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Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system

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ABSTRACT **Corticotropin (ACTH) and melanotropin (MSH) peptides (melanocortins) are produced not only in the pituitary but also in the brain, with highest concentrations in the arcuate nucleus of the hypothalamus and the commisural nucleus of the solitary tract. We have identified a receptor for MSH and ACTH peptides that is specifically expressed in regions of the hypothalamus and limbic system. This melanocortin receptor (MC3-R) is found in neurons of the arcuate nucleus known to express proopiomelanocortin (POMC) and in a subset of the nuclei to which these neurons send projections. The MC3-R is 43% identical to the MSH receptor present in melanocytes and is strongly coupled to adenylyl cyclase. Unlike the MSH or ACTH receptors, MC3-R is potently activated by γ-MSH peptides, POMC products that were named for their amino acid homology with α- and β-MSH, but lack melanotropic activity. The primary biological role of the γ-MSH peptides is not yet understood. The location and properties of this receptor provide a pharmacological basis for the action of POMC peptides produced in the brain and possibly a specific physiological role for γ-MSH.**

The large proopiomelanocortin (POMC) protein is processed into three main families of peptides with adrenocorticotropic, melanotropic, or opiate activities. The melanocortins, which include all POMC peptides except β-endorphin, are primarily known for their role in the regulation of adrenal steroid production [corticotropin (ACTH)] and pigmentation [α melanotropin (α-MSH)]. In addition to these well-known effects, administration of melanocortin peptides has been reported to increase retention of learned behaviors (1, 2), induce grooming behavior (3), decrease fever (4, 5), stimulate nerve regeneration (6, 7), and increase heart rate, blood pressure, and natriuresis (8, 9). Many of these biological activities have been demonstrated to result from direct action of the melanocortin peptides in the brain.

Recently, the cloning of the MSH (10, 11) and ACTH receptors (10) (MSH-R and ACTH-R) has provided probes for the examination of MSH-R and ACTH-R mRNA expression. Thus far, MSH-R and ACTH-R mRNA expression has only been detected in melanocytes and in the adrenal cortex, respectively. Although specific high-affinity MSH and ACTH binding has been reported in brain, with highest levels in the hypothalamus (12, 13), no MSH-R or ACTH-R mRNA was detected in the brain by either Northern hybridization or *in situ* hybridization (14, 15). Additionally, melanocortin binding and biological action in the brain display pharmacological profiles that do not match those of either the adrenal ACTH-R or the melanocyte MSH-R. These data suggested the existence of unique melanocortin receptor(s) expressed specifically in the brain.

We report here the cloning and characterization of a neural-specific melanocortin receptor from a rat hypothalamus cDNA library. This is the third melanocortin receptor reported and has been termed the melanocortin 3 receptor (MC3-R) to distinguish it from the peripheral MSH-R and ACTH-R.

MATERIALS AND METHODS

Isolation of a MC3-R cDNA Clone. A 400-bp *Eco*RI/*Sal* I PCR fragment from the human MSH-R was used to screen a λgt10 rat hypothalamic cDNA library (kindly provided by R. H. Goodman, Vollum Institute) at low stringency [30% formamide, 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 0.1% sodium pyrophosphate, 0.2% SDS, 100 μg of salmon sperm DNA per ml, and 10% Denhardt's solution] at 42°C for 18 hr. A single 1-kb cDNA clone was characterized by dideoxy sequencing (16), and the sequence was compared to the reported MSH-R and ACTH-R sequences (10). The sequence revealed that the 1-kb partial clone was a novel G-protein-linked receptor highly related to the MSH-R and ACTH-R. The 1-kb *Eco*RI insert from this cDNA clone was isolated and used to probe the same library at moderately high stringency (45% formamide), and a 2.0-kb clone was isolated, which encoded a full-length receptor. The 2.0-kb fragment was subcloned into the plasmid vector pBluescript SK− (Stratagene) and analyzed by dideoxy sequencing. Amino acid sequences were aligned using the GENALIGN program version 5.4 (IntelliGenetics).

Functional Coupling of the MC3-R to Adenylyl Cyclase and Ligand Binding. A 2.0-kb *Bam*HI/*Xho* I fragment of the full-length MC3-R cDNA clone was inserted in the expression vector pcDNA I Neo (Invitrogen), and human 293 kidney cells were transfected with the vector alone or the vector plus MC3-R insert by a modified calcium phosphate procedure (17). Stable populations of transfected cells were selected in medium containing 1 mg of G418 per ml (GIBCO). For analysis of functional coupling of the MC3-R to adenylyl cyclase, duplicate plates of cells (≈5×10^6) were stimulated with the peptides shown, and intracellular cAMP was measured with an assay (Amersham) that measures displacement of [5,8-^{3}H]cAMP from a high-affinity cAMP binding protein (18); this has been described in detail (19). Binding of melanocortin peptides to transfected 293 cells was assessed by inhibition of binding of ^{125}I-labeled Nle^4,D-Phe^7-α-MSH,

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Abbreviations: ACTH, corticotropin; ACTH-R, ACTH receptor; MSH, melanotropin; MSH-R, MSH receptor; POMC, proopiomelanocortin.

an α-MSH analogue, which, unlike α-MSH, can be iodinated without significantly decreasing its potency. Suspended cells (2×10^5 per assay) were incubated at 37°C with 50,000 cpm of label for 10 min in binding buffer (Ham's F10 medium/10 mM Hepes, pH 7.3/0.25% bovine serum albumin/500 kallikrein inhibitor units of aprotinin per ml/100 μg of bacitracin per ml/1 mM 1,10-phenanthroline) in the absence (control) or presence of the indicated concentrations of peptides. Label was prepared, incubations were performed, and cells were washed as described (20). The KALEIDAGRAPH software program (Synergy Software, Reading, PA) was used for fitting curves to the data and calculating EC_{50} and K_i values by the method of least squares.

Northern Analysis. RNA was prepared from various rat brain sections, rat adrenals, and the S91 Cloudman melanoma cell line using the guanidinium thiocyanate procedure (21) followed by poly(A)$^+$ purification using the PolyATrack mRNA isolation system (Promega). Poly(A)$^+$ RNAs (4 μg) were analyzed by electrophoresis on a 2.2 M formaldehyde/1.2% agarose gel, transferred by capillary blotting to a nylon membrane (Micron Separations, Westboro, MA), and hybridized with the 1-kb *Eco*RI MC3-R cDNA fragment under stringent conditions (50% formamide/5× SSC/0.1% sodium pyrophosphate/0.2% SDS/100 μg of salmon sperm DNA per ml/10% Denhardt's solution) at 42°C for 18 hr.

***In Situ* Hybridization.** Male Sprague–Dawley rats (200 g) were anesthetized and perfused with 1000 ml of 4% paraformaldehyde in borate buffer, pH 9.5 at 4°C (fixation buffer). Brains were dissected and incubated in fixation buffer for 8 hr and then incubated overnight in fixation buffer with 10% sucrose. Brains were sectioned serially into 10 series of 30-μm slices with a sliding microtome. Sections were prepared and hybridized as described (22). A 400-bp *Eco*RI/*Sal* I fragment of the MC3-R cDNA in the pBluescript KS vector (Stratagene) was used to synthesize an antisense cRNA probe. 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/13 mM Tris/1.3 mM EDTA/13% dextran sulfate, pH 8, at 65°C for 24 hr. Slides were washed in 4× SSC, digested with RNase (20 μg/ml for 30 min at 37°C), and 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.

RESULTS

Sequence Analysis and Functional Coupling of the MC3-R. To identify the pharmacological site(s) of action of the melanocortins in the brain we screened a rat hypothalamus cDNA library at low stringency using human MSH-R DNA as a hybridization probe. A 2.0-kb clone was identified that encoded a G-protein-coupled receptor of 323 amino acids, with ≈43% amino acid identity to the murine MSH-R, identifying the receptor as a member of the melanocortin receptor family (Fig. 1). MC3-R also shares several of the structural features reported for the MSH-R and ACTH-R (10), including the absence of a conserved cysteine residue in the first extracellular loop, thought to form a disulfide bond with the second extracellular loop in most G-protein-coupled receptors (23).

The 2.0-kb MC3-R cDNA was then inserted into the pcDNA NEO I expression vector and transfected into 293 cells for functional characterization of the receptor. Because the two known melanocortin receptors, the MSH-R and ACTH-R, both couple to adenylyl cyclase, we tested the ability of various melanocortin peptides to stimulate adenylyl cyclase activity in transfected cells. Every melanocortin peptide tested (Fig. 2) containing the core MSH sequence, His-Phe-Arg-Trp, produced a potent stimulation of adenylyl cyclase, elevating cAMP levels up to 60-fold, with EC_{50} values ranging from 1 to

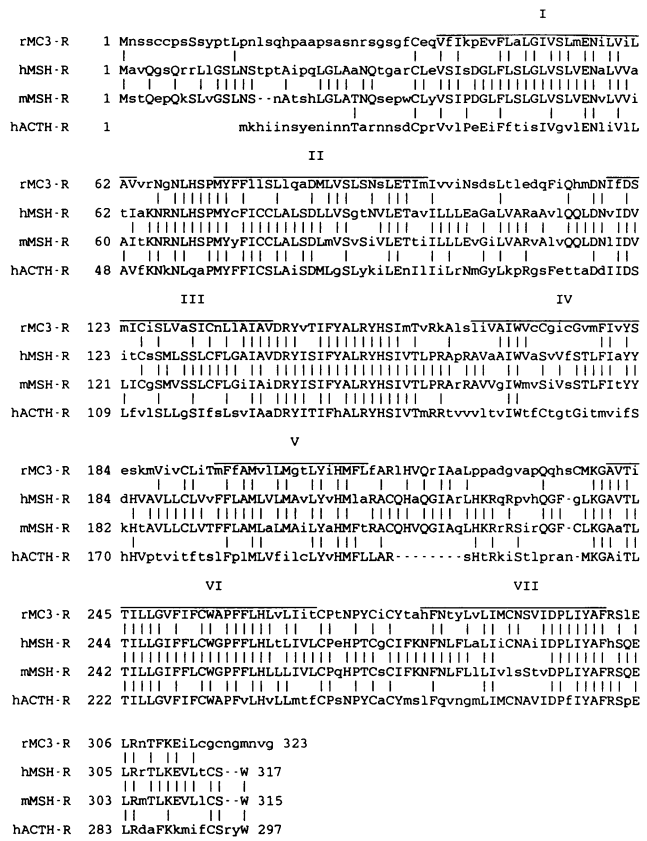

FIG. 1. Sequence alignment of the rat MC3-R with other members of the melanocortin receptor family. The rat MC3-R (rMC3-R), human and mouse MSH-R (hMSH-R and mMSH-R), and human ACTH-R (hACTH-R) amino acid sequences were aligned with the GENALIGN program, version 5.4 (IntelliGenetics). Bars indicate transmembrane regions I–VII, predicted by hydropathy analysis and by comparison with other members of the G-protein-coupled receptor family. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Nucleotide sequences are available through the GenBank/European Molecular Biology Laboratory data bases under accession nos. X65633 (mouse MSH-R), X65634 (human MSH-R), X65635 (human MSH-R), and X70667 (rat MC3-R).

100 nM (Fig. 3 *A–C*). Similar maximal levels of cyclase stimulation were obtained with each active peptide. Surprisingly, the most potent naturally occurring ligands were γ1- and γ2-MSH as well as α-MSH, thus identifying MC3-R as a melanocortin receptor that is responsive to physiological levels of γ-MSH peptides. The order of potency for synthetic ([Nle^4,D-Phe^7]-α-MSH, $ACTH_{4-10}$) and naturally occurring melanocortins was [Nle^4,D-Phe^7]-α-MSH > γ2-MSH–γ1-MSH–α-MSH–$ACTH_{1-39}$ > γ3-MSH > desacetyl α-MSH ≫ $ACTH_{4-10}$. γ1- and γ2-MSH were somewhat more potent than

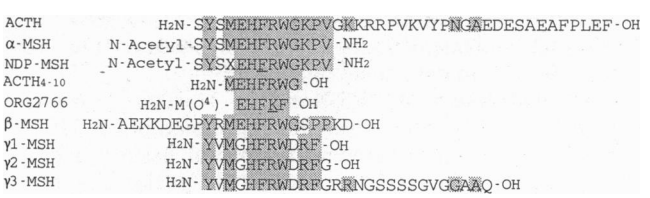

FIG. 2. Structure of the melanocortin peptides. Melanocortins used in this study are aligned with shading indicating amino acid residues conserved between two or more of the ACTH/α-MSH, β-MSH, and γ-MSH peptide families. Underlined residues are D-amino acid enantiomers and X indicates norleucine. NDP-MSH, [norleucine^4,D-Phe^7]-α-MSH.

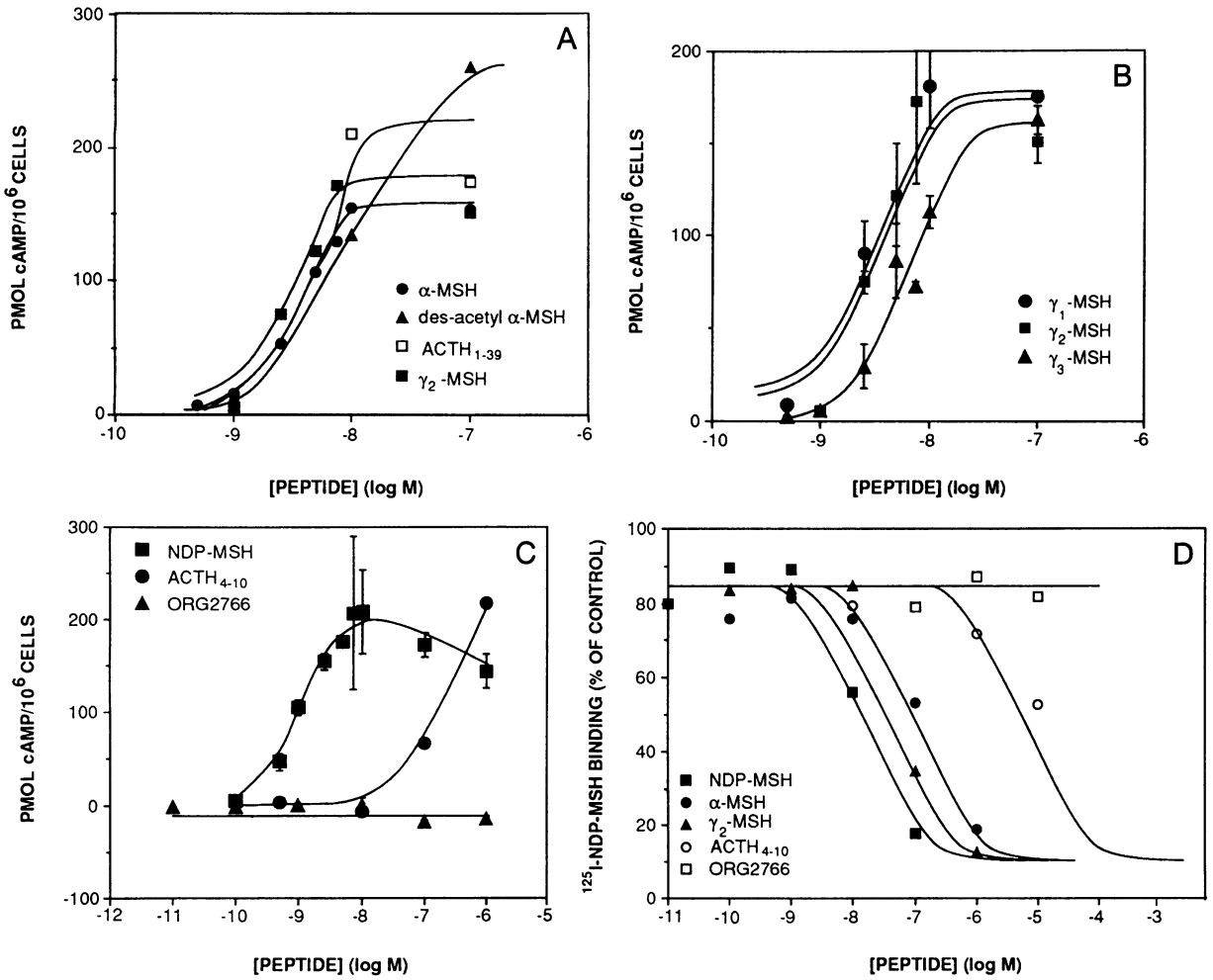

FIG. 3. Pharmacological characterization of the MC3-R. (*A–C*) Functional coupling of the MC3-R to adenylyl cyclase. 293 cells permanently transfected with a MC3-R expression vector (pcDNA/MC3-R) were incubated for 1 hr with various concentrations of the melanocortin peptides shown (Bachem). Curves show intracellular cAMP concentration as a function of increasing concentrations of representative melanocortin peptides: peptides found *in vivo* (*A*), γ-MSH variants (*B*), and synthetic melanocortin analogues (*C*). Points indicate the mean of two independent experiments and bars indicate standard deviation. Error bars in *A* did not exceed 15% of the mean and were left out for clarity. EC_{50} values were as follows: Nle^4,D-Phe^7-α-MSH ($1.6 \pm 0.27 \times 10^{-9}$ M), γ_2-MSH–γ_1-MSH–α-MSH–$ACTH_{1-39}$ ($3.8 \pm 1.45 \times 10^{-9}$ M), γ_3-MSH ($8.0 \pm 1.6 \times 10^{-9}$ M), desacetyl α-MSH ($13.8 \pm 1.7 \times 10^{-9}$), and $ACTH_{4-10}$ ($>10^{-7}$ M). (*D*) Binding of melanocortin peptides to the MC3-R. Points represent the mean of two separate experiments in which each measurement was performed in quintuplicate. K_i values were as follows: Nle^4,D-Phe^7-α-MSH ($1.0 \pm 0.18 \times 10^{-8}$ M), γ_2-MSH ($4.4 \pm 0.62 \times 10^{-8}$ M), α-MSH ($5.2 \pm 4.4 \times 10^{-8}$ M), and $ACTH_{4-10}$ ($>10^{-6}$ M). NDP-MSH, as in Fig. 2.

γ_3-MSH (Fig. 3*B*). ORG 2766, an $ACTH_{4-9}$ analogue (methionylsulfone-Glu-His-Phe-D-Lys-Phe) and $ACTH_{4-10}$, synthetic melanocortin peptides with little adrenocorticotropic activity but potent activity in various assays involving the central and peripheral nervous systems, either did not stimulate the MC3-R (ORG 2766) or had very reduced ability to activate adenylyl cyclase ($ACTH_{4-10}$) (Fig. 3*C*). ORG 2766 had no activity as an antagonist either (data not shown). Competition binding studies demonstrated approximate K_i values for the melanocortin peptides from 1×10^{-8} to $>10^{-6}$ M with an order of affinity (Nle^4,D-Phe^7–α-MSH > γ_2-MSH–α-MSH >> $ACTH_{4-10}$) equivalent to their order of potency (Fig. 3*D*). ORG 2766 had no detectable binding affinity.

Tissue Distribution of MC3-R mRNA. Northern hybridization analysis was used to examine expression of the MC3-R in brain regions and in several peripheral tissues (Fig. 4). Using 4 μg of poly(A)$^+$ RNA, two RNA species of approximately 2.0 and 2.5 kb were detected specifically in the hypothalamus, but in no other brain area. No MC3-R mRNA was detected in melanocytes or in the adrenal glands, the sites of expression of the MSH-R and ACTH-R. Additionally, in an experiment utilizing 20 μg of total cellular RNA, no MC3-R mRNA was detected in heart, liver, lung, testes, or thyroid (data not shown). A more sensitive technique, *in situ* hybridization, confirmed that MC3-R mRNA is found primarily in the hypothalamus but is present in small amounts in other brain regions as well (Fig. 5 and Table 1).

Anatomical Localization of MC3-R mRNA in the Brain. The detailed distribution of neurons that express MC3-R mRNA was determined by *in situ* hybridization using a 400-bp ^{35}S-labeled cRNA antisense probe (Fig. 5 and Table 1). The greatest density of MC3-R mRNA-containing neurons was found in the hypothalamus with heavily labeled cells in the arcuate nucleus, which contains all of the forebrain POMC-containing neurons. In addition, labeled cells were found in a subset of the presumptive terminal fields of arcuate POMC neurons (24) but were not present in the nucleus of the solitary tract, the other major site of POMC-positive cell bodies. This restricted pattern of expression corresponded remarkably well with that of γ-MSH immunoreactivity in the diencephalon and telencephalon (25) and represents a distinct subset of the melanocortin binding sites demonstrated by ^{125}I-labeled Nle^4,D-Phe^7-α-MSH binding to rat brain slices (13).

DISCUSSION

The identification and characterization of the MC3-R demonstrate the existence of a unique class of neural melanocor-

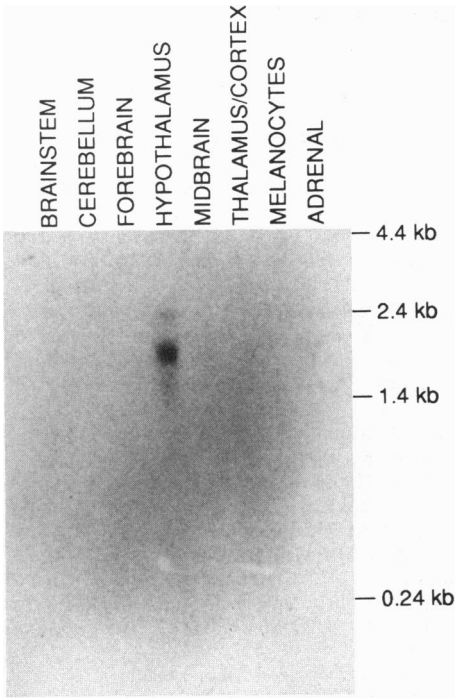

FIG. 4. Northern hybridization analysis of MC3-R mRNA distribution. Each lane contained 4 μg of poly(A)$^+$ RNA from the brain region or tissue indicated. Brain and adrenal tissues were from the rat, and the mouse Cloudman S91 melanoma cell line was used as a source of melanocyte RNA. The gel was transferred to a nylon membrane and probed with a ^{32}P-labeled 1-kb *Eco*RI fragment from the rat MC3-R cDNA. The hybridization and washes were performed under stringent conditions. The 0.24- to 9.5-kb RNA ladder (BRL) was used for size markers. The equivalent loading of RNA in each lane was demonstrated by methylene blue staining of the nylon filter prior to hybridization.

Table 1. MC3-R mRNA distribution in the brain

Region	Signal	Specifically labeled nuclei
Hypothalamus	++	Anteroventral preoptic nucleus
	+	Anteroventral periventricular nucleus
	+	Preoptic periventricular nucleus
	+(+)	Anterior hypothalamic nucleus
	+(+)	Lateral hypothalamic area
	++	Arcuate nucleus
	+++	Dorsomedial part of the ventromedial nucleus
	++	Posterior hypothalamic area
	+	Posterior periventricular nucleus
	+(+)	Ventral part of the premammillary nucleus
	(+)	Dorsal part of the premammillary nucleus
	+	Supramammillary nucleus
	+(+)	Medial preoptic nucleus
	+(+)	Lateral preoptic area
Septum	++	Intermediate part of the lateral nucleus
	+	Dorsomedial nucleus of the bed nuclei of the stria terminalis
	+	Anterolateral nucleus of the bed nuclei of the stria terminalis
Hippocampus	+	CA1-3
Olfactory cortex	+	Piriform cortex
Thalamus	+	Central medial nucleus
	+	Rhomboid nucleus
	+(+)	Paraventricular nucleus
	++	Medial habenular nucleus
Amygdala	+	Anterior amygdaloid area
Other	+	Periaqueductal gray
	++(+)	Ventral tegmental area
	++	Interfasicular nuclei
	++(+)	Central linear nucleus of raphe
	(+)	Substantial inominata

The complete distribution of MC3-R mRNA was detected in various brain regions by *in situ* hybridization. Semiquantitative estimates of the signal are indicated: + (weak), ++ (moderate), and +++ (strong), with parentheses indicating intermediate levels.

tin receptors. The distribution of MC3-R mRNA in the arcuate nucleus as well as terminal fields of arcuate neurons implies that melanocortin peptides produced in the hypothalamus may function as neuromodulators and neurotransmitters. The distribution of MC3-R mRNA in the brain is also interesting in light of the postulated role of melanocortin peptides in neuroendocrine (26–28), cardiovascular (8, 9), and thermoregulatory functions (4, 5). Thus, a moderate density of neurons expressing MC3-R mRNA was found in rostral and medial parts of the medial preoptic area and in the arcuate and posterior periventricular hypothalamic nuclei, which mediate various cardiovascular and neuroendocrine processes (29). Furthermore, the posterior nucleus of the hypothalamus and the lateral preoptic and lateral hypotha-

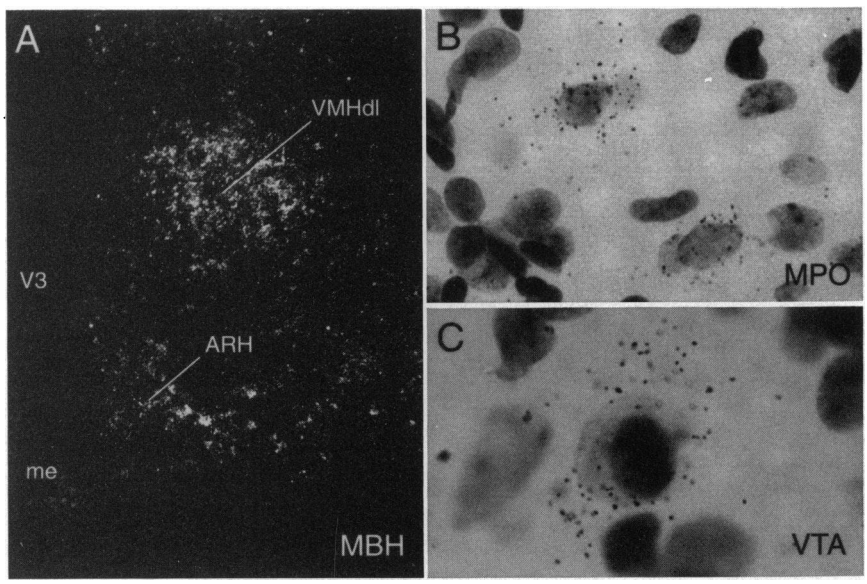

FIG. 5. Localization of MC3-R mRNA in the rat brain using *in situ* hybridization. (*A*) Dark-field microscopy of the medial basal part of the tuberal hypothalamus (MBH), showing MC3-R-positive cell bodies in the arcuate nucleus of the hypothalamus (ARH) and the dorsomedial portion of the ventromedial nucleus of the hypothalamus (VMHdl). (×25.) (*B* and *C*) Bright-field microscopy of representative MC3-R-positive neurons in the medial preoptic area (MPO) of the hypothalamus and the ventral tegmental area (VTA). (*B*, ×800; *C*, ×1500.) V3, third ventricle; me, median eminence. No hybridization was detected using a sense probe synthesized from the same MC3-R cDNA fragment.

lamic areas all contained MC3-R mRNA-containing neurons and are thought to play key roles in coordinating thermoregulatory responses (30). Other regions with significant numbers of neurons that express MC3-R mRNA include the medial habenula, the anterior and ventromedial nuclei of the hypothalamus, the lateral septal nucleus, and the ventral tegmental area, indicating that this receptor may participate in the regulation of complex motivational behaviors.

γ-MSH, originally named because of its amino acid homology with the α- and β-MSH forms, has little or no demonstrated ability to stimulate mouse melanocytes (31, 32). We have shown here that MC3-R is a melanocortin receptor that is responsive to γ-MSH peptides, suggesting unique physiological role(s) for these peptides at this receptor site. The presence of the MC3-R mRNA in the medial preoptic area and arcuate and posterior periventricular hypothalamic nuclei suggests that some of the cardioacceleratory, pressor, and natriuretic effects reported for the γ-MSH peptides may occur via the MC3-R in these brain regions (8, 9, 33). The affinity of MC3-R for γ_1- and γ_2-MSH is not significantly greater than the affinity for α-MSH. However, several forms of γ-MSH remain to be tested, including forms containing an amino-terminal lysine residue (34), which may predominate *in vivo*, and large molecular weight forms containing the first 76 amino-terminal residues of POMC (35). A more detailed characterization of the POMC peptides present in these brain regions will be necessary before a specific role for γ-MSH can be proven.

MC3-R mRNA was not found in the nucleus of the solitary tract and was only found in a subset of the presumptive terminal fields of the hypothalamic POMC neurons. Likewise, the MC3-R-positive nuclei represented only a subset of the brain regions binding ^{125}I-labeled Nle^4,D-Phe^7-α-MSH (13). ORG 2766 and $ACTH_{4-10}$ are melanocortin analogues that demonstrate significant activity in stimulation of retention of learned behavior and in stimulation of neural regeneration (1, 2, 6, 7). ORG 2766 has been used clinically for the prevention of neurotoxicity associated with cisplatin chemotherapy (36). The inability of ORG 2766 and $ACTH_{4-10}$ to significantly bind or stimulate the MC3-R suggests that this receptor does not mediate the behavioral or regenerative activities of the melanocortins. Consequently, it is likely that there exists a family of neural melanocortin receptors making up the complex pattern of MSH binding in the brain and accounting for the diverse physiological effects of melanocortin peptides in the brain. MC3-R and other neural melanocortin receptors will be valuable tools in elucidating the functions of POMC in the nervous system.

Note: The cloning of a human homologue of the MC3-R was recently reported by Gantz *et al.* (37).

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