Localization of the Melanocortin-4 Receptor (MC4-R) in Neuroendocrine and Autonomic Control Circuits in the Brain

Kathleen G. Mountjoy*, Marty T. Mortrud, Malcolm J. Low, Richard B. Simerly, and Roger D. Cone

Vollum Institute for Advanced Biomedical Research (K.J.M., M.T.M., M.J.L., R.D.C.)
Oregon Health Sciences University
Portland, Oregon 97201–3098

Oregon Regional Primate Research Center (R.B.S.)
Beaverton, Oregon 97006

POMC, the precursor of ACTH, MSH, and β-endorphin peptides, is expressed in the pituitary and in two sites in the brain, in the arcuate nucleus of the hypothalamus and the commissural nucleus of the solitary tract of the brain stem. Little is known regarding the functions of melanocortin (ACTH and MSH) peptides in the brain. We report here the detailed neuroanatomical distribution of the MC4-R mRNA in the adult rat brain. The melanocortin 3 receptor (MC3-R), characterized previously, was found to be expressed in arcuate nucleus neurons and in a subset of their presumptive terminal fields but in few regions of the brainstem. The highly conserved MC4-R is much more widely expressed than MC3-R and is pharmacologically distinct. MC4-R mRNA was found in multiple sites in virtually every brain region, including the cortex, thalamus, hypothalamus, brainstem, and spinal cord. Unlike the MC3-R, MC4-R mRNA is found in both parvicellular and magnocellular neurons of the paraventricular nucleus of the hypothalamus, suggesting a role in the central control of pituitary function. MC4-R is also unique in its expression in numerous cortical and brainstem nuclei. Together, MC3-R and/or MC-4R mRNA are found in every nucleus reported to bind MSH in the adult rat brain and define neuronal circuitry known to be involved in the control of diverse neuroendocrine and autonomic functions. The high degree of conservation, distinct pharmacology, and unique neuronal distribution of the MC4 receptor suggest specific and complex roles for the melanocortin peptides in neuroendocrine and autonomic control. (Molecular Endocrinology 8: 1298–1308, 1994)

0888-8810/94/1298-1308$03.00/0
Molecular Endocrinology

INTRODUCTION

In addition to its expression and processing into hormonal peptides in the pituitary, POMC is expressed in two sites in the brain, the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract in the brainstem (1–6). POMC is one of the earliest neuropeptide precursors expressed in the brain, appearing as early as embryonic day 12.5 in the rat (7). Processed POMC peptides begin to appear approximately 1 day later and, with a few exceptions, largely resemble the peptides found in the intermediate lobe of the pituitary: des-acetyl-α-melanotropin, β-endorphin, and N-POMC-(1–49) (for review see Ref. 8).

Two lines of evidence have suggested a functional role for POMC peptides in the brain. First, a complex pattern of specific high affinity binding sites for β-endorphin and melanocortin (ACTH and MSH) peptides has been demonstrated (9–11). Second, a number of biological responses have been seen upon intracerebroventricular introduction of these peptides. For example, central administration of melanocortin peptides has been reported to have thermoregulatory (12), pressor (13, 14), behavioral (15, 16), and neuroendocrine effects (17).

Recent cloning experiments have identified two distinct subfamilies of G protein-coupled receptors mediating the actions of the opioid (18–21) and melanocortin peptides (22–31), respectively. The first two melanocortin receptors cloned were the melanocyte MSH receptor (MSH-R or MC1-R) (22, 23) and the adrenocortical ACTH receptor (ACTH-R or MC2-R) (23). Subsequently, three additional melanocortin receptor genes have also been cloned; however, the functions of these receptors remain unknown. Two of these melanocortin receptors, termed the melanocortin-3 and melanocortin-4 receptors (MC3-R and MC4-R), have now been shown to be expressed primarily in the brain (24–26,

28). A fifth melanocortin receptor, originally termed MC2-R (27), appears to be expressed in numerous peripheral organs as well as in the brain (27–30). The consensus that has developed is that this receptor should be named the MC5-R to adhere to a numerical nomenclature assigned according to the order of discovery (28–30).

Neuroanatomical mapping of rat MC3-R expression demonstrated this receptor mRNA to be expressed in approximately 30 different nuclei, including arcuate nucleus POMC neurons and a subset of their respective terminal fields (25). A recent report of the cloning and characterization of the MC4-R has documented expression of this receptor in five regions of the mouse brain (hippocampus, dentate gyrus, cortex, ventromedial hypothalamus, and amygdala) by low resolution autoradiography (26). The detailed neuroanatomical distribution of expression of the rat MC4-R mRNA is presented here. MC4-R mRNA is found in more than 100 nuclei, including the remainder of POMC-positive arcuate nucleus termini and several sites in the brainstem.

RESULTS

Cloning of the Human and Rat MC4-Rs

A fragment of the coding sequence of the rat MC4-R was isolated by polymerase chain reaction (PCR) with deeenerate oligonucleotides based on conserved amino acid sequences found in transmembrane domains II and VII in the previously reported sequences of the MSH and ACTH receptors (22). First strand cDNA prepared from total rat brain RNA served as the template. This fragment was found to encode a novel G protein-coupled receptor spanning from the beginning of the second transmembrane domain through the beginning of the seventh transmembrane domain. This fragment was then used as a probe to screen a human genomic DNA library and identify a human MC4 receptor (hMC4-R) homolog. A single exon containing the entire 332 amino acid coding sequence of the hMC4-R was found by DNA sequencing within a 1.9 kilobase (kb) genomic *Hin*dIII fragment. While this work was in progress, cloning and characterization of the hMC4-R were reported by Gantz *et al.* (26), and the sequence and pharmacological data reported here largely confirm this earlier report.

The MC4 receptor is highly conserved, with approximately 95% overall amino acid identity between the human and partial rat receptor sequences (Fig. 1A). This receptor is identical in amino acid sequence to the hMC4-R reported by Gantz *et al.* (26), with the exception of codon 169, at which Gantz *et al.* report an isoleucine residue. The MC4-R is most closely related to the other two newly described melanocortin receptors, MC3-R (24, 25) and MC5-R (27–30), with 55–61% overall amino acid identity, and demonstrates slightly less overall amino acid identity (46–47%) with the MSH (MC1) and ACTH (MC2) receptors (Fig. 1B).

Functional Expression of the hMC4-R

A full length coding fragment of the hMC4-R gene was amplified by PCR using a 20-kb phage insert containing the genomic hMC4-R sequence as template and oligonucleotides designed against the 5′- and 3′-untranslated regions of the gene. The amplified DNA was subcloned into pcDNA1NEO vector (Invitrogen, San Diego, CA) and stably transfected into human embryonic kidney 293 cells. Adenylyl cyclase activity was then determined directly by measuring the ability of cells to convert [^{3}H]adenine to [^{3}H]cAMP after exposure of the cells to various natural and synthetic POMC peptides (Fig. 2). MC4-R couples to adenylyl cyclase and is most potently activated by Nle^4, D-Phe^7-α-MSH (NDP-MSH), a superpotent synthetic analog of α-MSH (32). The order of potency for the native melanocortin peptides was des-acetyl-α-MSH >/= $ACTH_{1-39}$ >/= α-MSH > > γ_2-MSH = $ACTH_{4-10}$. ORG2766 (methionyl-sulfone-Glu-His-Phe-D-Lys-Phe), a synthetic $ACTH_{4-9}$ analog demonstrated to induce a number of behavioral responses upon administration in the rat (33), produced no detectable activation of adenylyl cyclase in this MC4-R expression system at concentrations up to 1 μM. Likewise, neither corticotropin-like intermediate lobe peptide nor β-endorphin activated the hMC4-R.

Localization of MC4-R mRNA in Rat Brain

Northern hybridization analysis using a rat MC4-R probe failed to detect MC4-R mRNA in stomach, spleen, salivary gland, lung, heart, kidney, liver, adrenal, melanoma, or lymphocytes, while a faint signal was observed in all brain regions examined (data not shown). *In situ* hybridization using the rat MC4-R probe was then used to demonstrate the density and distribution of MC4-R mRNA-containing cells in the central nervous system of the rat (Table 1 and Fig. 3). MC4-R mRNA expression showed a widespread distribution with at least moderate levels of labeling in every major division of the central nervous system. Within regions of isocortex, the highest levels of labeling were found in auditory regions that included the primary auditory area, agranular insular, and anterior cingulate areas of isocortex. In addition, a high density of labeled cells was found in the ventral part of the orbital area with fewer labeled cells in primary and secondary motor areas. Somatosensory and visual sensory regions of isocortex lacked detectable levels of MC4-R mRNA, yet the ventral temporal association areas had moderate to high levels of labeling. Regions of cortex involved in olfactory responses, such as the anterior olfactory nucleus, the taenia tecta, olfactory tubercle, and the piriform area, also contained labeled cells.

MC4-R mRNA in the hippocampus was generally sparse, although high levels of labeling were found in the lateral part of the entorhinal area and ventral part of the subiculum. Lower levels of labeling were detected in the parasubiculum and fields CA1, CA2, and CA3 of Ammon's horn. Extensive labeling was found over sev-

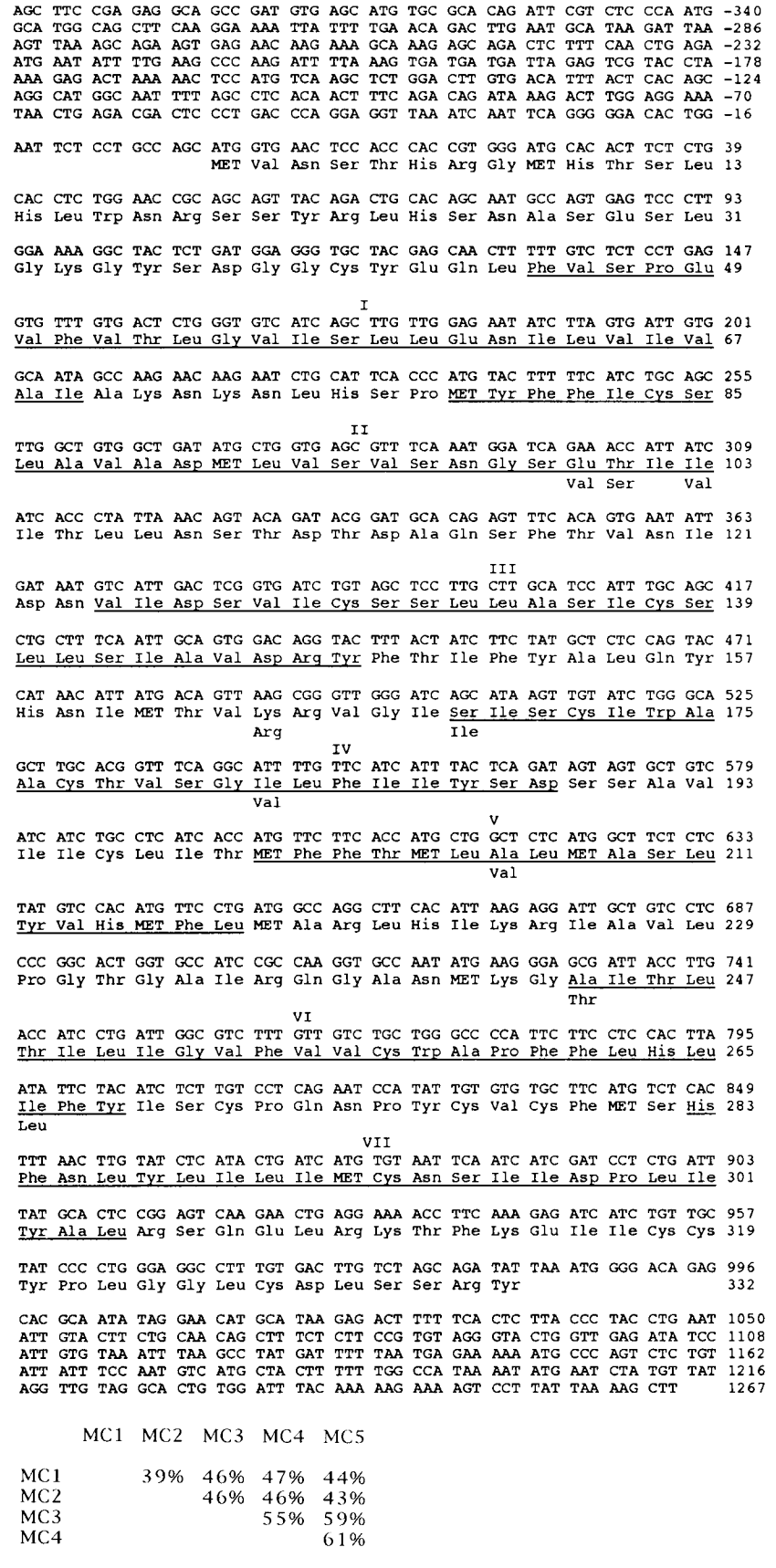

Fig. 1. Cloning of the Human and Rat MC4-Rs

A, Sequence of the rat and human MC4-Rs. Approximate location of transmembrane domains are *underlined*. Sequence of the rat receptor was determined from codons 78–293; amino acid differences in the rat MC4-R are shown *underneath* the human sequence. B, Relative overall amino acid identities among the melanocortin receptors.

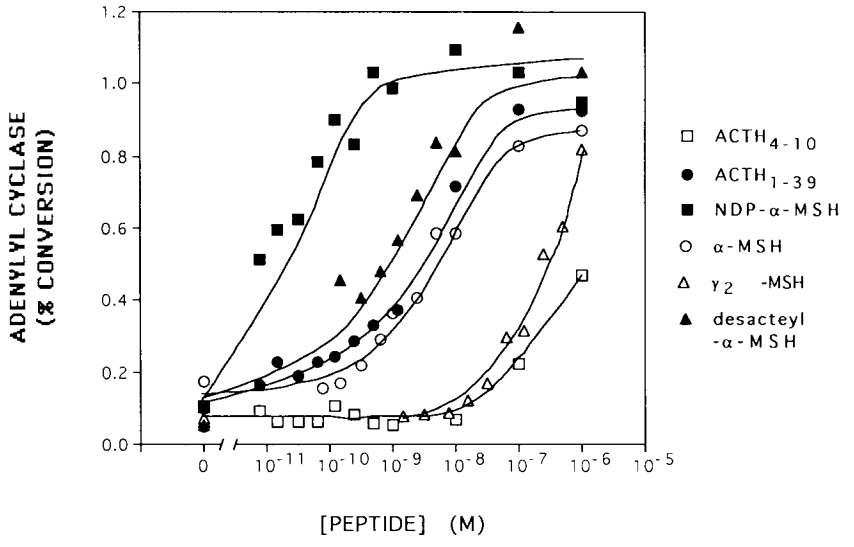

Fig. 2. Functional Coupling of the hMC4-R to Adenylyl Cyclase

The X axis indicates the concentration of each hormone, and the Y axis indicates the percent of [^{3}H]adenine converted to [^{3}H] cAMP after a 1-h stimulation at 37 C. *Curves* are representative of three separate experiments. *Points* indicate the mean of duplicate incubations, and errors did not exceed 20% for any of the data points. EC_{50} values calculated for each curve are Nle^4, D-Phe^7-α-MSH ($1.1 \pm 0.2 \times 10^{-11}$ M), des-acetyl-α-MSH ($4.9 \pm 1.8 \times 10^{-10}$ M), $ACTH_{1-39}$ ($6.8 \pm 2.8 \times 10^{-10}$ M), α-MSH ($1.5 \pm 0.5 \times 10^{-9}$ M), γ_2-MSH ($> 10^{-7}$ M), $ACTH_{4-10}$ ($> 10^{-7}$ M).

eral nuclei of the amydgala including the anterodorsal and posterodorsal part of the medial nucleus of amygdala, the anterior cortical nucleus, and the medial zone of the posterior part of the cortical nucleus (Fig. 3B). In addition, all three divisions of the central nucleus of the amygdala contained at least a few MC4-R mRNA-containing cells, and the posterior part of the basolateral nucleus, and basomedial nucleus of the amygdala were also labeled.

The lateral septal nucleus was among those regions with high densities of MC4-R mRNA-containing cells, especially in the intermediate part. The nucleus of the diagonal band and medial septal nuclei also contained high densities of labeled neurons. Moderate levels of labeling were found throughout the bed nuclei of the stria terminalis, including all three parts of its anterior division, but the highest density of labeled cells was in the principal nucleus. In addition, high densities of labeled cells were found in the septohippocampal nucleus and subfornical organ. A possible role for MC4-R in mediating motor responses is indicated by the moderate density of MC4-R mRNA-containing cells found in the striatum and substantia innominata. In general, thalamic nuclei were unlabeled, although a few labeled neurons were found in nucleus reuniens, and a high density of MC4-R mRNA-containing cells was found in the supragenicuiate nucleus. In the ventral thalamus the zona incerta, peripeduncular nucleus, and magnocellular part of the subparafascicular nucleus contained labeled neurons.

MC4-R mRNA-containing neurons show an extensive distribution throughout all of the major parts of the hypothalamus. In the periventricular zone of the hypothalamus, moderate to high levels of labeling were observed in the median preoptic nucleus, suprachiasmatic preoptic nucleus, and all parts of the periventricular nucleus of the hypothalamus. The highest density of MC4-R mRNA-containing cells in the periventricular zone were found in the anteroventral periventricular nucleus (Fig. 3A) and supraoptic nucleus (Fig. 3E). In addition, the nucleus circularis was heavily labeled. A cluster of labeled neurons was found in the dorsal zone of the medial parvicellular part of the paraventricular nucleus of the hypothalamus (PVH), which contains neuroendocrine cells that project to the median eminence. Moreover, many MC4-R mRNA-containing cells were found throughout the lateral parvicellular part of the PVH and ventral zone of the medial parvicellular part, both of which have descending projections to regions that mediate autonomic responses. Within the magnocellular division of the paraventricular nucleus, a low to moderate density of MC4-R mRNA-containing cells was localized to the anterior magnocellular part.

Moderate to high densities of labeled cells were found in each of the major nuclei that occupy the medial zone of the hypothalamus, including the medial preoptic nucleus, the anterior hypothalamic nucleus, ventromedial hypothalamic nucleus, and dorsomedial nucleus of the hypothalamus. In addition, both the dorsal and ventral parts of the tuberomammillary nucleus were labeled, and a moderate density of MC4-R mRNA-containing cells were found within the medial mammillary nucleus and posterior hypothalamic nucleus. Moderate to high densities of MC4-R mRNA-containing cells were distributed throughout the lateral zone of the hypothalamus.

In the brainstem, the highest densities of MC4-R mRNA-containing cells were localized to regions involved in relaying sensory information, most notable

Table 1. Distribution of MC4-R mRNA in the Rat Central Nervous System

I. Forebrain (FB)	
A. Isocortex (ISO)	
1. Motor areas (MO)	
a. Primary motor area (MOp)	++
b. Secondary motor areas (MOs)	++
2. Agranular insular area (AI)	
a. Dorsal part (AId)	++(+)
b. Ventral part (AIv) (layers 2, 3 & 5)	++(+)
c. Posterior part (AIp)	++(+)
3. Anterior cingulate area (ACA)	
a. Dorsal part (ACAd) (layer 6A)	++
b. Ventral part (ACAv)	+(+)
4. Auditory areas (AUD) (Primary, dorsal, ventral)	+++
5. Infralimbic area (ILA)	++
6. Orbital area (ORB)	
a. Ventral part (ORBv)	+++
b. Ventrolateral part (ORBvl)	++
7. Retrosplenial area (RSP)	
a. Dorsal part (RSPd) (deeper layers)	+(+)
8. Ventral temporal association areas (TEv)	+++
9. Claustrum (CLA)	+(+)
B. Olfactory cortex (OLF)	
1. Accessory olfactory bulb (AOB	
a. Mitral layer (AOBmi)	+
2. Anterior olfactory nucleus (AON)	
b. Lateral part (AONl)	++
c. Medial part (AONm)	+
d. Posteroventral part (AONpv)	+
3. Taenia tecta (TT)	
a. Dorsal part (TTd)	+++(+)
4. Olfactory tubercle (OT)	
a. Pyramidal layer (OT2)	+++
5. Piriform area (PIR)	
a. Pyramidal layer (PIR2)	+(+)
6. Postpiriform transition area (TR)	+
C. Hippocampal formation (cortex) (HPF)	
1. Retrohippocampal region (RHP)	
a. Entorhinal area (ENT)	
(1) Lateral part (ENTl) (layer 2)	+++
b. Parasubiculum (PAR)	++
c. Subiculum (SUB)	
(1) Ventral part (SUBv)	++(+)
2. Hippocampal region (HIP)	
a. Ammon's horn (CA)	
(1) Field CA1 (CA1)+(+)	
(2) Field CA2 (CA2)	++
(3) Field CA3 (CA3)	+++
D. Amygdala (AMY)	
1. Bed nucleus of the accessory olfactory tract (BA)	+++
2. Medial nucleus of the amygdala (MEA)	
a. Anterodorsal part (MEAad)	+++
b. Posterodorsal part (MEApd)	++
c. Posteroventral part	++
3. Cortical nucleus of the amygdala (COA)	
a. Anterior part (COAa)	++
b. Posterior part (COAp)	
(1) Medial zone (COApm)	+++
4. Anterior amygdaloid area (AAA)	++
5. Central nucleus of the amygdala (CEA)	
a. Medial part (CEAm)	++
b. Lateral part (CEAl)	+
c. Capsular part (CEAc)	+++
6. Basolateral nucleus of the amygdala (BLA)	
a. Posterior part (BLAp)	++

Table 1. Distribution of MC4-R mRNA in the Rat Central Nervous System

7. Basomedial nucleus of the amygdala (BMA)	
a. Anterior part (BMAa)	++(+)
b. Posterior part (BMAp)	(+)
8. Posterior nucleus of the amygdala (PA)	+
E. Septal region (SEP)	
1. Lateral septal nucleus	
a. Dorsal part (LSd)	++
b. Intermediate part (LSi)	++++
c. Ventral part (LSv)	++
2. Medial septal complex (MSC)	
a. Medial septal nucleus (MS)	++++
b. Nucleus of the diagonal band (NDB)	+++
3. Bed nuclei of the stria terminalis (BST)	
a. Anterior division (BSTa)	
(1) Anterodorsal area (BSTad)	++(+)
(2) Anterolateral area (BSTal)	++(+)
(3) Anteroventral area (BSTav)	++(+)
(4) Rhomboid nucleus (BSTrh)	++(+)
(5) Dorsomedial nucleus (BSTdm)	++(+)
(6) Dorsolateral nucleus (BSTdl)	++(+)
(7) Ventral nucleus (BSTv)	++(+)
(8) Magnocellular nucleus (BSTmg)	++(+)
b. Posterior division (BSTp)	
(1) Principal nucleus (BSTpr)	+++
(2) Interfascicular nucleus (BSTif)	++(+)
(3) Transverse nucleus (BSTtr)	++(+)
4. Septohippocampal nucleus (SH)	+++(+)
5. Subfornical organ (SFO)	+++(+)
F. Corpus striatum (CSTR)	
1. Striatum (STR)	
a. Caudoputamen (CP)	++
b. Nucleus accumbens (ACB)	++
c. Fundus of the striatum (FS)	+++
2. Pallidum (PAL)	
a. Magnocellular preoptic nucleus (MA)	+
G. Thalamus (TH)	
1. Dorsal thalamus (DOR)	
a. Midline group of the dorsal thalamus (MID)	
(1) Nucleus reuniens (RE)	+
b. Lateral group of the dorsal thalamus (LAT)	
(1) Suprageniculate nucleus (SGN)	+++
2. Ventral thalamus (VNT)	
a. Zona incerta (ZI)	++
b. Peripeduncular nucleus (PP)	++(+)
c. Subparafascicular nucleus (SPF)	
(1) Magnocellular part (SPFm)	++
H. Hypothalamus (HY)	
1. Periventricular zone of the hypothalamus (PVZ)	
a. Suprachiasmatic preoptic nucleus (PSCH)	+++
b. Median preoptic nucleus (MEPO)	++
c. Anteroventral periventricular nucleus (AVPv)	++++
d. Preoptic periventricular nucleus (PVpo)	+(+)
e. Supraoptic nucleus (SO)	+++(+)
(1) Accessory supraoptic group (ASO)	
(a) Nucleus circularis (NC)	+++
f. Paraventricular nucleus of the hypothalamus (PVH)	
(1) Descending division (PVHd)	
(a) Medial parvicellular part, ventral zone (PVHmpv)	++(+)
(b) Lateral parvicellular part (PVHlp)	++(+)
(2) Magnocellular division (PVHm)	
(a) Anterior magnocellular part (PVHam)	++
(b) Posterior magnocellular part (PVHpm)	++

Table 1. Distribution of MC4-R mRNA in the Rat Central Nervous System

(3) Parvicellular division (PHVp)	
(a) Anterior parvicellular part (PHVap)	++
(b) Medial parvicellular part, dorsal zone (PVHmpd)	+++
(c) Periventricular part (PHVpv)	+
(h) Arcuate nucleus of the hypothalamus (ARH)	+
(i) Posterior periventricular nucleus of the hypothalamus (PVp)	++
2. Medial zone of the hypothalamus (MEZ)	
a. Medial preoptic area (MPO)	
(1) Medial preoptic nucleus (MPN)	
(a) Lateral part (MPNl)	++
(b) Medial part (MPNm)	++++
(c) Central part (MPNc)	+++
b. Anterodorsal preoptic nucleus (ADP)	+
c. Anteroventral preoptic nucleus (AVP)	+++(+)
d. Posterodorsal preoptic nucleus (PD)	
e. Anterior hypothalamic area (AHA)	
(1) Anterior hypothalamic nucleus (AHN)	
(a) Anterior part (AHNa)	++(+)
(b) Central part (AHNc)	++(+)
(c) Posterior part (AHNp)	++(+)
f. Tuberal area of the hypothalamus (TUA)	
(1) Ventromedial nucleus of the hypothalamus (VMH)	
(a) Dorsomedial part (VMHdm)	+
(b) Ventrolateral part (VMHvl)	+++
(2) Dorsomedial nucleus of the hypothalamus (DMH)	
(a) Anterior part (DMHa)	+++
(b) Posterior part (DMHp)	+
(c) Ventral part (DMHv)	+
(3) Ventral premammillary nucleus (PMv)	++
g. Mammillary body (MBO)	
(1) Tuberomammillary nucleus (TM)	
(a) Dorsal part (TMd)	+++
(b) Ventral part (TMv)	++
(2) Medial mammillary nucleus (MM)	++
h. Posterior hypothalamic nucleus (PH)	++(+)
3. Lateral zone of the hypothalamus (LZ)	
a. Lateral preoptic area (LPO)	++(+)
b. Lateral hypothalamic area (LHA)	+++
II. Brainstem (BS)	
A. Sensory	
1. Visual	
a. Superior colliculus (SC)	
(1) Optic layer (SCop)	++++
(2) Intermediate gray layer (SCig)	++
(3) Deep gray layer (SCdg)	++
b. Pretectal region (PRT)	
(1) Nucleus of the optic tract (NOT)	++++
(2) Posterior pretectal nucleus (PPT)	+
(3) Nucleus of the posterior commissure (NPC)	++(+)
(4) Anterior pretectal nucleus (APN)	+
(5) Medial pretectal area (MPT)	+
c. Medial terminal nucleus of the accessory optic tract (MT)	++
2. Somatosensory	
a. Spinal nucleus of the trigeminal (SPV)	
(1) Caudal part (SPVC)	++(+)
3. Auditory	
a. Nucleus of the lateral lemniscus (NLL)	+
b. Inferior colliculus (IC)	
(1) External nucleus (ICe)	+
4. Gustatory	
a. Nucleus of the solitary tract, rostral zone of medial part (NTSm)	+
5. Visceral	
a. Nucleus of the solitary tract (NTS)	
(1) Medial part, caudal zone (NTSm)	+
b. Parabrachial nucleus (PB)	
(1) Medial division (PBm)	
(a) Medial part (PBmm)	++
(2) Lateral division (PBl)	+
(a) Central lateral part (PBlc)	+
(b) External lateral part (PBle)	++
B. Motor	
1. Viscera	
a. Inferior salivatory nucleus (ISN)	+
b. Dorsal motor nucleus of the vagus nerve (DMX)	++++
c. Nucleus ambiguus, ventral division (AMBv)	++
2. Extrapyramidal	
a. Substantia nigra (SN)	
(1) Compact part (SNc)	++
(2) Reticular part (SNr)	++
b. Ventral tegmental area (VTA)	+
C. Pre- and postcerebellar nuclei	
1. Red nucleus (RN)	+++
D. Reticular core	
1. Central gray of the brain (CGB)	
a. Periaqueductal gray (PAG)	++
b. Interstitial nucleus of Cajal (INC)	+
c. Dorsal tegmental nucleus (DTN)	+
2. Raphé (RA)	
a. Superior central nucleus raphé (CS)	
(1) Medial part (CSm)	+(+)
(2) Lateral part (CSl)	+(+)
b. Dorsal nucleus raphé (DR)	+
c. Nucleus raphé magnus (RM)	+
d. Nucleus raphé pallidus (RPA)	++
3. Reticular formation (RET)	
a. Mesencephalic reticular nucleus (MRN)	++
(1) Retrorubral area (RR)	++
b. Pedunulopontine nucleus (PPN)	++
c. Pontine reticular nucleus (PRN)	
(1) Caudal part (RPNc)	+
d. Gigantocellular reticular nucleus (GRN)	++
e. Paragigantocellular reticular nucleus (PGRN)	
(1) Lateral part (PGRNl)	++(+)
f. Magnocellular reticular nucleus (MARN)	++(+)
g. Supratrigeminal nucleus (SUT)	+(+)
h. Parvicellular reticular nucleus (PARN)	+++
i. Medullary reticular nucleus (MDRN)	
(1) Dorsal part (MDRNd)	++
(2) Ventral part (MDRNv)	++
III. Spinal cord (SP)	
A. Dorsal horn of the spinal cord (DH)	
1. Substantia gelatinosa of the spinal cord (SGE)	++(+)

Semiquantitative estimates of the signals are indicated: + (weak); ++ (moderate), +++ (strong), with parentheses indicating intermediate levels.

among these being cell groups involved in processing visual information. Thus, very high levels of labeling were localized to the optic layer of the superior colliculus (Fig. 3C) and nucleus of the optic tract. Moderate to high levels of labeling were also observed over the nucleus of the posterior commissure, medial terminal nucleus of the accessory optic tract, and intermediate and deep gray layers of the superior colliculus. The only somatosensory brainstem region that contained labeled cells was the caudal part of the spinal nucleus of the

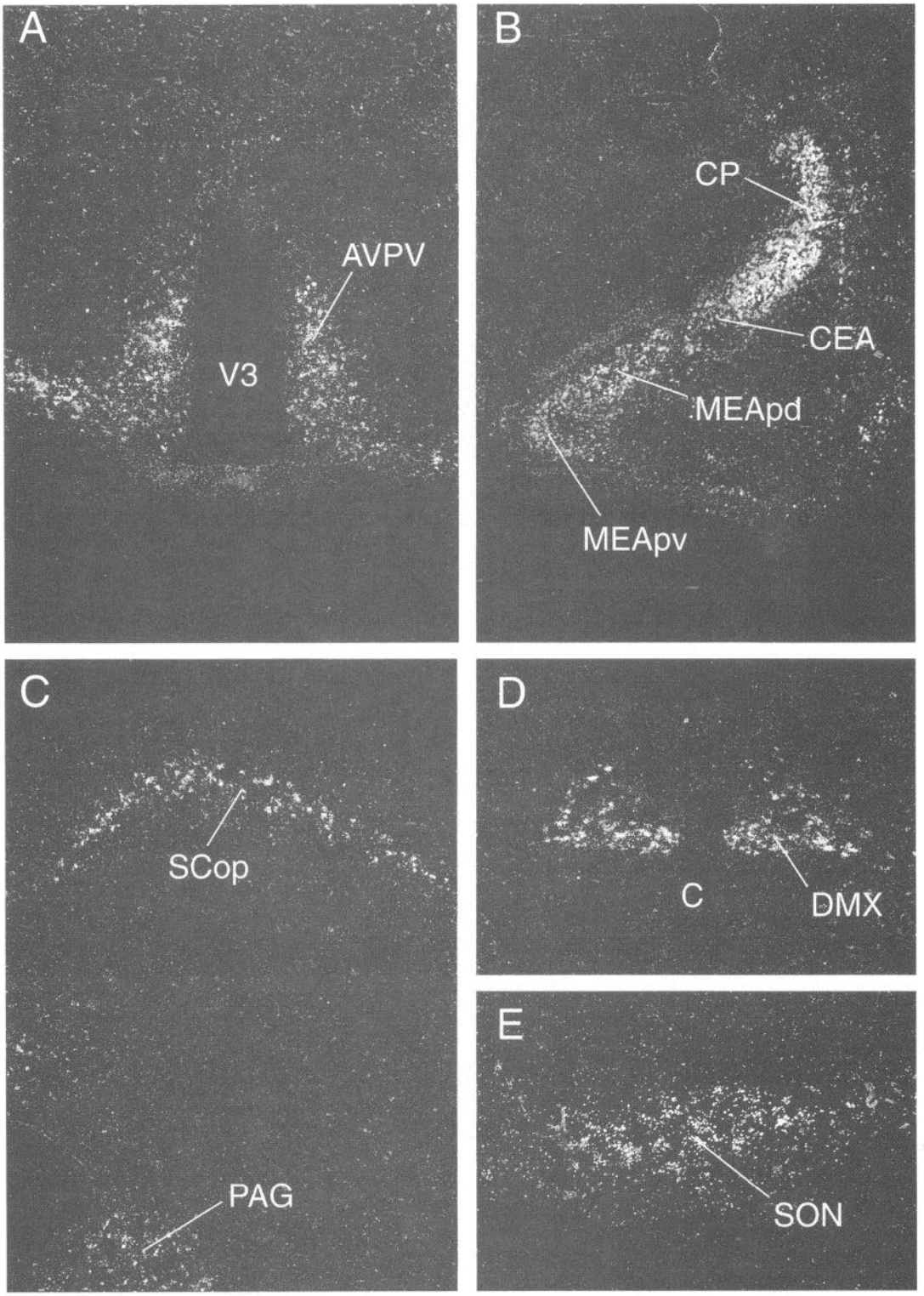

Fig. 3. Expression of MC4-R mRNA in Anteroventral Periventricular Nucleus (A), Amygdala (B), Superior Colliculus and Periaqueductal Gray (C), Dorsal Motor Nucleus of the Vagus (D), and Supraoptic Nucleus (E)

V3, Third ventricle; AVPV, anteroventral periventricular nucleus; CP, caudupatamen; CEA, central nucleus of the amygdala; MEApd, medial nucleus of the amygdala, posterodorsal part; MEApv, medial nucleus of the amygdala, posteroventral part; SCop, superior colliculus, optic layer; PAG, periaquaductal grey; C, central cavity; DMX, dorsal motor nucleus of the vagus nerve; SON, supraoptic nucleus. Photos show MC4-R-positive cell bodies using darkfield microscopy. Magnifications are ×100 (A-D) or ×150 (E). No hybridization was detected using a sense cRNA probe synthesized from the same MC4-R fragment.

trigeminal nerve. In contrast to the widespread distribution of MC4-R mRNA-containing cells in auditory regions of isocortex, only a few labeled neurons were detected in brainstem auditory pathways. Thus, only a few labeled neurons were detected in the nucleus of the lateral lemniscus and external nucleus of the inferior collicus. Similarly, labeling was sparse throughout brainstem regions that relay visceral sensory information, but a few cells were detected in the medial part of the nucleus of the solitary tract and in the medial part of the medial division of the parabrachial nucleus.

The most intensely labeled cells in the rat brain were contained in the dorsal motor nucleus of the vagus nerve (Fig. 3D), with fewer cells in the ventral division of the nucleus ambiguus, which provides preganglionic parasympathetic innervation to the heart. A role for MC4-R in somatomotor function is suggested by the moderate to high levels of labeling detected in the red nucleus. A moderate number of labeled cells were localized to the substantia nigra, with fewer cells distributed throughout the ventral tegmental area. Low to moderate densities of MC4-R mRNA-containing neurons were distributed throughout several regions that can be considered collectively as part of the reticular core of the brainstem. These include labeled neurons in the periaqueductal gray (Fig. 3C), dorsal tegmental nucleus, the superior central and dorsal nuclei of the raphé as well as the nucleus raphé magnus and pallidus. In addition, the mesencephalic reticular nucleus and pedunculopontine and gigantocellular nuclei contained moderate numbers of labeled cells as did the magnocellular supratrigeminal and parvicellular reticular nuclei of the brainstem. Labeling was generally absent from all regions of spinal cord, one notable exception being a band of labeled cells at the border between marginal zone and the substantia gelatinosa.

DISCUSSION

The discovery and characterization of the MC4-R demonstrate the existence of a previously unknown family of melanocortin receptors in addition to the well characterized MSH and ACTH receptors. These newly described receptors, MC3-R (24, 25), MC4-R (26, 31), and MC5-R (27–30), may be considered a subfamily of the melanocortin receptors in that they are significantly more closely related to one another (55–61% amino acid identity) than they are to the MSH-R (MC1-R) and ACTH-R (MC2-R) (43–47% identity).

While the neural MC3 and MC4 receptors are highly related, they are unique both in their pharmacological properties and in their distribution of expression in the brain. The peripheral melanocortin receptors demonstrate significant ligand specificity; in most mammals the ACTH and MSH receptors respond somewhat selectively to ACTH and α-MSH, respectively. For example, α-MSH is 10,000 times less potent than ACTH in inducing adrenal steroidogenesis in the rat (34), but 10-fold more potent in stimulating melanocytes (35). In contrast, the MC4 receptor is similar to the other neural receptor, MC3-R, in that it responds equally well to ACTH and α-MSH melanocortin peptides. Human MC4-R is distinguishable from the rat and human MC3-R in one potentially interesting property: both MC3-Rs are activated as potently by the γ-MSH peptides as they are by the α-MSH or ACTH peptides while hMC4-R responds only very poorly to γ-MSH ($EC_{50} = 3 \times 10^{-7}$ M) (Fig. 2). Thus far, all of the melanocortin receptors have been demonstrated to couple to G_s and activate adenylyl cyclase; however, the potential activation of other signal transduction pathways has not yet been examined in detail.

Neurons that express MC4-R mRNA have a widespread distribution that includes more than 100 different discrete structures that extend through each major division of the brain. This is in marked contrast to MC3-R mRNA-containing neurons, which are largely localized to the hypothalamus (arcuate nucleus and forebrain-projecting POMC neurons), restricted parts of the thalamus, and only a few brainstem nuclei (25). This distribution corresponds quite well with the published distribution of γ-MSH immunoreactivity (36) and is interesting given the unique ability of MC3-R to bind γ-MSH. MC4-R is activated only poorly by γ-MSH, and the present results indicate that the distribution of MC4-R mRNA-containing neurons is quite distinct from that of γ-MSH immunoreactivity or that of MC3-R mRNA-containing neurons in the rat brain. However, the combined distribution of MC3-R plus MC4-R mRNA-containing cells corresponds quite well with that of NDP-MSH binding sites (10), consistent with the high affinity of both receptors for this ligand. The presence of either MC4-R and/or MC3-R in every brain nucleus known to bind NDP-MSH suggests that there may not be any other additional neural melanocortin receptors. However, several questions remain to be resolved. These include the nature of the ORG2766 binding sites and the observation that, while there is apparent heterogeneity of NDP-MSH binding sites in the rat brain as assayed with ligand competition binding studies using brain sections, the heterogeneity does not clearly reproduce the distribution of MC3-R and MC4-R mRNAs (37). Anti-receptor antibodies may help resolve some of these issues; they will allow one to examine actual receptor distribution rather than the distribution of receptor mRNA-containing cell bodies.

MC4-R mRNA-containing neurons are found in several hypothalamic regions, but generally they are in greater abundance and are located in different cell groups than those with MC3-R mRNA. Melanocortin peptides have been implicated in the central control of CRH release (17, 38), and the presence of MC4-R mRNA in the PVH suggests a unique role for this receptor in neuroendocrine control. Thus, the expression of MC4-R mRNA in the medial parvicellular part of the PVH is consistent with a role for POMC peptides in regulating certain aspects of stress responses, but portions of the PVH that provide descending projections

to autonomic preganglionic neurons also appear to express MC4-R mRNA, suggesting a role for the receptor in regulating autonomic responses as well. Likewise, only MC4-R mRNA appears to be expressed by magnocellular neurons that project to the posterior pituitary, which may be indicative of yet another functional distinction between these two receptor systems.

The apparent overlap between the distribution of MC3-R and MC4-R neurons in nuclei such as the anteroventral periventricular, ventral premammilary, and posterior hypothalamic nuclei raises the possibility that both receptors are coexpressed within individual neurons, but this remains to be demonstrated directly. Nevertheless, in most regions containing both receptor mRNAs their distribution does not overlap. For example, MC3-R mRNA is expressed by many of the cells in the dorsomedial part of the ventromedial hypothalamic nucleus, yet most of the MC4-R mRNA-containing neurons are localized to its ventrolateral part. These distinct distributions may have behavioral consequences since the dorsomedial and ventrolateral parts of the ventromedial nucleus of the hypothalamus appear to be involved in separate pathways related to the neural control of feeding and reproductive pathways, respectively (39).

Regions such as the superior colliculus and auditory regions of the isocortex lack MC3 mRNA but contain high densities of MC4-R mRNA containing neurons suggesting a role for MC4-R in modulating the flow of visual and auditory sensory information. The unique expression of MC4-R mRNA in regions such as the caudoputamen, nucleus accumbens, substantia nigra, and red nucleus suggests that MC4-R may also coordinate aspects of somatomotor control. The expression in discrete regions of the isocortex implicates MC4-R in higher cortical processes, and the high number of positive neurons in the entorhinal cortex suggests that MC4-R may play a role in mediating processes underlying learning and memory.

Interestingly, MC4-R mRNA is also uniquely abundant in brainstem nuclei such as the dorsal motor nucleus of the vagus and ventral division of the nucleus ambiguus and may, therefore, participate in the modulation of autonomic outflow to the heart.

The presence of MC4-R mRNA in regions of the cortex involved in learning and memory would seemingly provide a pharmacological basis for the many reports of effects of the melanocortin peptides on the retention of learned avoidance behaviors (for review see Ref. 33). Unfortunately, a pharmacological paradox remains. In parallel with MC3-R, MC4-R does not respond well to $ACTH_{4-10}$ and does not respond at all to the synthetic $ACTH_{4-9}$ analog ORG2766. These compounds are often more active than the native melanocortins in behavioral assays involving the retention of learned behaviors (40), and in neural regeneration assays (41, 42). Additional work will be necessary to definitively link the neural MC3 and MC4 receptors with reported neuroendocrine, cardiovascular, and behavioral activities of the melanocortin peptides.

MATERIALS AND METHODS

Isolation of a Partial Rat MC4-R Clone by PCR

Total RNA from rat brain was reverse transcribed using oligo dT and superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD) at 42 C for 90 min. This cDNA was used as a template for PCR with degenerate oligonucleotides based upon sequence from transmembrane domains II and VII of the human ACTH and MSH receptors. The transmembrane domain II oligonucleotide was 5′-GAGTCGACC(AG)CCCATGTA-(CT)T(AGT)(CT)TTCATCTG-3′, and the transmembrane VII oligonucleotide was 5′-CAGAATTCGGAA(AG)GC(AG)TA(GT)-ATGA(AG)GGGGTC-3′. Parentheses indicate variable nucleotide positions containing one of the species indicated. These oligonucleotides encode restriction sites for *Eco*RI and *Sal*I at their 5′-ends to facilitate subcloning of the amplified DNA. The PCR conditions were 94 C, 1 min; 42 C, 2 min; 72 C, 2 min; for 40 cycles, and then 72 C for 10 min. The amplified DNA products were separated on a 1.2% agarose gel and stained with ethidium bromide. Regions in the gel between 800 and 200 bp were isolated using NA40 paper (Shleicher & Schuell, Keene, NH), subcloned into pBKS- (Stratagene, La Jolla, CA), and sequenced by dideoxy sequencing (43). An 800-bp amplified DNA fragment was identified that encoded a novel melanocortin receptor which was related to the ACTH and MSH receptors.

Isolation of a Full Length Human MC4-R Clone

The *Eco*RI-*Sal*I 800-bp rat MC4-R PCR clone was labeled with ^{32}P by random priming (Pharmacia, Piscataway, NJ) and used as a probe to screen a human genomic DNA library in the EMBL3 vector (kindly provided by Dr. C. Passavant). The library was hybridized under moderate hybridization conditions to allow cross-reactivity of the rat probe with the hMC4-R. The hybridization conditions were 40% formamide, 1 mM NaCl, 50 mM Tris (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), salmon sperm DNA (100 μg/ml), and 10× Denhardt's at 42 C for 18 h. Twenty-nine independent clones were isolated, and phage DNA from each one was prepared. The genomic inserts were digested from the phage, run on a 1.2% agarose gel, and Southern blotted. By sequential probing of this Southern blot with rat MC4-R, human ACTH-R, human MSH-R, and rat MC3-R probes, we identified 28 phage of which 18 encoded for the hMC4-R, three for the human MSH-R, two for the human ACTH-R, and two for the human MC3-R. One phage containing hMC4-R was restriction mapped, subcloned, and sequenced with the dideoxy sequencing method (43). A full length coding hMC4-R was found in a 1.9-kb *Hin*dIII fragment.

Functional Coupling of hMC4-R to Adenylyl Cyclase

A full length coding fragment of the hMC4-R gene was amplified by PCR using a 20-kb phage insert containing the complete coding exon of the gene as template and oligonucleotides designed against the 5′- and 3′-untranslated regions (UTR) of the gene. Vent polymerase (New England Biolabs, Beverly, MA) was used in order to reduce the misincorporation frequency. The 5′-UTR oligonucleotide was 5′-CAG-GATCCTAAATCAATTCAGGGGAC-3′, and the 3′-UTR oligonucleotide was 5′-AGCTCGAGCTGTTGCAGAAGTACAAT-3′. These oligonucleotides encode restriction sites for *Bam*H I and *Xho*I at their 5′-ends to facilitate subcloning of the amplified DNA. The PCR conditions were 94 C, 1 min; 53 C, 1.5 min; 72 C, 1.5 min; for 30 cycles followed by 72 C for 10 min. The amplified DNA was subcloned into pcDNAI/Neo (Invitrogen, San Diego, CA) and stably transfected into human embryonic kidney 293 cells using a modified $CaPO_4$ procedure (44), and populations of stably transfected cells were selected by growth in medium containing 1 mg/ml G418 (GIBCO, Grand

Island, NY). Cells (~10^5) were plated in 24-well dishes and grown in Dulbecco's modified essential medium (DMEM) containing 10% calf serum for 3 days. Adenylyl cyclase activity was then determined directly by measuring the ability of cells to convert [^{3}H]adenine to [^{3}H]cAMP (45) after exposure of the cells to various natural and synthetic POMC peptides (Bachem, Torrance, CA). Duplicate wells were incubated for 2 h with 2.5 μCi [^{3}H]adenine in DMEM containing 10% calf serum. Medium was aspirated and the cells were washed once with PBS warmed to 37 C. The cells were then exposed for 1 h to varying concentrations of NDPα-MSH, α-MSH, ACTH$_{1-39}$, desacetyl α-MSH, ACTH$_{4-10}$, γ2-MSH, CLIP, β-endorphin, and ORG2766 in the presence of DMEM containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine. The medium with peptides was aspirated and the cells solubilized with 1 ml 2.5% perchloric acid, 0.1 mM cAMP. Lysate (0.8 ml) was removed, neutralized with 80 μl 4.2 N KOH, and 0.42 ml H_2O. The samples were mixed, and the sediment was allowed to settle. Lysate was counted in a β-counter to determine the total amount of [^{3}H]adenine incorporated into cells. cAMP was separated from the lysate after sequential chromatography over Dowex and alumina columns (46). cAMP was eluted from the alumina columns in 4 ml Tris (pH 7.4) and counted in a β-counter. Relative cyclase activity was calculated by determining the percentage of [^{3}H]adenine converted into [^{3}H]cAMP. The Kaleidograph software package (Synergy Software, Reading, PA) was used for fitting curves to the data and calculating EC_{50} values.

In Situ Hybridization Analysis of the Expression of MC4-R mRNA in Rat Brain

Sequencing of the 800-bp rat MC4-R fragment indicated the presence of an unidentified approximately 200-bp DNA insert with no significant homology to any known sequence in the Genbank data base, possibly an artifact of the PCR. This necessitated the isolation of a fragment colinear with the rat genomic MC4-R sequence for *in situ* hybridization studies. Degenerate olionucleotides from transmembrane domains II and VII of the human ACTH and MSH receptors were used for the first round of PCR using conditions described above for isolation of the 800-bp partial rat MC4-R clone. Ten microliters of this reactions were then amplified by PCR using oligonucleotides specific to the rat MC4-R sequence in transmembranes III and and VII. The transmembrane III oligonucleotide was 5′-GGGTCAGTTTCCATCGT-3′, and the transmembrane VII oligonucleotide was 5′-TTAAAATGAGACATGAAG-3′. The PCR conditions were 94 C, 1 min; 50 C, 2 min; 72 C, 2 min; for 40 cycles followed by 72 C for 10 min. The amplified DNA was run on a 1.5% agarose gel and stained with ethidium bromide. A 600-bp fragment was isolated using NA40 paper, subcloned into pBKS-, and sequenced by the dideoxy method to confirm the presence of an intact MC4-R sequence fragment. Male Sprague-Dawley rats (Simonsen Labs, Gilroy CA) were anesthetized and perfused with 1000 ml 4% paraformaldehyde in borate buffer, pH 9.5, at 4 C (fixation buffer). Brains were dissected and incubated in fixation buffer for 8 h and then overnight in fixation buffer with 10% sucrose. After this the brains were serially sectioned into 10 series of 30-μM slices with a sliding microtome. The 600-bp fragment of the rat MC4-R in pBKS was used to synthesize an antisense [^{35}S]cRNA probe. Sections were prepared and hybridized as described previously (47). Sections were hybridized with ^{35}S-labeled probe (~ 1×10^7 cpm/ml) in 65% formamide, 0.26 M NaCl, 1.3× Denhardt's solution, 1.3 mM EDTA, 13% dextran sulfate, 13 mM Tris, pH 8. Slides were then digested with RNase (20 μg/ml for 30 min at 37 C), washed in 4× SSC, then rinsed to a final stringency of 0.1 × SSC at 65 C for 30 min. Sections were dehydrated, dipped in NTB-2 emulsion (Kodak), and developed after 21 days.

Acknowledgements

The authors thank Anne Carr for technical assistance and Robin Chedester and June Shiigi for assistance in the preparation of the manuscript.

Received February 21, 1994. Re-revised manuscript received and accepted July 8, 1994.

Address requests for reprints to: Roger D. Cone, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97201-3098.

The work was supported by NIH Grants HD-30236 (to M.J.L., R.B.S., and R.D.C.), NS-26723 (to R.B.S.), RR-00163 (to R.B.S), and DK-43859 (to R.D.C.).

*Present Address: Research Centre for Developmental Medicine and Biology, Department of Pediatrics, School of Medicine, University of Auckland, Park Road, Auckland 1, New Zealand

REFERENCES

1. Watson SJ, Akil H, Richard CW, Barchas JD 1978 Evidence for two separate opiate peptide neuronal systems and the coexistence of beta-lipotropin, beta-endorphin, and ACTH immunoreactivities in the same hypothalamic neurons. Nature 275:226–228
2. Jacobowitz DM, O'Donohue TL 1978 α-melanocyte stimulating hormone; Immunohistochemical identification and mapping in neurons of rat brain. Proc Natl Acad Sci USA 75:6300
3. Nilaver G, Zimmerman EA, Defendi R, Liotta A, Krieger DT, Brownstein MJ 1979 Adrenocorticotropin and beta-lipotropin in the hypothalamus. Localization in the same arcuate neurons by sequential immunocytochemical procedures. J Cell Biol 81:50–58
4. Joseph SA, Pilcher WH, Bennet-Clarke C 1983 Immunocytochemical localization of ACTH parikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. Neurosci Lett 38:221
5. Palkovits M, Mezey E, Eskay RL 1987 Pro-opiomelanocortin-derived peptides (ACTH/beta-endorphin/alpha-MSH) in brainstem baroreceptor areas of the rat. Brain Res 436:323–328
6. Bronstein DM, Schafer MK, Watson SJ, Akil H 1992 Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. Brain Res 587:269–275
7. Khatchaturian H, Alesi NE, Munfakh N, Watson SJ 1983 Ontogeny of opioid and related peptides in the rat CNS and pituitary: an immunocytochemical study. Life Sci 33:61–64
8. Smith AI, Funder JW 1988 Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. Endocr Rev 9:159–179
9. Hnatowich MR, Queen G, Stein D, LaBella FS 1989 ACTH receptors in nervous tissue. High affinity binding-sequestration of [125I][Phe^2,Nle^4]ACTH 1–24 in homogenates and slices from rat brain. Can J Physiol Pharmacol 67:568–576
10. Tatro JB 1990 Melanotropin receptors in the brain are differentially distributed and recognize both corticotropin and α-melanocyte stimulating hormone. Brain Res 536:124–132
11. Lichtensteiger W, Hanimann B, Schlumph M, Siegrist W, Eberle AN 1993 Pre- and postnatal ontogeny of [^{125}I]Nle^4, D-Phe^7-α-MSH binding sites in rat brain. Ann NY Acad Sci 680:652–654
12. Feng JD, Dao T, Lipton JM 1987 Effects of preoptic

microinjections of α-MSH on fever and normal temperature control in rabbits. Brain Res 18:473–477
13. Lin SY, Chaves C, Wiedemann E, Humphreys MH 1987 A γ-melanocyte stimulating hormone-like peptide causes reflex natriuresis after acute unilateral nephrectomy. Hypertension 10:619–627
14. Gruber KA, Callahan MF 1989 ACTH (4–10) through γ-MSH: Evidence for a new class of central autonomic nervous system-regulating peptides. Am J Physiol 257:R681-R694
15. Murphy JV, Miller RE 1955 The effect of adrenocorticotrophic hormone (ACTH) on avoidance conditioning in the rat. J Comp Physiol Psychol 48:47–49
16. Garrud P, Gray JA, DeWied D 1974 Pituitary-adrenal hormones and extinction of rewarded behavior in the rat. Physiol Psychol 112:109–119
17. Wiegant VM, Jolles J, Colbern DL, Zimmermann E, Gispen WH 1979 Intracerebroventricular ACTH activates the pituitary-adrenal system: Dissociation from a behavioral response. Life Sci 25:1791–1796
18. Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG 1992 The δ-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89:12048–12052
19. Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH 1992 Cloning of a delta opioid receptor by functional expression. Science 258:1952–1955
20. Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI 1993 Cloning and functional comparison of κ and δ opioid receptors from mouse brain. Proc Natl Acad Sci USA 90:6736–67740
21. Chen Y, Mestek A, Liu J, Hurley JA, Yu L 1993 Molecular cloning and functional expression of a μ-opioiod receptor from rat brain. Mol Pharmacol 44:8–12
22. Mountjoy KG, Robbins LS, Mortrud MT, Cone RC 1992 The cloning of a family of genes that encode the melanocortin receptors. Science 257:543–546
23. Chhajlani V, Wikberg JES 1992 Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett 309:417–420
24. Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J, Yamada T 1993 Molecular cloning of a novel melanocortin receptor. J Biol Chem 268:8246–8250
25. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB, Cone RD 1993 Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc Natl Acad Sci USA 90:8856–8860
26. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, Yamada T 1993 Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 268:15174–15179
27. Chhajlani V, Muceniece R, Wikberg JES 1993 Molecular cloning of a novel human melanocortin receptor. Biochem Biophys Res Commun 195:866–873
28. Griffon N, Mignon V, Facchinetti P, Diaz J, Schwartz J-C, Sokoloff P 1994 Molecular cloning and characterization of the rat fifth melanocortin receptor. Biochem Biophys Res Commun 200:1007–1014
29. Gantz I, Shimoto Y, Konda Y, Miwa H, Dickinson CJ, Yamada T 1994 Molecular cloning, expression, and characterization of a fifth melanocortin receptor. Biochem Biophys Res Commun 200:1214–1220
30. Labbe O, Desarnaud F, Eggerickx D, Vassart G, Parmentier M 1994 Molecular cloning of a mouse melanocortin 5 receptor gene widely expressed in peripheral tissues. Biochemistry 33:4543–4549
31. Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD, Cloning, characterization of a neural melanocortin receptor. Program of the 75th annual meeting of The Endocrine Society, Las Vegas, NV, 1993, p 471 (Abstract 1681)
32. Sawyer TK, San Filippo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB, Hadley ME 1980 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: a highly potent α-melanotropin with ultralong biological activity. Proc Natl Acad Sci USA 77:5754–5758
33. DeWied D, Jolles J 1982 Neuropeptides derived from proopiocortin: behavioral, phsyiological, and neurochemical effects. Physiol Rev 62:977–1059
34. Baumann JB, Eberle AN, Christen E, Ruch W, Girard J 1986 Steroidogenic activity of highly potent melanotropic peptides in the adrenal cortex of the rat. Acta Endocrinol (Copenh) 113:396–402
35. Eberle AN 1988 The Melanotropins. Chemistry, Physiology, and Mechanisms of Action. S. Karger Publishers, Basel, p 334
36. Kawai Y, Inagaki S, Shiosaka S, Shibasaki T, Ling N, Tohyama M, Shiotani Y 1984 The distribution and projection of gamma-melanocyte-stimulating hormone in the rat brain: an immunohistochemical analysis. Brain Res 297:21–32
37. Tatro JB, Entwistle ML 1994 Heterogeneity of brain melanocortin receptors suggested by differential ligand binding *in situ*. Brain Res 635:148–158
38. Calogero AE, Gallucci WT, Gold PW, Chrousos GP 1988 Multiple feedback regulatory loops upon rat hypothalamic corticotropin-releasing hormone secretion. J Clin Invest 82:767–774
39. Canteras NS, Simerly RB, Swanson LW 1994 Organization of projections from the ventromedial hypothalamic nucleus: a PHAL study in the rat. J Comp Neurol, in press
40. DeWied D, Wolterink G 1988 Structure-activity studies on the neuroactive and neurotropic effects of neuropeptides related to ACTH. Ann NY Acad Sci 525:130–140
41. Bijlsma WA, Jennekens FGI, Schotman P, Gispen WH 1981 Effects of corticotrophin (ACTH) on recovery of sensorimotor function in the rat: structure-activity study. Eur J Pharmacol 76:73
42. Strand FL, Kung TT 1980 ACTH accelerates recovery of neuromuscular function following crushing of peripheral nerves. Peptides 1:135–138
43. Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
44. Chen C, Okayama H 1987 High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752
45. Salomon Y 1991 Cellular responsiveness to hormones and neurotransmitters: conversion of [^{3}H]adenine to [^{3}H]cAMP in cell monolayers, cell suspensions, and tissue slices. Methods Enzymol 195:22–28
46. Johnson RA, Salomon Y 1991 Assay of adenylyl cyclase catalytic activity. Methods Enzymol 195:3–21
47. Arriza JL, Simerly RB, Swanson LW, Evans RM 1988 The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron 1:887–900