sequencing. A forward primer, 643bH, based on the new upstream sequence was synthesized: 5′-GAGCTTAAGCTTGAACAATGAATCCATTTCATGC-3′. The primer pair 643bH/643K was used to PCR-amplify the entire coding region of the new GPCR (S643b) with cDNA prepared from human brain mRNA. PCR thermal cycling conditions used were as follows: 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. The resulting PCR product was then subcloned into the pcDNA3.1 vector. The recombinant plasmid, designated pS643b, was sequenced on both strands by automated sequencing.

Gene Expression Analysis by RT-PCR. Poly(A)$^+$ RNA from human tissues were obtained from CLONTECH except for human hypothalamus, which was obtained from Analytical Biological Services (Wilmington, DE). RNA were reversed transcribed using oligo(dT)$_{12–18}$ and reverse transcriptase Superscript II (Invitrogen). The first strand cDNA (corresponding to 1 μg of total RNA) was amplified using a program consisting of 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, with a pre- and postincubation of 94°C for 1 min and 72°C for 5 min, respectively. PCR amplification used the forward primers 643bH and the reverse primer 643E 5′-GCACAACTCTCAACACCGTC-3′. PCR products were separated by agarose (1%) gel electrophoresis and transferred onto Hybond N$^+$ membrane (Amersham Pharmacia Biotech, Saclay, France). Hybridization was performed at 42°C with an internal specific ^{32}P-labeled oligonucleotide probe, 5′-GTCCCTGACATCTATATCTGC-3′. The blots were washed twice at 50°C in 2× SSC containing 0.1% (w/v) SDS for 30 min each and exposed to X-ray film overnight.

Northern Blot Analysis. Multiple tissue Northern Blot (MTN blot; CLONTECH) carrying mRNA purified from brain and various peripheral tissues was prehybridized for 4 h in hybridization solution containing 5× SSC, 10× Denhardt's solution, 100 μg of salmon sperm DNA, 2% SDS, and 50% deionized formamide. The *Hin*dIII/*Kpn*I digestion fragment from the pS643b-plasmid was randomly labeled to a specific activity of 2×10^9 cpm/μg with [α-^{32}P]dCTP. The blot was then hybridized with 2×10^6 cpm/ml of probe at 65°C overnight. The blot was washed twice in 2× SSC and 0.1% SDS at room temperature, followed by one wash in 0.1× SSC and 0.1% SDS for 40 min at 50°C and exposed to X-ray film at −80°C in the presence of an intensifying screen.

Chromosome Mapping. The GeneBridge 4 human/hamster radiation hybrid panel (Invitrogen) was used for PCR amplification of the human S643b-receptor gene using the forward primer 5′-GAATGTTTCCTCTGCAGCTG-3′ and the reverse primer 5′-TGTGATTTCAGAGTGTTTCCC-3′ designed according to the GenBank published sequence (accession number AC027643). PCR reactions were performed with the *Taq* PCR Core Kit according to the manufacturer's instructions (QIAGEN, Courtaboeuf, France) with a 35-cycle program of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 3 min. The PCR products (353 bp) were analyzed by 1.5% (w/v) agarose gel electrophoresis. The results were analyzed via the GeneBridge 4 internet site at http://www.genome.wi.mit.edu. The cytogenetic location was calculated using MapView software at http://www.gdb.org.

Stable CHO-K1 Cell Lines. CHO-K1-Gα16 cells were maintained in Ham-F12 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 500 IU/ml penicillin and 100 μg/ml streptomycin. The coding regions of the human S643a and S643b (MCH2) receptor isoforms, containing the flag epitope sequence (DYKDDDDK) at their 3′-end, were subcloned into the pcDNA3.1-neo expression vector (Invitrogen, France) and transfected into CHO-K1-Gα16 cells, stably expressing the G-protein Gα16 subunit, using LipofectAMINE as described by the manufacturer (Invitrogen). Stably transfected cells were selected with Geneticin (800 μg/ml) and

tested by immunofluorescence for their ability to bind the M5 antibody.

Ca^{2+} Mobilization Assay Stable CHO-K1-Gα16 cells expressing either the S643a or S643b (MCH2) receptor were seeded (30,000 cells per well with a plating volume of 100 μl) into D-lysine–coated 96-well plates 24 h before assay. Cells were then loaded with a fluorescence-imaging plate reader (FLIPR) calcium kit assay buffer (Molecular Devices, Sunnyvale, CA) containing 2.5 mM final probenecid and incubated at 37°C for 1 h in 6% CO_2 atmosphere. The fluorescence emission caused by intracellular calcium mobilization elicited by agonists of the expressed receptor was determined with a FLIPR (Molecular Devices, Sunnyvale, CA). Compounds were added to the assay after 10 s.

Results

Identification and Cloning of a Novel Human MCH-Receptor. As a part of our ongoing search for novel GPCRs, we queried the GenBank database (HTGS) with known GPCR sequences. An unfinished human genomic sequence (accession number AC027643) was identified showing 35% identity with the human SLC-1 receptor. Although this genomic sequence was unordered, a virtual 789-bp open reading frame encoding a 263-amino-acid protein was assembled using the Fgene software. This sequence was used to design primers based upon the predicted start and stop codons. A PCR experiment performed on human brain cDNA amplified a 912-bp fragment. Sequence analysis of the cloned fragment confirmed its identity to the predicted transcript, except for an additional 123-bp in-frame insertion. This cDNA sequence, designated S643a, indicated an open reading frame encoding 304 amino acids (Fig. 1). The hydrophilicity profile of the predicted protein indicated the presence of seven hydrophobic regions, consistent with a seven-transmembrane structure typical of G-protein-coupled receptors.

Comparison of the S643a-amino acid sequence with that of the hSLC-1 receptor revealed a significant difference in the length of the two receptors. As shown in Fig. 1, the terminal extracellular part of the SLC-1 receptor is longer by 50 amino acids, suggesting that the S643a sequence might be incomplete. To test this hypothesis, 5′ RACE was conducted using a human brain cDNA library as a template. PCR was performed with the forward PE1 primer based on the sequence of the pEXP1 vector and a reverse primer based on the S643a sequence. The resulting PCR products were cloned and sequenced. Unexpectedly, all the isolated clones displayed a 3′ end identical to the S643a sequence but showed a divergent 5′ end (Fig. 2). This is of particular interest because it suggests that alternative splicing of the S643 gene can give rise to the expression of two splice variants with different N-terminal regions.

PCR primers were designed based upon the new predicted open reading frame and a cDNA, designated S643b, was subsequently obtained. As shown in Fig. 1, the 1023-bp ORF encodes a 340-amino-acid protein. The nucleotide sequence surrounding the initiation codon agrees well with Kozak's consensus sequence for optimal translation initiation (Kozak, 1981). Moreover, the DNA sequence of the 5′-UTR shows an in-frame stop codon upstream of the predicted translation initiation methionine, suggesting that the proper start codon was identified. The S643b putative protein contains several features common to most members of the GPCRs superfamily, including seven putative transmembrane domains, two putative *N*-linked glycosylation sites in the N-terminal region, a DRY motif located at the end of the TM3 (Asp 111-Tyr 113), an aspartate residue (Asp 94) in TM3, a NPXXXY motif in TM7, and a potential palmitoylation site in the C-terminal region. The comparison of the amino acid sequence with known GPCRs revealed that S643b showed the greatest overall identity (32%) with the human SLC-1 receptor.

Functional Characterization of S643a/b Receptors. To determine whether S643a and S643b were novel MCH receptors, we investigated their ability to mediate an increase in intracellular Ca^{2+} when stimulated by MCH. Transiently transfected HEK-293 cells expressing either the S643a or the S643b receptor were incubated with Fluo-3 AM and challenged with MCH using a FLIPR. As shown in Fig. 3, cells expressing the S643b receptor responded to MCH with a robust, dose-dependent, transient elevation of intracellular Ca^{2+}, with an EC_{50} value of 5 nM. In contrast, cells expressing S643a did not induce an intracellular Ca^{2+} mobilization by MCH. No response was detected in untransfected cells (data not shown). To confirm the specific activation of the S643b receptor by MCH, a number of other peptides, including α-melanocyte stimulating hormone, somatostatin-14, somatostatin-28, rat atrial natriuretic peptide (ANP) (1–28),

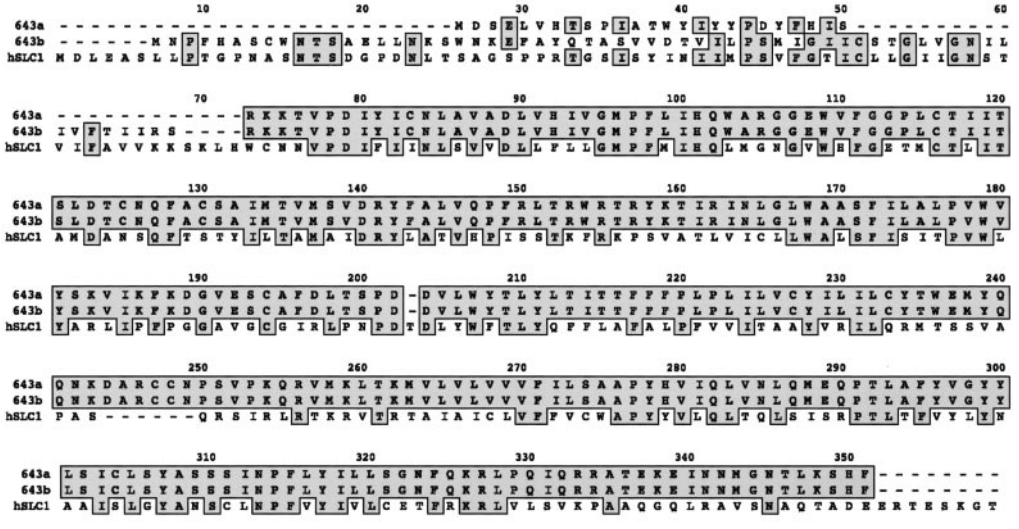


Fig. 1. Amino acid alignments of the human S643a, S643b, and SLC-1 receptors. The deduced amino acid sequence of S643a and S643b were aligned to the human SLC-1 receptor. Hyphens represent added spaces necessary for proper alignment. Shaded residues represent identical amino acid among the receptors. Numbers correspond to amino acid positions in the alignment.

and human ANP (3–28), were tested at concentrations up to 10 μM (Burgaud et al., 1997). Cells expressing the S643b receptor did not respond to any of these peptides (data not shown). Taken together, these results demonstrated that the S643b sequence encodes a new MCH receptor.

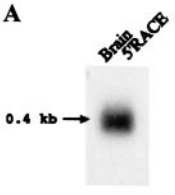
Gene Structure and Chromosome Mapping of the Human S643-Receptor Gene. The chromosomal localization of human S643-receptor gene was assessed using the GeneBridge 4 human-hamster radiation hybrid panel. The cell hybrid clones were screened for the presence or absence of a PCR-amplifiable marker for the S643 receptor locus. The radiation hybrid analyses indicated that the S643-receptor gene (using the GeneBridge 4 panel, data vector: 00100001011010000100010001000100011000001110001000101101000001101000101111101110001000100 1001) was located between the two markers CHLC.GATA5C03.939 and D6S424 on chromosome 6q14.3-q15.

The schematic organization of the S643-receptor gene deduced from sequence comparison between the S643a and S643b cDNAs and the human genomic sequence AC027643 is shown in Fig. 4. This gene spans more than 23 kb and contains six exons and five introns. Analysis of the exon/intron boundaries revealed that consensus splice donor and acceptor sequences were found at all splice donor and acceptor sites in the S643-receptor gene. Exon 1B encodes the 5′ end of the S643b-receptor coding region (amino acids 1–60), whereas exon 1A encodes the 5′end of the S643a coding region. Furthermore, four additional exons encode regions encompassing amino acids 61 to 130, 131 to 196, 197 to 236, and 237 to 340 of the S643b putative protein. The sizes of introns were estimated based on the genomic sequence: 718 bp for intron I, 7203 bp for intron II, 695 bp for intron IV, and 9469 bp for intron V. The size of intron III could not be predicted because the genomic sequence was unfinished.

Distribution of the S643b-Receptor mRNA. The distribution and expression level of the human S643b-receptor mRNA was first determined by Northern blot analysis using the S643b-cDNA coding region as a probe. As shown in Fig. 5A, a single 4.0-kb band was detected in the brain. A smaller 1.0-kb band was also detected in the placenta. No expression was seen in other peripheral tissues, even after extended exposure. To further analyze the distribution of the S643b mRNA, expression of this transcript was examined in several human brain regions and peripheral tissues using RT-PCR. The primer set, namely 643Hb and 643E, allowed the amplification of a PCR product with the expected size of 559 bp (Fig. 5b). Southern blotting with an internal oligonucleotide probe indicated the authenticity of the amplicons. In human brain, S643b-receptor mRNA expression was observed in hippocampus, caudate nucleus, and amygdala. No signal could be detected in cerebellum, thalamus, or hypothalamus. Aside from the expression in central nervous system structures, S643b-receptor mRNA was detected in small intestine, but not in other peripheral tissues such as heart, skeletal muscle, colon, thymus, spleen, kidney, liver, or lung (not shown). No signal was observed when either mRNA or reverse transcriptase was omitted from the first-strand cDNA conversion, which demonstrated that the signals observed were not caused by contaminating genomic DNA. A weakly hybridizing band with an estimated size of 400 bp was also detected in hippocampus, caudate nucleus, and amygdala. This PCR product could not be further characterized because we were unable to clone it.

Comparative Pharmacology between the SLC-1 and S643b Receptors. To analyze the structural requirements of MCH for the SCL1 and S643b-receptor activation, several MCH derivatives (Table 1) were tested for their ability to mobilize intracellular Ca^{2+} in CHO cells stably expressing either the SLC-1 or S643b receptors. MCH was able to activate both receptors with EC_{50} values of 1.8 nM and 9.5 nM, respectively (Table 2). [Phe^{13},Tyr^{19}]-MCH and salmon MCH were as potent ligands as the native MCH at both receptors. The linear MCH analog (compound C1), in which the cystine was replaced by two serine residues, was 170-fold less efficient at SLC-1 receptor (EC_{50} = 313 nM) compared with the native MCH and was inactive at the S643b receptor even at micromolar concentrations. The dodecapeptide MCH_{6-17} was 4-fold more potent than native MCH at the S643b receptor (EC_{50} = 2.3 nM) whereas this MCH derivative was equipotent to the native peptide at the SLC-1 receptor.

Because MCH_{6-17} has been described as the minimal MCH structure retaining potent activity at the SLC-1 receptor (Audinot et al., 2001a), we then tested a group of MCH_{6-17} derivatives in which single amino acids were modified by



B

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643b   -99 ggatagacctgcagtcccgcagagaggaagggcgcccgggctgtccgccgcgcggggaggacgcgagcacccagctttaatccctggaaagtccacgaaca   0
643b     1 ATGAATCCATTTCATGCATCTTGTTGGAACACCTCTGCCGAACTTTTAAACAAATCCTGGAATAAAGAGTTTGCTTATCAAACTGCCAGTGTGGTGGATA  100

643b   101 CAGTCATCCTCCCTTCCATGATTGGGATTATCTGTTCAACAGGGCTGGTTGGCAACATCCTCATTGTATTCACTATAATAAGATCCAGGAAAAAAACAGT  200
643a     1            ATGGATTCAGAATTAGTGCACACTAGCCCTATAGCTACTTGGTATATTTACTATCCTGATTATTTTCACATATCCAGGAAAAAAACAGT   89
                      * **  *** *     **   *   *  ***  **** *   *    **  *  * * * * *  ******************

643b   201 CCCTGACATCTATATCTGCAACCTGGCTGTGGCTGATTTGGTCCACATAGTTGGAATGCCTTTTCTTATTCACCAATGGGCCCGAGGGGGAGAGTGGGTG  300
643a    90 CCCTGACATCTATATCTGCAACCTGGCTGTGGCTGATTTGGTCCACATAGTTGGAATGCCTTTTCTTATTCACCAATGGGCCCGAGGGGGAGAGTGGGTG  189
           ****************************************************************************************************
                                      <-------- Primer 643R
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Fig. 2. Analysis of the 5′end region of the S643a and S643b cDNAs. A, PCR product analysis. The 5′RACE experiment was performed with primers PE1 and 643R and a human brain cDNA library as described under *Experimental Procedures*. The PCR products were run on a 1% agarose gel, transferred onto Hybond N^+ membrane, and hybridized with an internal specific ^{32}P-labeled oligonucleotide probe. B, nucleotide sequence alignment of the 5′end region of S643a and S643b cDNAs. 5′ Untranslated region appears in lower case and coding region in upper case letters. An in-frame stop codon (TAA) located beyond the predicted translation initiation methionine of S643b sequence is indicated in bold. The reverse primer used in 5′RACE experiment is indicated by an arrow.

Ala-scanning. The potency of the peptide with Met^8 substitution (C2) was reduced 700-fold at the SLC-1 receptor (EC_{50} = 1280 nM) compared with the parent compound MCH_{6-17} (EC_{50} = 2.1 nM). However, this peptide displayed only a 40-fold decrease in its ability to stimulate Ca^{2+} response at the S643b receptor (EC_{50} = 96.5 nM) compared with the parent compound. Compounds obtained by substitution of Val^{12} (C3) or Arg^{14} (C5) kept their ability to mediate Ca^{2+} response at both receptors, but their potencies were reduced 3- to 10-fold at the SLC-1 receptor and 10- to 30-fold at the S643b receptor compared with the parent compound. Peptides with Arg^{11} or Tyr^{13} substitution were unable to induce Ca^{2+} mobilization through the S643b receptor at concentrations up to 10 μM. Finally, peptides with deletion(s) within the cystine loop (C7, C8) did not produce any measurable Ca^{2+} response even at concentrations up to 10 μM.

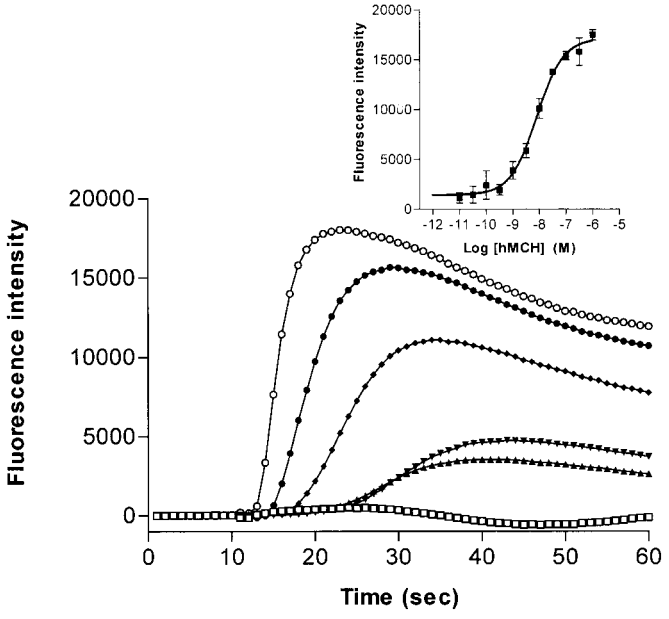

Fig. 3. Intracellular Ca^{2+} mobilization induced by MCH in cells expressing the S643b receptor. HEK-293 cells transiently transfected with S643b cDNA were loaded with Ca^{2+}-sensitive dye Fluo 3-AM, and the changes in fluorescence in response to a range of MCH concentrations (□, 0.01 nM; ▲, 0.1 nM; ▼, 1 nM; ♦, 10 nM; ●, 100 nM; and ○, 10,000 nM) were monitored in a FLIPR as described under *Experimental Procedures*. The data are expressed as arbitrary fluorescence units normalized to the initial baseline level. Inset, HEK-293 cells transiently transfected with S643b cDNA responded in a dose-dependent fashion to MCH with robust increases in intracellular calcium. The intracellular Ca^{2+} response was measured from the maximum fluorescence intensity after the addition of hMCH (maximum response − basal response). The data shown are from three independent experiments performed in triplicate.

Discussion

Using a genomics-based approach, we have identified and cloned a novel MCH receptor designated S643b. The S643-receptor gene has been mapped by radiation hybrid experiments to chromosome 6q14.3-q15. Its deduced protein sequence is encoded by five exons. The S643b receptor displays the greatest overall identity with the SLC-1 receptor (32%) and to a lesser extent to the somatostatin receptor subtypes.

MCH induced a strong, dose-dependent (EC_{50} = 9.5 nM), transient elevation of intracellular calcium in HEK293 cells transiently transfected with S643b receptor. To confirm the specificity of S643b activation by MCH, we tested a variety of representative peptides, including somatostatin and natriuretic peptides, and found them to be inactive. Cell lines expressing endogenous MCH binding sites, different from the SLC-1 receptor, have been described previously by different groups (Drozdz et al., 1995; Burgaud et al., 1997). The binding of [Phe^{13},Tyr^{19}]-MCH to these cells was weakly displaced by a number of natriuretic peptides. These peptides were found to have no functional activity at the S643b receptor, suggesting the existence, in these cell lines, of an other subtype of MCH receptor different from SLC-1 and S643b receptors.

To compare the pharmacological profiles of SLC-1 and S643b receptors and to assess the structural features of MCH required for S643b receptor activation, 11 MCH derivatives (Audinot et al., 2001a) were tested for their ability to mobilize intracellular Ca^{2+} in CHO cells stably expressing either SLC-1 or S643b receptors. According to their potencies, these peptides could be classified into three groups: highly active, moderately active, or inactive compounds. The first set of compounds, including MCH, [Phe^{13},Tyr^{19}]-MCH, salmon MCH, and the dodecapeptide MCH_{6-17}, were full agonists at both receptors. Although MCH, [Phe^{13},Tyr^{19}]-MCH, and salmon MCH were equipotent at both receptors, MCH_{6-17} behaved differently, because it was 4-fold more potent than native MCH at the S643b receptor and equipotent to the native peptide at the SLC-1 receptor. Recently, an extensive structure-activity relationships study was performed at the SLC-1 receptor, demonstrating that MCH_{6-17} was the minimal MCH structure retaining potent activity at the SLC-1 receptor (Audinot et all. 2001a). A second group of active peptides included compounds with high to moderate potency at both receptors. Val^{12} or Arg^{14} (compounds C3 and C5) did not seem to be essential because substitution of these amino acids by Ala only decreased their potency 3- to 30-fold at both receptors compared with MCH_{6-17}. In contrast, the substitution of Met^8 by Ala (C2) seemed to be more deleterious at the

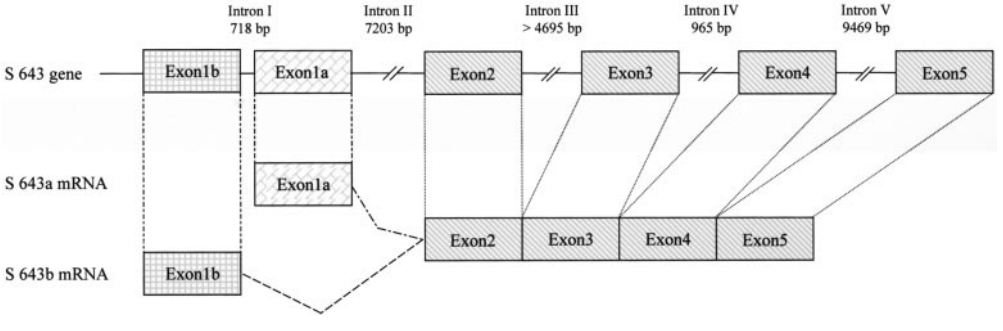

Fig. 4. Schematic organization of the S643-receptor gene. Schematic organization of the S643-receptor gene was deduced from sequence comparison between S643a and S643b cDNAs and the human genomic sequence AC027643. Exons (E_{1-5}) are shown by boxes and introns (I_{1-5}) are shown by horizontal lines. The sizes of introns are estimated based on the genomic sequence.

SLC-1 than at S643b receptor because the potency of this compound was decreased 700- and 40-fold, respectively. Amino acid replacement at this position should allow the design of new peptides with increased specificity for S643b receptor versus SLC-1. Finally, compounds of the third group, including peptides with Arg^{11} (C6), Tyr^{13} (C4) substitution or deletion inside the loop (C7 and C8) were inactive as agonists or antagonists (data not shown) at both receptors,

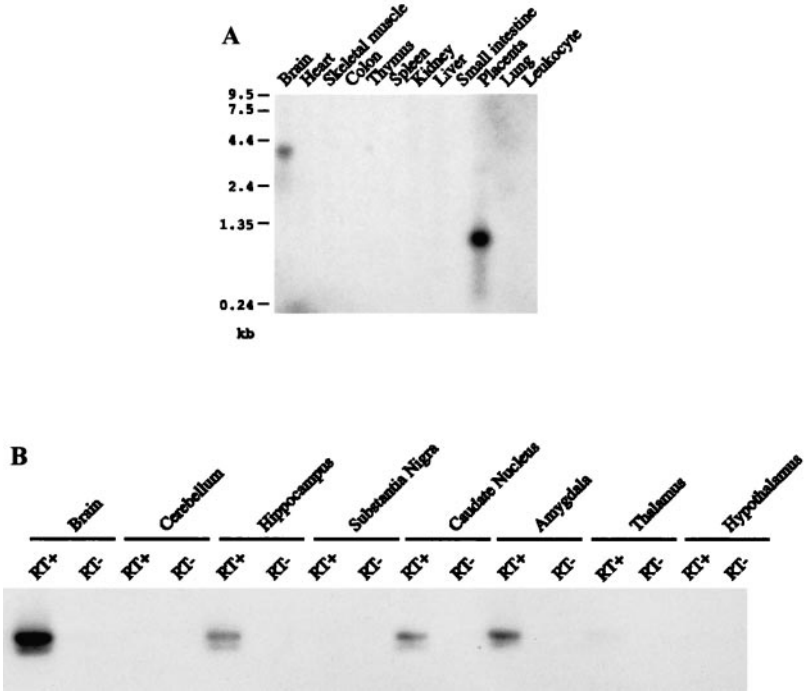

Fig. 5. Tissue distribution of the S643b mRNA in human tissues. A, Northern blot analysis. A multiple-tissue Northern (MTN) blot membrane (CLONTECH), containing 2 μg of poly(A^+) RNA per lane, was hybridized with a probe corresponding to the entire coding region of the S643b-receptor cDNA. B, detection of the S643b mRNA by RT-PCR. cDNAs from different brain regions were used as templates for PCR amplification with primers 643bH and 643E. The PCR products were run on a 1% agarose gel, transferred onto Hybond N^+ membrane, and hybridized with a specific ^{32}P-labeled oligonucleotide probe.

TABLE 1

Structure of MCH analogs

All compounds except C1 are cyclic peptides between Cys^7 and Cys^{16}: Substituted residues are in **bold.**

Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
MCH[a]	Asp	Phe	Asp	Met	Leu	Arg	Cys	Met	Leu	Gly	Arg	Val	Tyr	Arg	Pro	Cys	Trp	Gln	Val
salmon MCH[a]			Asp	**Thr**	**Met**	Arg	Cys	Met	**Val**	Gly	Arg	Val	Tyr	Arg	Pro	Cys	Trp	**Glu**	Val
[Phe^{13},Tyr^{19}]-MCH[a]	Asp	Phe	Asp	Met	Leu	Arg	Cys	Met	Leu	Gly	Arg	Val	**Phe**	Arg	Pro	Cys	Trp	Gln	Tyr
C1	Asp	Phe	Asp	Met	Leu	Arg	Ser	Met	Leu	Gly	Arg	Val	Tyr	Arg	Pro	Ser	Trp	Gln	Val
MCH_{6-17}						Arg	Cys	Met	Leu	Gly	Arg	Val	Tyr	Arg	Pro	Cys	Trp		
C2						Arg	Cys	Ala	Leu	Gly	Arg	Val	Tyr	Arg	Pro	Cys	Trp		
C3						Arg	Cys	Met	Leu	Gly	Arg	Ala	Tyr	Arg	Pro	Cys	Trp		
C4						Arg	Cys	Met	Leu	Gly	Arg	Val	Ala	Arg	Pro	Cys	Trp		
C5						Arg	Cys	Met	Leu	Gly	Arg	Val	Tyr	Ala	Pro	Cys	Trp		
C6						Arg	Cys	Met	Leu	Gly	His	Val	Tyr	Arg	Pro	Cys	Trp		
C7						Arg	Cys				Arg	Val	Tyr	Arg	Pro	Cys	Trp		
C8						Arg	Cys	Met	Leu		Arg	Val	Tyr	Arg	Pro	Cys	Trp		

TABLE 2

Activation of intracellular calcium mobilization by CHO cells expressing either SLC-1 or S643b receptors

Changes in intracellular Ca^{2+} mobilization were measured in intact CHO cells, expressing either SLC-1 or S643b receptor, loaded with Fluo 3 as described under *Experimental Procedures.* Maximum responses produced by peptides are expressed as percent of the maximum response produced by r, h, m MCH. Potencies are given as means of EC_{50} values in nanomolar ± S.E.M. obtained from at least three separate concentration-response curves.

Compound	SLC-1		S643b	
	EC_{50}	Ca^{2+} Response	EC_{50}	Ca^{2+} Response
	nM	%	*nM*	%
r, h, m MCH	1.8 ± 0.8	100	9.5 ± 2.2	100
Salmon MCH	3.2 ± 0.7	100	15.2 ± 0.6	100
[Phe^{13},Tyr^{19}]-MCH	3.2 ± 0.7	100	11.7 ± 1.7	97
MCH_{6-17}	2.1 ± 0.1	100	2.3 ± 0.8	91
C1	313 ± 43.6	85	>10,000	0
C2	1,280 ± 150	50	96.5 ± 19.6	81
C3	20.7 ± 4.7	95	23.7 ± 0.1	59
C4	>10,000	0	>10,000	0
C5	7.3 ± 0.1	94	78.2 ± 11.6	70
C6	>10,000	0	>10,000	0
C7	>10,000	0	>10,000	0
C8	>10,000	0	>10,000	0

even at micromolar concentrations. In a previous study, Macdonald et al. (2000) reported that both Arg^{11} in MCH and Asp^{123} in the SLC-1 receptor were required for agonist-mediated receptor activation. Interestingly, the Asp residue is also conserved in S643b receptor (Asp^{113}), suggesting that it may have a similar function in both MCH receptors. Alanine substitution of the Tyr^{13} led to the loss of agonistic activity, whereas substitution by phenylalanine maintained the agonistic activity of the peptide (see [Phe^{13},Tyr^{19}]-MCH). These results demonstrated the importance of the phenolic structure for the activation of both MCH receptors. Deletions inside the cyclic structure led to inactive compounds, suggesting that the size of the loop was also crucial for agonistic activity at both receptors. The linear MCH analog (compound C1), in which the cystine had been replaced by two serine residues, was inactive at S643b receptor, suggesting the importance of the ring structure for agonistic activity. Taken together, these data clearly demonstrated that Met^{8}, Arg^{11}, Tyr^{13}, and the disulfide ring are crucial structural requirements that enable MCH derivatives to behave as agonists at this new MCH receptor.

To further study the functional implications of the S643b receptor, we investigated its distribution in various human tissues. Our results indicated that this receptor is expressed predominantly in the brain. The corresponding mRNA was found in hippocampus, amygdala, and caudate nucleus, indicating that the S643b receptor may be functionally related to the action of MCH in cognition, learning (McBride et al., 1994), and anxiety (Gonzalez et al., 1996). In contrast to SLC-1, no expression was detected in hypothalamus, cerebellum, or substantia nigra. However, within these tissues, expression of S643b might be restricted to minor populations of cells. Thus, further studies, such as in situ hybridization, will be needed to examine S643b distribution in detail. Although predominantly expressed in brain, the S643b transcript was also found in intestine. Expression of MCH mRNA and peptide itself have also been localized to discrete areas of the rat digestive tract (Hervieu and Nahon, 1995), suggesting that S643b might be involved in the regulation of nutritional homeostasis by MCH.

During our attempts to clone the S643b receptor, a splice variant form of this receptor was isolated. RT-PCR experiments (results not shown) indicated that the S643a transcript was expressed at a very low level in human brain only. In addition, S643a transiently expressed in HEK293 cells did not mediate intracellular calcium mobilization when stimulated by MCH. These data suggested either that this splice variant was an inappropriately spliced transcript or that the N-terminal part of the receptor was missing and therefore prevented MCH activation of the S643a receptor. These two hypotheses are currently under investigation in our laboratory. As stated earlier, two other reports described the discovery of a new MCH receptor subtype (Hill et al., 2001; Mori et al., 2001), tentatively named MCH2, whose sequence is identical to that of S643b.

In conclusion, we have cloned and pharmacologically characterized a novel MCH receptor that has many properties in common with the SLC-1 receptor. There are also some differences between the two receptors in terms of their pharmacology and tissue distribution. In combination with SLC-1, S643b (MCH2) will provide further insights into the physiological implication of MCH in modulating brain functions and behaviors, including food intake.

References

Audinot V, Beauverger P, Lahaye C, Suply T, Rodriguez M, Ouvry C, Lamamy V, Imbert J, Rique H, Nahon JL, et al. (2001a) Structure-activity relationship studies of melanin-concentrating hormone (MCH)-related peptide ligands at SLC-1, the human MCH receptor. *J Biol Chem* **276:**13554–62.

Audinot V, Lahaye C, Suply T, Beauverger P, Rodriguez M, Galizzi JP, Fauchere JL, and Boutin JA (2001b) [I]-S36057 a new and highly potent radioligand for the melanin-concentrating hormone receptor. *Br J Pharmacol* **133:**371–378.

Bachner D, Kreinkamp H, Weise C, Buck F, and 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.

Bittencourt JC, Press F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W, and Sawchenko PE (1992) The melanin concentrating hormone system of the rat brain: an immuno and hybridization histochemical characterization. *J Comp Neurol* **319:**218–245.

Bradley RL, Kokkotou EG, Maratos-Flier E, and Cheatham B (2000) Melanin-concentrating hormone regulates leptin synthesis and secretion in rat adipocytes. *Diabetes* **49,** 1073–1077.

Burgaud JL, Poosti R, Fehrentz JA, Martinez J, and Nahon JL (1997) Melanin-concentrating hormone binding sites in human SVK14 keratinocytes. *Biochem Biophys Res Commun* **241:**622–629.

Chambers J, Ames RB, Bergsma D, Muir A, Fitzgerald LR, Hervieu G, Dytko GM, Foley JJ, Martin J, Liu WS, et al. (1999) Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature (Lond)* **400:**261–265.

Drozdz R, Siegrist W, Baker BI, Chluba-De Tapia J, and Eberle AN (1995) Melanin-concentrating hormone binding to mouse melanoma cells in vitro. *FEBS Lett* **359:**199–202.

Gonzalez MI, Vaziri S, and Wilson CA (1996) Behavioral effects of alpha-MSH and MCH after central administration in the female rat. *Peptides* **17:**171–177.

Hervieu G and Nahon JL (1995) Pro-melanin concentrating hormone messenger ribonucleic acid and peptide expression in peripheral tissues of the rat. *Neuroendocrinology* **61:**348–364.

Hervieu GJ, Cluderay JE, Harrisson D, Meakin J, Maycox P, Nasir S, and Leslie RA (2000) The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, slc-1, in the central nervous system of the rat. *Eur J Neurosci* **12:**1194–216.

Hill J, Duckworth M, Murdock P, Rennie G, Sabido-David C, Ames RS, Szekeres P, Wilson S, Bergsma DJ, Gloger IS, et al. (2001) Molecular cloning and functional characterization of hMCH2, a novel human MCH receptor. *J Biol Chem* **276:** 20125–20129.

Jezova D, Bartanutsz V, Westergren I, Johansson B, Rivier J, Vale W, and Rivier C (1992) Rat melanin-concentrating hormone stimulates adrenocorticotropin secretion: evidence for a site of action in brain regions protected by the blood brain barrier. *Endocrinology* **130:**1021–1029.

Kozak M (1981) Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. *Nucleic Acids Res* **9:**5233–5262

Lembo PM, Grazzini E, Cao J, Hubatsch DA, Pelletier M, Hoffert C, St-Onge S, Pou C, Labrecque J, Groblewski T, et al. (1999) The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. *Nat Cell Biol* **1:**267–271.

Ludwig DS, Mountjoy KG, Tatro JB, Gillette JA, Frederich RC, Flier JS, and Maratos-Flier E (1998) Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *Am J Physiol* **274:**E627–E633.

Macdonald D, Murgolo N, Zhang R, Durkin JP, Yao X, Strader CD, and Graziano MP (2000) Molecular characterization of the melanin-concentrating hormone/receptor complex: identification of critical residues involved in binding and activation. *Mol Pharmacol* **58:**217–225.

McBride RB, Beckwith BE, Swenson RR, Sawyer TK, Hadley ME, Matsunaga TO, and Hruby VJ (1994) The actions of melanin-concentrating hormone on passive avoidance in rats: a preliminary study. *Peptides* **15:**757–759.

Miller CL, Hruby VJ, Matsunaga TO, and Bickford PC (1993) alpha MSH and MCH are functional antagonists in a CNS auditory gating paradigm. *Peptides* **14:**431–440.

Mori M, Harada M, Terao Y, Sugo T, Watanabe T, Shimomura Y, Abe M, Shintani Y, Onda H, Nishimura O, et al. (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.

Qu D, Ludwig DS, Gammeloft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, and Maratos-Flier E (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature (Lond)* **380:**243–247.

Rossi M, Choi SJ, O'Shea D, Miyoshi T, Gathei MA, and Bloom SR (1997) Melanin concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. *Endocrinology* **138:**351–355.

Saito Y, Nothacker HP, and Civelli O (2000) Melanin-concentrating-hormone receptor an orphan receptor fits the key. *Trends Endocrinol Metab* **11:**299–303.

Saito Y, Nothacker HP, Wang Z, Lin SH, Leslie F, and Civelli O (1999) Molecular characterization of the melanin-concentrating-hormone receptor. *Nature (Lond)* **400:**265–269.

Salamov AA, and Solovye VV (2000) Ab initio gene finding in Drosophila genomic DNA. *Genome Res* **10:**516–522.

Sanchez M, Baker BI, and Celis M (1997) Melanin-concentrating hormone (MCH) antagonizes the effects of alpha-MSH and neuropeptide E-I on grooming and locomotor activities in the rat. *Peptides* **18:**393–396.

Shimada M, Tritos NA, Lowel B, Flier JS, and Maratos-Flier E (1998) Mice lacking

melanin-concentrating hormone are hypophagic and lean. *Nature (Lond)* **380:**243–247.
Shimomura Y, Mori M, Sugo T, Ishibashi Y, Abe M, Kurokawa T, Onda H, Nishimura O, Sumino Y, and 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.
Suply T, Della Zuana O, Audinot V, Rodriguez M, Beauverger P, Duhault J, Canet E, Galizzi JP, Nahon JL, Levens L, et al. (2001) SLC-1 receptor mediates the effect of melanin concentrating hormone on feeding behavior in the rat: a structure activity study. *J Pharmacol Exp Ther* **299**, In press.
Tadayyon M, Welters HJ, Haynes AC, Cluderay JE, and Hervieu G (2000) Expression of melanin-concentrating hormone receptors in insulin-producing cells: MCH stimulates insulin release in RINm5F and CRI-G1 cell-lines. *Biochem Biophys Res Commun* **275:**709–712.
Tsukamura H, Thompson RC, Tsukahara S, Ohkura S, Maekawa F, Moriyama R, Niwa Y, Foster DL, and Maeda K (2000) Intracerebroventricular administration of melanin-concentrating hormone suppresses pulsatile luteinizing hormone release in the female rat. *J Neuroendocrinol* **12:**529–534.

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