

SLC-1 Receptor Mediates Effect of Melanin-Concentrating Hormone on Feeding Behavior in Rat: A Structure-Activity Study

THOMAS SUPPLY, ODILE DELLA ZUANA, VALÉRIE AUDINOT, MARIANNE RODRIGUEZ, PHILIPPE BEAUVERGER, JACQUES DUHAULT, EMMANUEL CANET, JEAN-PIERRE GALIZZI, JEAN-LOUIS NAHON, NIGEL LEVENS, and JEAN A. BOUTIN

Division de Pharmacologie Moleculaire et Cellulaire, Institut de Recherches Servier, Croissy/Seine, France (T.S., V.A., M.R., P.B., E.C., J.-P.G., J.A.B.); Division Métabolisme, Institut de Recherches Servier, Suresnes, France (O.D.Z., J.D., N.L.); and Institut de Pharmacologie Moleculaire et Cellulaire, Unité Mixte de Recherche Center National de la Recherche Scientifique, Valbonne-Sophia-Antipolis, France (T.S., J.-L.N.)

Received March 29, 2001; accepted June 4, 2001 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Several studies have shown that melanin-concentrating hormone (MCH) is an orexigenic peptide in rat. In the present study, a structure-activity relationship with MCH analogs was performed in rat, both in vitro and in vivo. On rat recombinant SLC-1 receptor, both cAMP inhibition and [¹²⁵I]S36057 binding were measured. In vivo, these analogs were injected intracerebroventricularly in rats and their effects were evaluated upon food intake. First, data obtained with the rat recombinant receptor were highly correlated with those obtained from its human counterpart. Second, agonist

potencies in the cAMP assay were also highly correlated with binding affinities. These peptides could be classified into several groups according to their potency at the SLC-1 receptor (from subnanomolar activity to complete inactivity). Indeed, there was a strong correlation between their effects upon food intake and the results obtained at the rat SLC-1 receptor. The present report describes for the first time the rat SLC-1 receptor pharmacology and clearly establishes the relevance of the SLC-1 receptor in feeding behavior.

The rat/human melanin-concentrating hormone (MCH) consists of a 19 amino acid sequence presenting a high homology with the fish peptide originally described (Kawauchi et al., 1983; Vaughan et al., 1989; Nahon, 1994). Immunolocalization and/or in situ hybridization studies in rat have revealed the existence of MCH in the perikarya of lateral hypothalamus and the zona incerta, and fibers innervating the entire brain (Bittencourt et al., 1992). MCH mRNA and hormone precursor expression were also found in peripheral tissues (Hervieu and Nahon, 1995; Hervieu et al., 1996a,b), suggesting implication of this peptide in various physiological functions (Nahon, 1994). In the rat brain, MCH is expressed in the lateral hypothalamus, an area playing a central role in the control of energy homeostasis, feeding behavior, and body weight. Indeed, hypothalamic MCH messenger expression is increased by fasting in wild-type as well as in *ob/ob* mice, and intracerebroventricular injection of MCH stimulates food intake in rats (Qu et al., 1996; Rossi et al., 1997, 1999; Sahu, 1998; Ludwig et al., 1998) and mice (O. Della Zuana, unpublished data). In addition, transgenic mice

that overexpress the MCH peptide are obese and developed particularly marked hyperphagia in response to high fat diet (Ludwig et al., 2001). Importantly, and, contrasting with other orexigenic factors such as neuropeptide Y or orexin (Erickson et al., 1996; Chemelli et al., 1999), MCH is the only peptide for which gene disruption results in hypophagic and lean mice (Shimada et al., 1998). There is thus a large body of evidence suggesting a critical role for MCH in food intake behavior and metabolism control in rodents (Tritos et al., 1998; Tritos and Maratos-Flier, 1999).

Most of the MCH functions have been investigated in the absence of information concerning its receptor (for review, see Nahon, 1994; Baker, 1994). Indeed, the MCH receptor has only been recently identified (Chambers et al., 1999; Lembo et al., 1999; Saito et al., 1999; for review, see Saito et al., 2000) through reverse pharmacology as the SLC-1 orphan G protein-coupled receptor (Kolakowski et al., 1996; Lakaye et al., 1998). This receptor is strongly and widely expressed in the rat brain and its distribution is in good agreement with that of the MCH peptide (Bittencourt et al., 1992; Hervieu et al., 2000). To characterize the MCH activity at its receptor, several structure-activity relationship studies based on salmon MCH analogs had been performed in the

T.S. was a recipient of a Convention CIFRE between the Association Nationale de la Recherche Technique, the Institut de Recherche SERVIER, and the Center National de la Recherche Scientifique.

ABBREVIATIONS: MCH, melanin-concentrating hormone; PCR, polymerase chain reaction; HEK, human embryonic kidney; CSF, cerebrospinal fluid; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance.

TABLE 1

Structure of MCH analogs

All compounds except C1 and S36077 are cyclic peptides between Cys⁷ and Cys¹⁶; 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
[F ¹³ ;Y 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		
S36077						Arg	Ser	Met	Leu	Gly	Arg	Val	Tyr	Arg	Pro	Ser	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	Met	Leu		Arg	Val	Tyr	Arg	Pro	Cys	Trp		
C8						Arg	Cys	Met	Leu		Arg	Val	Tyr	Arg	Pro	Cys	Trp		
S36057				Tyr(I)	ADO	Arg	Cys	Met	Leu	Gly	Arg	Val	Phe	Arg	Pro	Cys	Trp		

Tyr(I), 3-iodotyrosine; ADO, 8-amino-3,6-dioxo-octanoyl.

^a Commercially available.

past, using fish, reptilian, or batracian bioassays (Kawazoe et al., 1987; Wilkes et al., 1984; Matsunaga et al., 1989). Recently, the availability of, on one hand, the human cloned receptor, and, on the other, in vitro functional assays have allowed the description of a wide diversity of MCH analogs of various potencies (Audinot et al., 2001a). In this study, the MCH fragment MCH₆₋₁₇ (Table 1) was shown to be the minimal sequence keeping potent biological activity. Designed from this sequence, S36057 (Table 1) has been found to be the most powerful MCH agonist, which, as a radioligand, represents a more potent, less hydrophobic, and more stable tool than [¹²⁵I][3-iodo Tyr¹³]-MCH itself (Audinot et al., 2001b).

The aim of the present study was to investigate the implication of the SLC-1 receptor in mediating the effects of MCH upon food intake in rat. We thus selected MCH analogs and

tested them with rat recombinant SLC-1 receptor both for their functional activity (inhibition of intracellular cAMP) and their binding affinity using [¹²⁵I]S36057 as a radioligand. Furthermore, these analogs, injected i.c.v. in rats, were evaluated upon food intake. Indeed, there was a good correlation between their effects on feeding behavior and results obtained at the rat recombinant receptor, strongly suggesting that SLC-1 receptor mediates the central effects of MCH on food intake.

Experimental Procedures

Materials

Most of the natural and modified peptides used in these studies were custom-made by Neosystem SA (Strasbourg, France). They were all at least 95% pure as assessed by high-performance liquid

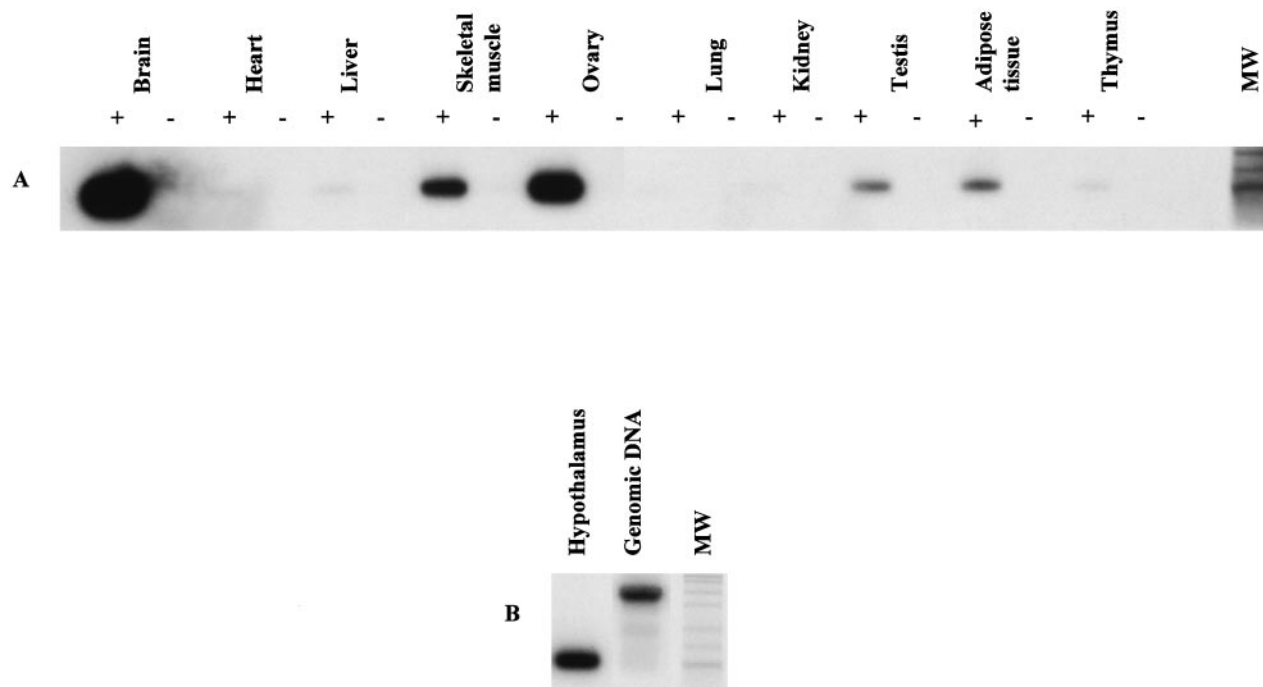


Fig. 1. Tissues distribution of SLC-1 receptor mRNA in the Sprague-Dawley rat. Analysis of total RNA from indicated rat tissues (A) and rat hypothalamus (B). Southern blot of RT-PCR amplicons from rat cDNA amplified with the 23 to 43 and 384 to 403 primers and revealed by the ³²P-labeled oligoprobe 214 to 236 (+). Negative reactions of RT-PCR in the absence of reverse transcriptase addition were also performed to test genomic DNA contamination (-). PCR product of genomic DNA obtained in the same conditions is larger than cDNA RT-PCR product.

chromatography and mass spectrometry (Audinot et al., 2001a). The amino acid structures of human, rat, mouse MCH, salmon MCH and MCH analogs used in these studies are shown in Table 1. Total RNA of heart, brain, lung, liver, and kidney obtained from a pool of Sprague-Dawley rats, as well as rat genomic DNA were obtained from CLONTECH (Palo Alto, CA). Total RNA of thymus, testis, and ovary were obtained from a pool of Sprague-Dawley rats, purchased from Ambion (Austin, TX).

Cloning of Rat SLC-1 Receptor

Poly(A⁺) RNA from a pool of rat hypothalami was reverse transcribed with oligo(dT)₁₂₋₁₈ using reverse Transcriptase Superscript II (Invitrogen, Cergy Pontoise, France). First-strand cDNA (corresponding to 1 µg of total RNA) was subjected to 35 cycles of amplification using primers based on GenBank entry (accession no. AF008650) describing the rat SLC-1 receptor (rSLC-1; forward primer 23–43 and the reverse primer 1065–1084). After an initial cycle of denaturation at 94°C for 1 min, PCR was carried out for 35 cycles with the following cycle conditions: 94°C, 1 min; 55°C, 1 min; and 72°C, 3 min with a postincubation of 72°C for 7 min. The expected 1061-base pair fragment was isolated and inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The recombinant plasmid pcDNA3.1-rSLC-11 was sequenced on both strands by automated sequencing (ABI 377; Applied Biosystems, Foster City, CA).

Tissue Expression of Rat SLC-1 Receptor Messenger RNA

First-strand cDNA from different rat tissues (corresponding to 1 µg of total RNA) was subjected to 30 cycles of amplification using the primers based on GenBank entries (accession no. AF008650) for the rat SLC-1 receptor (forward primer 23–43 and the reverse primer 384–403). PCR products were separated by agarose (1%) gel electrophoresis and transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech, Orsay, France). Hybridization was performed at 42°C using a ³²P-labeled oligoprobe (214–236).

Establishment of Stable Cell Line Expressing Rat SLC-1 Receptor

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. They were seeded at 5 × 10⁶ cells in a 75-cm² culture flask. Twenty-four hours later, the cells were transfected with 10 µg of the recombinant plasmid pcDNA3.1-rSLC-1, using LipofectAMINE as described by the manufacturer (Invitrogen). The day after transfection, cells were trypsinized, re-suspended in complete Dulbecco's modified Eagle's medium containing 800 µg/ml of geneticin, and seeded at different dilutions in 96-well plates, which were then kept for 2 to 3 weeks in a humidified CO₂ incubator. At the end of this selection period, isolated cellular clones were selected, amplified, and further characterized by cAMP experiments.

Intracellular cAMP Assay

Intracellular cAMP was measured using flashplates (SMP004; PerkinElmer Life Science Products, Boston, MA). In brief, forskolin (15 µM) and test peptides diluted in 0.1% bovine serum albumin were added to 96-well flashplates. Incubation was started with the addition of HEK293 cells stably expressing the rat SLC-1 receptor (35,000 cells/well). After 15 min at 37°C, the incubation was stopped by the addition of the revelation mix. Two hours later, plates were counted on a TopCount (Packard, Rungis, France).

Membrane Preparation

Cell lines stably expressing the rat SLC-1 receptor were grown to confluence, harvested in phosphate-buffered saline containing 2 mM EDTA, and centrifuged at 1000g for 5 min (4°C). The resulting pellet was suspended in 20 mM HEPES, pH 7.5, containing 5 mM EGTA

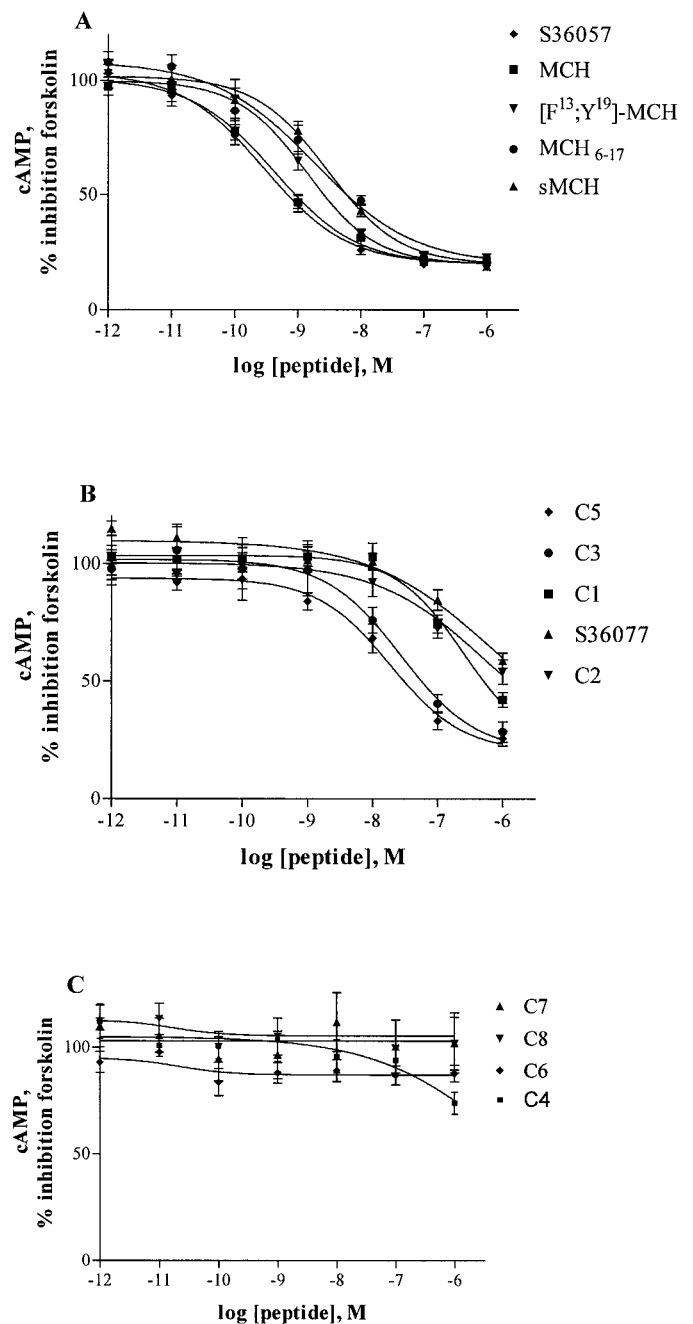


Fig. 2. Dose-dependent inhibition of forskolin-induced cAMP in HEK293 cells stably expressing the rat SLC-1 receptor. Effect of S36057, MCH, salmon MCH, linear MCH (C1), and minimal MCH agonist MCH₆₋₁₇ at rat recombinant SLC-1 receptor. Peptides were tested for their potency to inhibit the forskolin-elevated levels of cAMP in rSLC-1/HEK cells. Data were pooled from all the experiments and were expressed as means ± S.E.M.

and homogenized using a Kinematica Polytron. The homogenate was then centrifuged (95,000g, 30 min, 4°C) and the resulting pellet suspended in 50 mM HEPES, pH 7.5, 10 mM MgCl₂, and 2 mM EGTA. Determination of protein content was performed according to the method of Lowry et al. (1951). Aliquots of membrane preparations were stored at -80°C until use.

[¹²⁵I]S36057 Binding

Membranes (10–25 µg/ml) were incubated for 90 min at room temperature in binding buffer (25 mM HEPES, pH 7.4, 1 mM CaCl₂, 5 mM

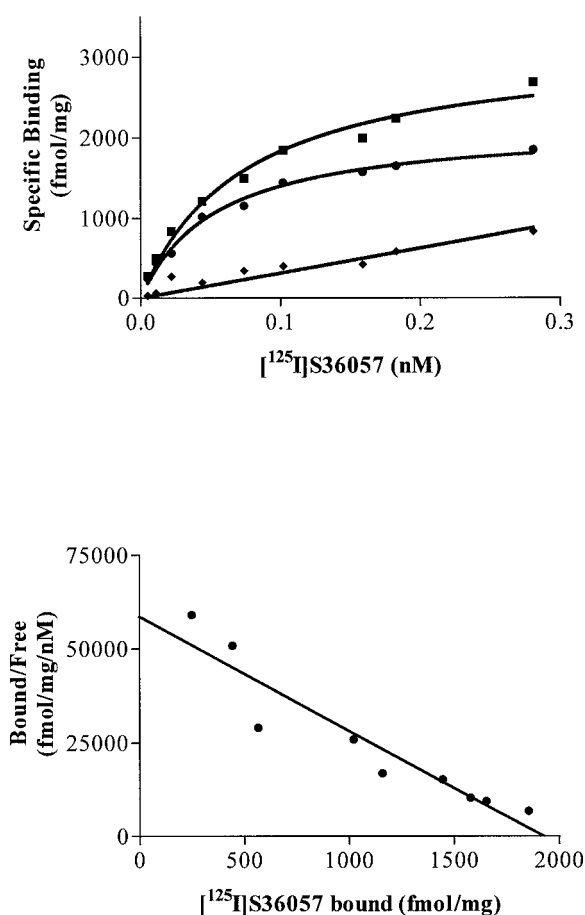


Fig. 3. Saturation binding experiments and Scatchard plot of the specific binding of $[^{125}\text{I}]\text{S36057}$ at membranes from HEK293 cells stably expressing the rat SLC-1 receptor. Saturation binding isotherm was obtained using $[^{125}\text{I}]\text{S36057}$ in the concentration range of 5 to 280 pM. A, total binding (■), nonspecific binding determined with 1 μM MCH (◆), and specific binding (●) are represented. B, Scatchard plot of the specific binding isotherm. Points shown are from a representative experiment performed in triplicate and repeated at least three times.

MgCl_2 , 0.5% bovine serum albumin) in a final volume of 250 μl containing, or where otherwise stated, 0.04 nM $[^{125}\text{I}]\text{S36057}$ (Amersham Pharmacia Biotech) and test peptides (Neosystem SA). Nonspecific binding was defined with 1 μM MCH. The reaction was stopped by rapid filtration through GF/B unifilters presoaked with 0.5% polyethyleneimine, followed by three successive washes with ice-cold buffer.

Central Peptide Administration

Animals. The feeding experiments in these studies were conducted using male Wistar rats weighing 325 to 350 g (Iffa Credo, L'arbresle, France). The animals were housed individually in wire-bottomed cages in a room with a 12-h light/dark cycle (lights on 7:30 AM) at $22 \pm 30^\circ\text{C}$ and 55% relative humidity. Six-millimeter-diameter food pellets of the following composition (67.5% food flour, 26.5% saccharose, 5% gum tragacanth, 1.25% magnesium stearate; A03 UAR, Orge, France) were available ad libitum. The pellets were available to the animals from a food hopper attached to the side of the cage. All animal procedures described in this study complied with French laws regulating animal experimentation (Decree no. 87-848 19 October 1987 and the ministerial Decree of 10 April 1988) and were also approved by the ethics committee of the Servier Research Institute.

Implantation of Intraventricular Cannulae. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.; Sanofi, Libourne, France) and a stainless steel guide cannulae (Plastic Products Co., Roanoke, VA) stereotaxically implanted into the right lateral ventri-

cle at the following coordinates relative to the bregma: AP, -0.8 mm; L, -1.2 mm; and V, -3.5 mm.

Peptide Injection. After a 7-day recovery period, during which the animals were handled every day to minimize nonspecific stress, they were lightly anesthetized with Forène (Abbott Laboratories, Queenborough, UK). MCH (4.4 nmol/kg; Bachem Biochimie SARL, Voisins-le-Bretonneux, France) was then slowly injected through the intraventricular cannulae over a 30-s period in a final volume of 5 μl . Immediately after injection, the animals quickly recovered and were returned to their home cages. Animals that exhibited a robust increase in food intake, i.e., indicating correct cannulae placement, were randomly assigned to different groups and studied in one of the following protocols.

Effect of Different Peptides on Spontaneous Food Intake. The injections were performed at 9:00 AM shortly after the beginning of the light phase. Each Wistar rat was injected i.c.v. either with artificial cerebrospinal fluid (aCSF, 5 μl) or with a single dose (1–10 nmol/kg in 5 μl) of peptide dissolved in aCSF. The animals were then returned to their home cage, which contained a known weight of food pellets in a spill-free cup. Measurements of food intake were made for 2 h after injection. For each animal, food spillage was quantified during each time period and food intake was corrected for this loss. At the end of the experiments, the animals were euthanized and the position of the cannulae assessed by the injection of 100 μl of Evans blue (2 mg/kg) followed by visual examination of the brain slices. Only data obtained from animals with correctly positioned cannulae were included in the final data analysis.

Data and Statistical Analysis

All statistical analyses were done with a 5% significance threshold. For in vitro data, IC_{50} and K_i were estimated on the overall values by nonlinear regression using the program PRISM (GraphPad Software Inc., San Diego, CA). For displacement experiments, inhibition constants (K_i) were calculated according to the Cheng-Prusoff equation: $K_i = \text{IC}_{50}/[1 + (L/K_D)]$, where IC_{50} is the inhibitory concentration₅₀, L is the concentration, and K_D the dissociation constant of the radioligand. Difference of potency between IC_{50} values or K_i values of two peptides can be statistically performed by comparing their confidence limits. For in vivo data, significance of effects was determined by a one way analysis of variance followed by a Newman-Keuls test for pairwise comparison.

Results

Expression of SLC-1 Receptor mRNA in Rat. We investigated the distribution of the rat SLC-1 receptor mRNA in several rat tissues using RT-PCR and Southern blotting (Fig. 1). By selecting the primers on either side of the splice junction, and performing a negative reaction consisting of RT-PCR without the addition of reverse transcriptase, it was possible to check that the samples were free of contamination with genomic DNA. A ^{32}P -labeled oligoprobe for the rat SLC-1 receptor transcript showed massive expression in brain, skeletal muscle, and ovary, and less expression in testis and adipose tissue (Fig. 1A). There was no detectable expression of the SLC-1 receptor mRNA in heart, liver, lung, kidney, and thymus. The hypothalamus also expressed mRNA for the SLC-1 receptor. This was not linked to genomic contamination, as attested by the higher molecular weight of the PCR product obtained with the rat genomic DNA (Fig. 1B).

Characterization of HEK293 Cell Line Stably Expressing Rat SLC-1 Receptor. To examine ligand binding properties and signal transduction through the rat SLC-1 receptor, the coding sequence was subcloned in the mammalian expression vector pcDNA3.1(+) and transfected into

TABLE 2

Characterization of MCH analogs in rat: in vitro potency and affinity at recombinant SLC-1 receptor and in vivo effects upon food intake

In vitro results were obtained from HEK293 cells stably expressing the rat SLC-1 receptor. IC₅₀ (inhibitory concentration₅₀) represents the concentration of peptide inducing 50% of inhibition of forskolin-induced intracellular cAMP level. K_i (inhibition constant) values from [¹²⁵I]S36057 binding experiments determined on membrane preparation. Results are expressed as the mean value of at least three separate determinations each performed in duplicate or triplicate and 95% confidence limits (CL). Effect upon food intake in rat, according to the statistical significance shown in Figs. 7 to 9, are represented in arbitrary units: very strong effect (++), strong effect (+), limited effect (+/-), or inactivity (-).

Compounds	cAMP IC ₅₀ ± S.E.M. (95% CL)	[¹²⁵ I]S36057 K _i (95% CL)	Effect on Food Intake (Arbitrary Units)
	<i>nM</i>		
S36057	0.28 (0.17–0.45)	0.06 (0.048–0.084)	++
MCH	0.41 (0.24–0.68)	0.12 (0.095–0.15)	++
[F ¹³ ;Y ¹⁹]-MCH	1.35 (0.74–2.46)	0.47 (0.36–0.62)	++
MCH _{6–17}	1.79 (0.86–3.69)	0.29 (0.20–0.42)	++
Salmon MCH	2.88 (1.78–4.68)	0.41 (0.32–0.53)	++
C5	18.70 (10.94–31.83)	2.03 (1.44–2.85)	+
C3	27.3 (10.49–71.10)	1.58 (1.16–2.17)	+/-
C1	254 (151–427)	131 (84–205)	+/-
C2	486 (224–1057)	95 (45–203)	+
S36077	629 (335–1180)	121 (64–230)	N.D.
C4	>1000	223	-
C6	>1000	>1000	-
C7	>1000	>1000	-
C8	>1000	>1000	-

N.D., not determined.

HEK293 cells. In HEK293 cells stably expressing the rat SLC-1 receptor coding sequence, MCH induced a dose-dependent inhibition of intracellular cAMP (Fig. 2), while no effect was observed in native cells (data not shown). A robust but transient elevation of intracellular calcium in the presence of MCH was also observed in transfected cells (data not shown). [¹²⁵I]S36057-specific binding to rat SLC-1 receptors was saturable (Fig. 3) and best fitted by a single-site analysis with a K_D of 0.029 ± 0.002 nM and a B_{max} of 1608 ± 122 fmol/mg of protein (*n* = 4), while, in contrast, no specific binding was detected in native cells (data not shown).

Characterization of MCH Analogs with Rat Recombinant SLC-1 Receptor: Effect on cAMP Levels. Peptides including the MCH analogs S36057, MCH, [F¹³;Y¹⁹]-MCH, salmon MCH, and the dodecapeptide MCH_{6–17} dose dependently inhibited the formation of intracellular cAMP (Fig. 2, A and B; Table 2) with potencies in the nanomolar range. A second group of five peptides also showed some activity in this test, but it was approximately 10- to 1000-fold less potent compared with the first set of active peptides (Fig. 2; Table 2). Indeed, substitution of Val¹² or Arg¹⁴ by Ala (C3 and C5) appeared to be less deleterious than Met⁸ substitution (C2) or Cys substitutions by Ser residues leading to linear peptides (C1 and S36077). Finally, other modifications such as Tyr¹³ by Ala (C4) or Arg¹¹ by His (C6) substitution or amino acid deletion inside the cysteine loop (C7 and C8) led to inactive compounds (Fig. 2C; Table 2). Results obtained in the cAMP assay at the rat receptor were highly correlated (*r* = 0.98, *P* < 0.0001, *n* = 10) with those previously reported with its human counterpart (Fig. 4).

Characterization of MCH Analogs at Rat Recombinant SLC-1 Receptor: Binding Affinities. Peptide compounds could be classified according to their binding affinity by a similar order to their functional efficacy in the cAMP assay. The first group, including the most potent peptides,

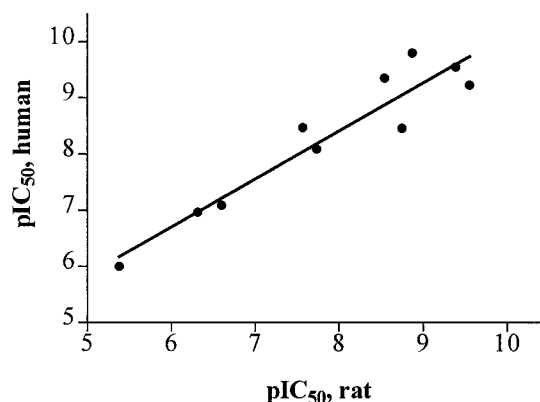


Fig. 4. Correlation plot of the MCH analogs potencies in the cAMP assay, at rat or human SLC-1 receptors stably expressed in HEK293 cells. Potencies expressed as pIC₅₀ (= -logIC₅₀) were calculated from Table 2 for the rat receptor and from Audinot et al. (2001a) for the human receptor. The correlation coefficient was *r* = 0.95 (*P* < 0.0001, slope = 0.85, *n* = 10).

displaced [¹²⁵I]S36057 binding (Fig. 5A; Table 2) with K_i values in the subnanomolar range. The second group of peptides (Fig. 5B; Table 2) exhibited affinities in the nanomolar range for C3 and C5 (Fig. 5B), while the less active peptides (C1, C2, and S36077) in the cAMP assay were also the less potent peptides in the [¹²⁵I]S36057 binding assay (Fig. 5B; Table 2). Finally, the third group of compounds included peptides that were inactive in the cAMP assay (C4, C6, C7, and C8) and also unable to displace [¹²⁵I]S36057 binding (Fig. 5C; Table 2). Data obtained from the cAMP assay were highly correlated (*r* = 0.97, *P* < 0.0001, *n* = 9) with those obtained from the binding test (Fig. 6).

Changes in Food Intake after Acute Central Administration of Different Peptides. All these MCH analogs (with the exception of S36077), were evaluated in rat, upon the cumulative food intake 2 h following i.c.v. injection. A significant effect of these peptides was searched in the dose range of 2 to 10 nmol/rat. MCH-induced stimulation of food intake was apparent 1 h after i.c.v. injection (data not shown) and was sustained for 2 h (Fig. 7A). Similarly to MCH, salmon MCH also increased cumulative food intake (Fig. 7B). These two natural peptides defined a group of particularly active compounds upon food intake, which also included the three MCH analogs [F¹³;Y¹⁹]-MCH (Fig. 7C), MCH_{6–17} (Fig. 7D), and S36057 (Fig. 7E). Effect of MCH_{6–17} appeared more pronounced at least at the lowest dose (Fig. 7B). Some peptides stimulated food intake, but with less effect than compounds of the first group (Fig. 8). In this second group of compounds, C2 and C5 presented limited but significant effects on food intake (Fig. 8, A and B), although compounds C1 and C3 nonsignificantly stimulated food intake (Fig. 8, C and D). Finally, analogs (C4, C6, C7, and C8) weakly potent or inactive in vitro were also inactive in vivo upon food intake (Fig. 9). At the highest doses (8–10 nmol/rat), some of the tested compounds (MCH_{6–17}, C2, C3, C4, C5, C7, and C8) induced apparent behavioral side effects like “barrel rolling”, hyperventilation, sedation, or epilepsy.

Discussion

Part of the evidences supporting an orexigenic role for MCH in food intake behavior came from the acute i.c.v.

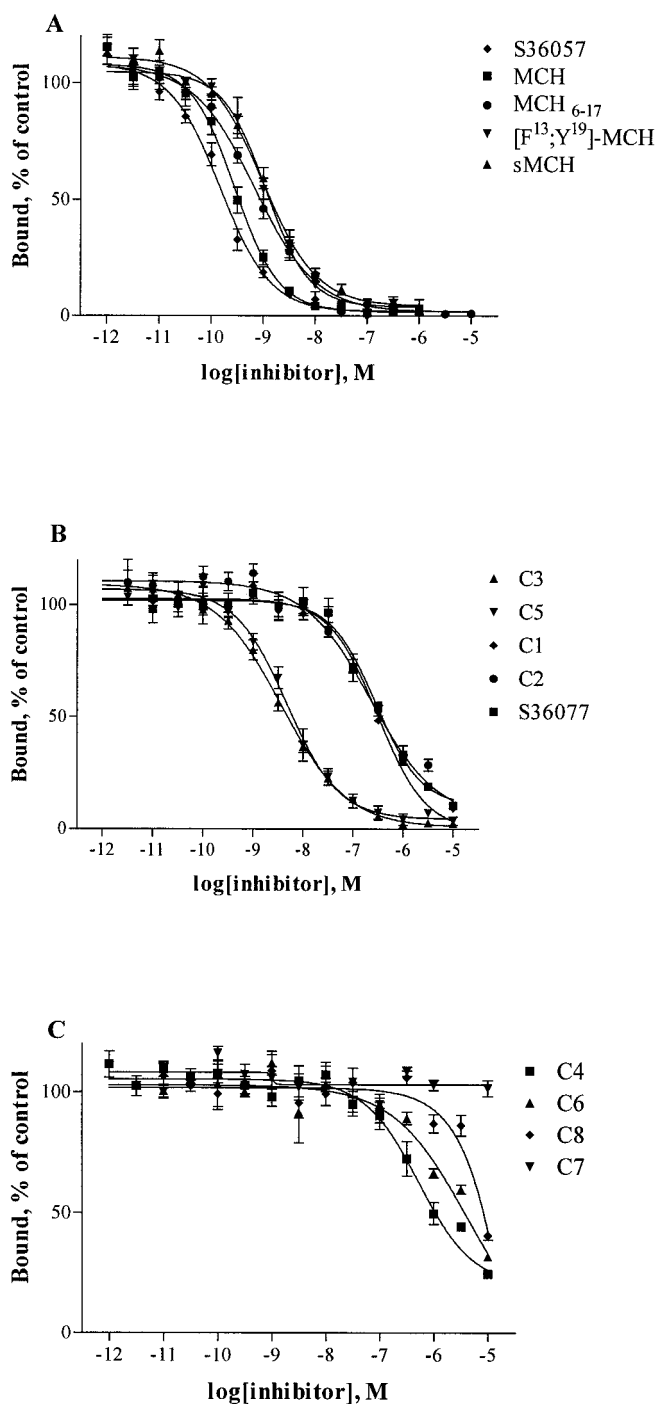


Fig. 5. Concentration isotherms of $[^{125}\text{I}]\text{S36057}$ -specific binding in the presence of MCH analogs at rat SLC-1 receptors stably expressed in HEK293 cells. Compounds are gathered according to their affinity: potent peptides (A), intermediate-to-weak peptides (B), and inactive peptides (C). Data were pooled from all the experiments and were expressed as means \pm S.E.M.

injections of MCH, its analog $[\text{F}^{13}, \text{Y}^{19}]\text{-MCH}$ (Drozd et al., 1995), or salmon MCH in rats and mice (Qu et al., 1996; Kokkotou et al., 2000; O. Della Zuana, unpublished data). In the present study we tried to determine whether the effects of MCH on feeding behavior were mediated through the described SLC-1 receptor. We first determined the functional and binding profiles of a range of MCH analogs in an in vitro model consisting of the rat SLC-1 receptor stably expressed

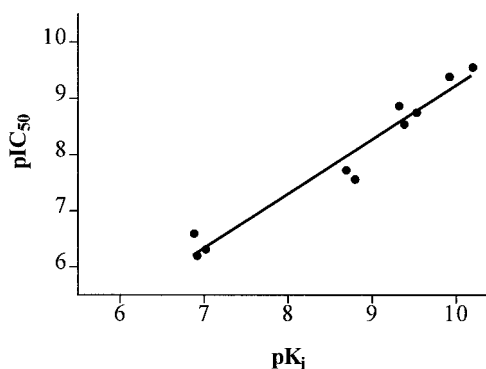


Fig. 6. Correlation plot of binding affinity and functional potency of MCH analogs at rat SLC-1 receptor stably expressed in HEK293 cells. Affinities expressed as pK_i ($= -\log K_i$) were determined in $[^{125}\text{I}]\text{S36057}$ displacement experiments and pIC₅₀ ($= -\log \text{IC}_{50}$) in cAMP assay. Data were calculated from Table 2. The correlation coefficient was $r = 0.98$ ($P < 0.0001$, slope = 1.30, $n = 10$).

in HEK293 cells. Effects of these MCH analogs were then evaluated upon food intake in rats. Finally, we studied the repartition of messenger RNA coding for the SLC-1 receptor in various rat tissues, and discuss the potential role of this receptor in the energy balance homeostasis, at the periphery.

SAR at Rat SLC-1 Receptor. Recently, a large study including 57 MCH analogs was performed with the human SLC-1 receptor, giving a great deal of information concerning MCH structure-activity relationships (Audinot et al., 2001a). There, it was shown that the minimal MCH sequence to retain potent biological activity was limited to the dodecapeptide MCH₆₋₁₇. Starting from this sequence, a large number of substitutions and/or deletions have been done to further assess MCH structure-activity relationships at the human receptor. In the present report some of these MCH analogs, from highly potent agonists to completely inactive compounds, were evaluated both at the recombinant rat SLC-1 receptor and in vivo upon food intake.

In cells stably expressing the rat SLC-1 receptor, there was indeed a highly significant correlation between the potencies of these analogs to inhibit intracellular cAMP at the rat receptors (this study) and those obtained at the human receptors (Audinot et al., 2001a). This reflected the high degree of homology between the human and rat receptor proteins (Saito et al., 2000), and the conservation of MCH peptide throughout evolution in mammals (for review, see Nahon, 1994).

The new MCH radioligand $[^{125}\text{I}]\text{S36057}$, designed from MCH₆₋₁₇ (Table 1) (Audinot et al., 2001b), was used to characterize the rat SLC-1 receptor. The K_D value of $[^{125}\text{I}]\text{S36057}$ (0.029 ± 0.002 nM) was comparable with the one obtained at the human receptor (Audinot et al., 2001b). To screen MCH analogs, binding affinities determined against $[^{125}\text{I}]\text{S36057}$ and potencies in the cAMP assay obtained at rat SLC-1 receptor were highly correlated. According to their potency (or affinity), these peptides could be classified in three classes: highly active, moderately active, or inactive compounds (Table 2). In vitro activity of these groups of compounds will be further discussed and compared with their effect upon food intake.

The first set of peptides, with subnanomolar binding affinities, includes MCH, salmon MCH, $[\text{F}^{13}, \text{Y}^{19}]\text{-MCH}$, the dodecapeptide MCH₆₋₁₇, and S36057, the latter exhibiting the

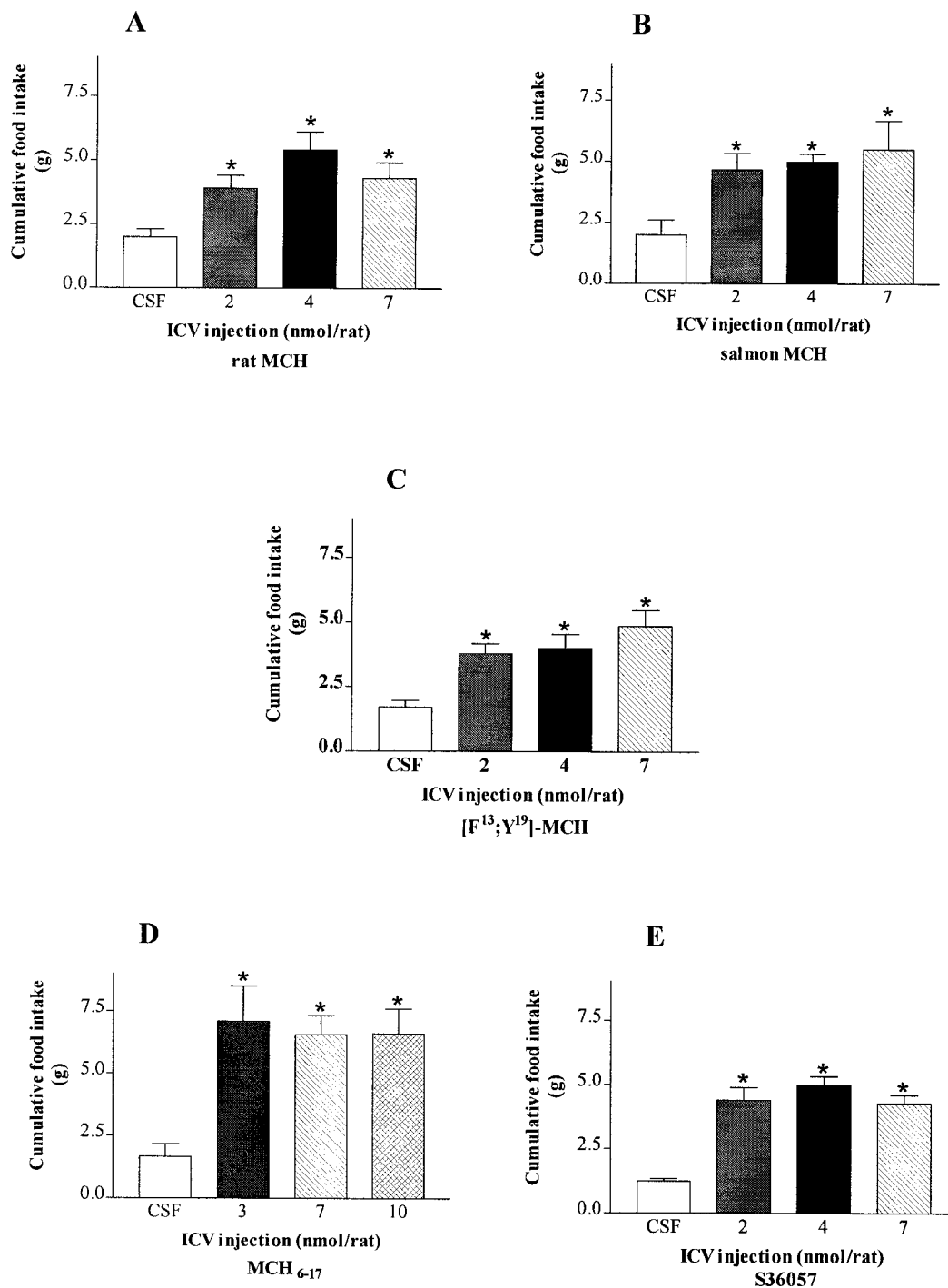


Fig. 7. Effect of “potent” MCH analogs upon cumulative food intake 2 h after acute i.c.v. administration. Vehicle (CSF-control), MCH (A), salmon MCH (B), $[F^{13};Y^{19}]$ -MCH (C), MCH_{6-17} (D), and S36057 (E) (2–10 nmol/rat) were injected at the beginning of the light period in satiated male Wistar rats. Cumulative food intake, represented in grams, was measured 2 h after injection. Results are expressed as mean \pm S.E. from 5 to 21 animals in each group. Statistics by one-way ANOVA followed by Newman-Keuls test at each time point: * $P < 0.05$ versus control group.

highest binding affinity. All these peptides potently stimulated cumulative food intake when injected i.c.v. in rats.

The second group of active peptides includes compounds with mild-to-weak in vitro activity at the SLC-1 receptor. This group could be further subdivided: those with IC_{50} values in the 10 nM range and those even less active in vitro, with IC_{50} values in the 100 nM range. Compared with MCH_{6-17} , substitution of Val¹² (C3) or Arg¹⁴ (C5) by Ala decreased ca. 5- to 10-fold both the potency and binding

affinity of these peptides. In contrast, Ala-substitution of the Met⁸ residue (C2) was more deleterious and serine substitutions of the two cysteine residues (in positions 7 and 16 upon MCH sequence, see compound C1 or upon MCH_{6-17} , S36077) leading to linear peptides, also diminished their potency and their binding affinity in vitro. It should be noticed that a cyclic structure of MCH is critical for activity as it was reported for salmon MCH in a fish skin assay (Kawazoe et al., 1987; B. Cardinaud, unpublished data) and for MCH at hu-

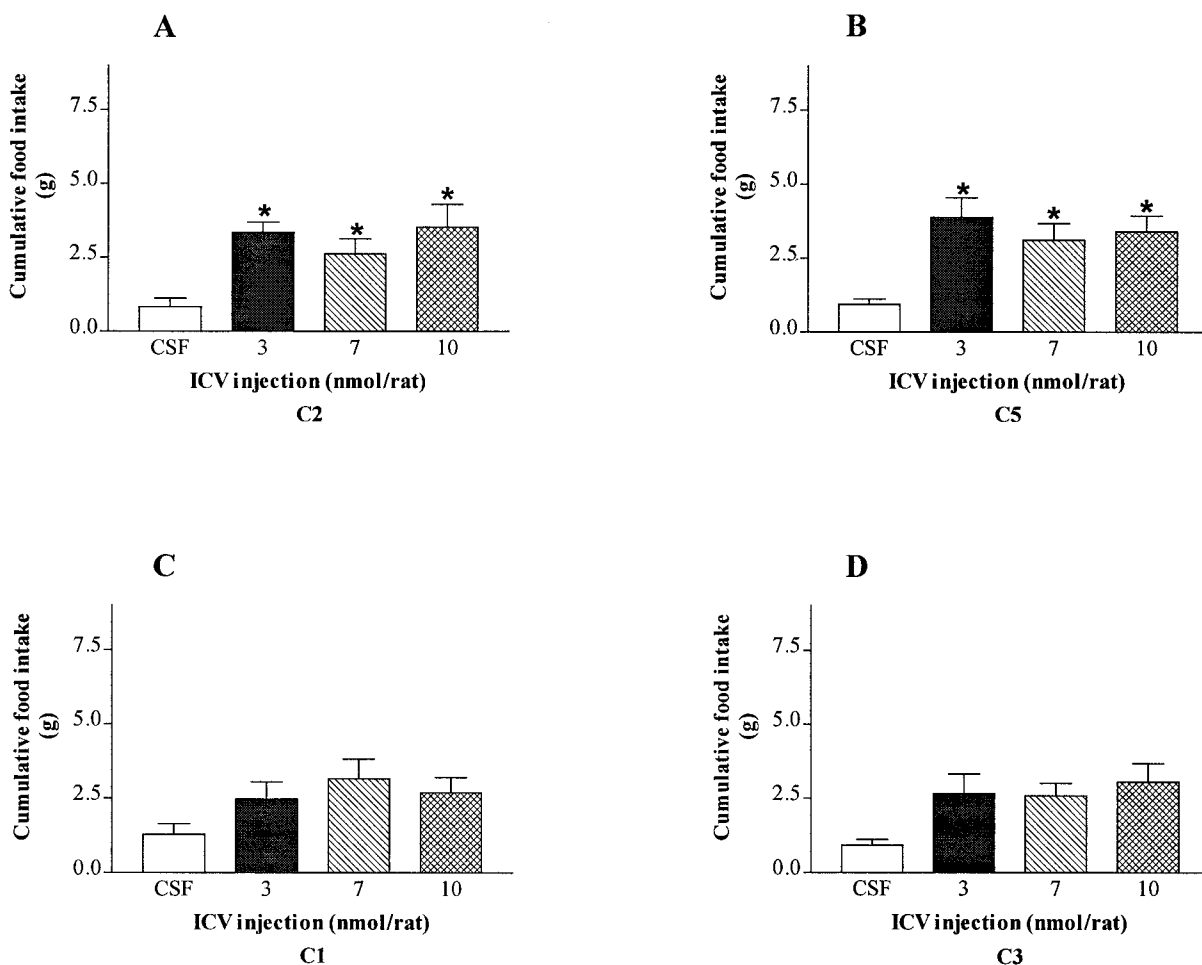


Fig. 8. Effect of “mild” MCH analogs upon cumulative food intake 2 h after acute i.c.v. administration. Vehicle (CSF-control) or weak agonist compounds C2 (A), C5 (B), C1 (C), and C3 (D) (2–10 nmol/rat) were injected at the beginning of the light period in satiated male Wistar rats. Cumulative food intake, represented in grams, was measured 2 h after injection. Results are expressed as mean \pm S.E. from 5 to 11 animals in each group. Statistics by one-way ANOVA followed by Newman-Keuls test at each time point: * $P < 0.05$ versus control group.

man SLC-1 receptor (Audinot et al., 2001a). Interestingly, peptides in this second group are effective upon food intake but with less effect compared with the first group, in the range of the tested doses. So, peptides C2 and C5 are significant food intake stimulators, while C1 and C3 are slightly less active. The nonsignificant but detectable effect of these two latter peptides may be due to their altered stability in the brain, which should be investigated, or by the behavioral effects observed after injection. In conclusion, this group of peptides with lower affinity than MCH caused lower effect on food intake.

Finally, the third group comprised inactive peptides *in vitro*. A critical role of Arg¹¹ has been shown previously for the functional activation of the MCH receptor (Macdonald et al., 2000; Audinot et al., 2001a). This activity was lost with substitutions by other charged or structurally related residues (His, see C6). Based on site-directed mutagenesis at the human MCH receptor, a critical interaction of this residue was proposed with the residue Asp¹²³ of the receptor (Macdonald et al., 2000). This position is conserved in several others G protein-coupled receptors and is involved in both agonist binding and activation. Alanine substitution of the Tyr¹³ residue was deleterious for activity (C4), while substitution by a phenylalanine maintained the biological activity

of the peptides (see [F¹³;Y¹⁹]-MCH and S36057), demonstrating the importance of the phenolic structure of the side chain of the amino acid. Deletions inside the cycle led to inactive compounds (C7 and C8). Other peptides bearing deletions have been tested at the human receptor, but they all lost their agonistic activity (Audinot et al., 2001a). This revealed the importance of the size of the loop for biological activity of the mammalian MCH, as previously shown for the salmon MCH in a fish skin assay (Lebl et al., 1988, 1989). Consistent with the data obtained with the cellular model, the “inactive” peptide failed to stimulate food intake. However, side effects such as barrel rolling and epilepsy were observed when a large amount of compounds was injected as reported for high doses of MCH (Rossi et al., 1997).

Taken together, these data clearly demonstrate a strong correlation between *in vitro* activity of MCH analogs at the rat SLC-1 receptor, and *in vivo* potency, upon stimulation of food intake. This suggests that the orexigenic effects of MCH are mediated by the SLC-1 receptor. Furthermore, the SLC-1 receptor mRNA is found in rat hypothalamus (Chambers et al., 1999; Lembo et al., 1999; Saito et al., 1999; Hervieu et al., 2000; this work), an area playing a key role in the control of feeding behavior.

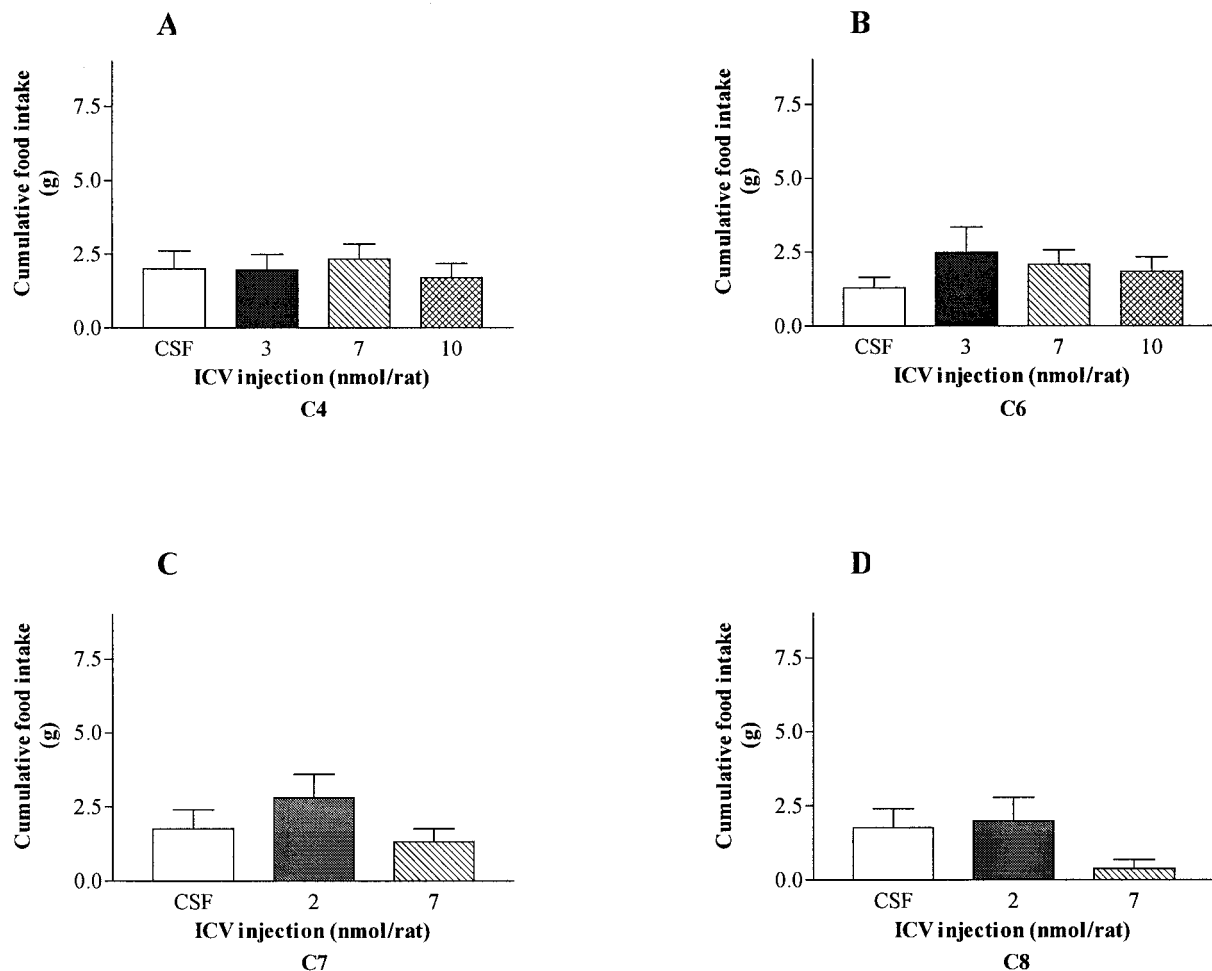


Fig. 9. Effect of “inactive” MCH analogs upon cumulative food intake 2 h after acute i.c.v. administration. Vehicle (CSF-control) or compounds (C4, C6, C7, and C8) (2–10 nmol/rat) were injected at the beginning of the light period in satiated male Wistar rats. Cumulative food intake, represented in grams, was measured 2 h after injection. Results are expressed as mean \pm S.E. from 5 to 11 animals in each group. Statistics by one-way ANOVA followed by Newman-Keuls test at each time point.

Potential Roles of SLC-1 Receptor at Periphery. We studied the expression of SLC-1 receptor in peripheral organs. SLC-1 receptor mRNA expression could not be detected in heart, liver, lung, kidney, or thymus. Contradictory to Saito et al. (1999), SLC-1 receptor expression was found in testis, probably because the PCR assay is more sensitive than the Northern blot approach. Expression of SLC-1 receptor found in reproductive organs reinforces the possible implication of MCH in reproductive functions and sexual behaviors (Hervieu et al., 1996a; Murray et al., 2000). We detected the presence of the SLC-1 receptor mRNA in adipose tissue and skeletal muscle. Indeed, a recent report has shown the presence of these receptor mRNAs in isolated adipocytes and the involvement of MCH peptide in the expression and secretion of leptin (Bradley et al., 2000). In addition, expression of both the peptide and the receptor in pancreas, as well as MCH regulation of insulin release in pancreatic cell lines, has been reported (Tadayon et al., 2000). Taken together, these results reinforce the hypothesis of the general implication of MCH and its cognate receptor in the regulation of energy balance with peripheral actions, in addition to the described effect in the central regulation of feeding (Tritos and Maratos-Flier, 1999).

In conclusion, we established the conserved molecular

pharmacological profile between human and rat SLC-1 receptors. We have provided mainly pharmacological evidence for the SLC-1 receptor involvement in mediating the central effect of MCH upon food intake in rat.

Acknowledgments

We thank M. Germain, F. Maupin, M. Sadlo, and J. Staczeck for expertise in contributing to the food intake studies. We also thank S. Dromaint, C. Lahaye, J. Imbert, and H. Rique from the Molecular and Cellular Pharmacology Department for help.

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 MCH-related peptide ligands of SLC-1, the human melanin-concentrating hormone receptor. *J Biol Chem* **276**:13554–13562.
- Audinot V, Lahaye C, Suply T, Beauverger P, Rodriguez M, Galizzi JP, Fauchère JL, and Boutin JA (2001b) [¹²⁵I]-S36057 a new and highly potent radioligand for the melanin-concentrating hormone receptor. *Br J Pharmacol* **133**:371–378.
- Baker BI (1994) Melanin-concentrating hormone updated functional considerations. *Trends Endocrinol Metab* **5**:120–126.
- Bittencourt JC, Presse 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.
- Chambers J, Ames RS, 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.
- Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, et al. (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**:437–451.
- 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.
- Erickson JC, Clegg KE, and Palmiter RD (1996) Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature (Lond)* **381**:415–418.
- Hervieu GJ, Cluderay JE, Harrison 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**:1194–1216.
- Hervieu G and Nahon JL (1995) Pro-melanin concentrating hormone messenger ribonucleic acid and peptides expression in peripheral tissues of the rat. *Neuroendocrinology* **61**:348–364.
- Hervieu G, Segretain D, and Nahon JL (1996a) Developmental and stage-dependent expression of melanin-concentrating hormone in mammalian germ cells. *Biol Reprod* **54**:1161–1172.
- Hervieu G, Volant K, Grishina O, Descroix-Vagne M, and Nahon JL (1996b) Similarities in cellular expression and functions of melanin-concentrating hormone and atrial natriuretic factor in the rat digestive tract. *Endocrinology* **137**:561–571.
- Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, and Baker BI (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature (Lond)* **305**:321–323.
- Kawazoe I, Kawauchi H, Hirano T, and Naito N (1987) Structure-activity relationships of melanin-concentrating hormone. *Int J Pept Protein Res* **29**:714–721.
- Kokkotou E, Mastaitis JW, Qu D, Hoersch D, Slieker L, Bonter K, Tritos NA, and Maratos-Flier E (2000) Characterization of [Phe¹³, Tyr¹⁹]-MCH analogues binding activity to the MCH receptor. *Neuropeptides* **34**:240–247.
- Kolakowski LF, Jung BP, Nguyen T, Johnson MP, Lynch KR, Cheng R, Heng HHQ, George SR, and O'Dowd BF (1996) Characterization of a human gene related to genes encoding somatostatin receptors. *FEBS Lett* **398**:253–258.
- Lakaye B, Minet A, Zorzi W, and Grisar T (1998) Cloning of the 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.
- Lebl M, Hruby VJ, Castrucci AM, and Hadley ME (1989) Melanin concentrating hormone analogues: contraction of the cyclic structure. II. Antagonist activity. *Life Sci* **44**:451–457.
- Lebl M, Hruby VJ, Castrucci AM, Visconti MA, and Hadley ME (1988) Melanin concentrating hormone analogues: contraction of the cyclic structure. I. Agonist activity. *J Med Chem* **31**:949–954.
- 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.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**:265–275.
- Ludwig DS, Mountjoy KG, Tatro JB, Gillette JA, Frederick RC, Flier JS, and Maratos-Flier E (1998) Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *Am J Physiol* **274**:E627–E633.
- Ludwig DS, Tritos NA, Mastaitis JW, Kulkarni R, Kokkotou E, Elmquist J, Lowell B, Flier JS, and Maratos-Flier E (2001) Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. *J Clin Invest* **107**:379–386.
- 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.
- Matsunaga TO, Castrucci AM, Hadley ME, and Hruby VJ (1989) Melanin concentrating hormone (MCH): synthesis and bioactivity studies of MCH fragment analogues. *Peptides* **10**:349–354.
- Murray JF, Adan RA, Walker R, Baker BI, Thody AJ, Nijenhuis WA, Yukitake J, and Wilson CAJ (2000) Melanin-concentrating hormone, melanocortin receptors and regulation of luteinizing hormone release. *J Neuroendocrinol* **12**:217–223.
- Nahon JL (1994) The melanin-concentrating hormone: from the peptide to the gene. *Crit Rev Neurobiol* **8**:221–262.
- Qu D, Ludwig DS, Gammeltoft S, Piper M, Pellemont 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, Beak SA, Choi SJ, Small CJ, Morgan DG, Ghatei MA, Smith DM, and Bloom SR (1999) Investigation of the feeding effects of melanin concentrating hormone on food intake—action independent of galanin and the melanocortin receptors. *Brain Res* **846**:164–170.
- Rossi M, Choi SJ, O'Shea D, Miyoshi T, Ghatei MA, and Bloom SR (1997) Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. *Endocrinology* **138**:351–355.
- Sahu A (1998) Leptin decreases food intake induced by melanin-concentrating hormone (MCH), galanin (GAL) and neuropeptide Y (NPY) in the rat. *Endocrinology* **139**:4739–4742.
- Saito Y, Nothacker H, 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.
- Shimada M, Tritos NA, Lowell BB, Flier JS, and Maratos-Flier E (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature (Lond)* **396**:670–674.
- Tadayon 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.
- Tritos NA and Maratos-Flier E (1999) Two important systems in energy homeostasis: melanocortins and melanin-concentrating hormone. *Neuropeptides* **33**:339–349.
- Tritos NA, Vicent D, Gillette J, Ludwig DS, Flier ES, and Maratos-Flier E (1998) Functional interactions between melanin-concentrating hormone, neuropeptide Y, and anorectic neuropeptides in the rat hypothalamus. *Diabetes* **47**:1687–1692.
- Vaughan JM, Fischer WH, Hoeger C, Rivier J, and Vale W (1989) Characterization of melanin-concentrating hormone from rat hypothalamus. *Endocrinology* **125**:1660–1665.
- Wilkes BC, Hruby VJ, Castrucci AM, Sherbrooke WC, and Hadley ME (1984) Synthesis of a cyclic melanotropic peptide exhibiting both melanin-concentrating and -dispersing activities. *Science (Wash DC)* **224**:1111–1113.

Address correspondence to: Dr. Jean A. Boutin, Division de Pharmacologie Moléculaire et Cellulaire, Institut de Recherches Servier, 125 chemin de Ronde, F-78290-Croissy/Seine, France. E-mail: jean.boutin@fr.netgrs.com
