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Morphine Down-regulates Melanocortin-4 Receptor Expression in Brain Regions that Mediate Opiate Addiction

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SUMMARY

Melanocortin peptides are reported to antagonize opiate dependence and tolerance, but the neural substrates underlying these actions are unknown. In this study, we characterize the rat melanocortin-4 receptor (MC4-R) and demonstrate that this receptor is regulated by opiate administration. The rat MC4-R is 95% identical to the human MC4-R, and the potency of melanocortin peptides to stimulate cAMP production is similar in these two species homologs (α-melanocyte-stimulating hormone = adrenocorticotropic hormone > γ-melanocyte-stimulating hormone). Expression of MC4-R mRNA was found to be enriched in the striatum, nucleus accumbens, and periaqueductal gray, all of which are regions implicated in the behavioral effects of opiates. In contrast, MC1-, MC3-, and MC5-R are expressed at very low or undetectable levels in these brain regions. Chronic administration of morphine (5 days) resulted in a time-dependent down-regulation of MC4-R mRNA expression in the striatum and periaqueductal gray. Expression of MC4-R mRNA was also decreased in the nucleus accumbens/olfactory tubercle, but this effect was observed after 1 or 3 days of morphine treatment. In the striatum, the reduction of MC4-R mRNA was accompanied by a concomitant decrease in melanocortin receptor levels, shown by quantitative radioligand binding and autoradiography. In contrast, morphine administration did not influence levels of MC4-R mRNA in several other brain regions, including frontal cortex, olfactory bulb, hypothalamus, and ventral tegmentum/substantia nigra. In light of previous findings that melanocortins antagonize opiate self-administration, analgesic tolerance, and physical dependence, we hypothesize that decreased melanocortin function, via down-regulation of MC4-R expression, may contribute to the development of these opiate-induced behaviors.

The melanocortin peptides α-melanocyte-stimulating hormone and ACTH have been implicated in a number of functions in the central nervous system, including induction of grooming (1), antagonism of cytokine action (2–5), thermoregulation (6), facilitation of learning (7, 8), and neurotropism (9–12). Furthermore, behavioral studies demonstrate that the administration of melanocortins antagonizes opiate tolerance and dependence (13, 14) and induces opiate withdrawal-like effects in opiate-naive animals (15). This suggests that down-regulation of melanocortin function may contribute to the development of opiate tolerance and dependence. However, the receptors that mediate these actions of melanocortins in the brain have not been characterized.

Five melanocortin receptor subtypes, all belonging to the G protein-coupled receptor superfamily, have been identified (16–28). We report the cloning and characterization of the full-length rat homolog of the human MC4-R. Furthermore, we demonstrate that expression of MC4-R mRNA is enriched in brain regions implicated in the behavioral actions of opiates and that morphine administration down-regulates MC4-R mRNA specifically in these regions. In contrast, the other MC-R subtypes are neither enriched nor regulated in these regions. These findings suggest that down-regulation of MC4-R, the major melanocortin receptor subtype in brain, may contribute to the development of opiate tolerance and dependence.

Materials and Methods

PCR. Degenerate oligonucleotide primers from the third and sixth transmembrane domains of cloned G protein-coupled receptors, as

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ABBREVIATIONS: MSH, melanocyte-stimulating hormone; MC4-R, melanocortin-4 receptor; ACTH, adrenocorticotropic hormone; RPA, RNase protection assay; NDP-MSH, Nle^4,D-Phe^7-α-melanocyte-stimulating hormone; SDS, sodium dodecyl sulfate; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair.

described previously (29), were used to PCR-amplify bovine locus ceruleus cDNA. PCR conditions were as follows: prewarming to 70° for 3 min, heating to 94° for 1 min 45 sec, and then cooling to 60°, at which time *Taq* polymerase (AmpliTaq, Perkin-Elmer Cetus, Norwalk, CT) was added. Cycling ensued with 35 cycles of denaturation at 95° for 30 sec, annealing at 46° for 30 sec, and extension at 70° for 60 sec. Reaction products were blunt-end ligated into pBluescript SK^- and introduced into $CaCl_2$-competent XL-1 Blue cells (Stratagene, La Jolla, CA); the bacteria were then plated onto Luria Broth plates. Stabs of individual colonies were placed in 50 μl of DNA extraction buffer (1% Triton X-100, 20 mM Tris, pH 8.3, 2 mM EDTA), heated to 95° for 10 min, and pulse spun in a microfuge. Five microliters of the supernatant was then reamplified using the same primers and PCR conditions as above. Colonies containing PCR-amplifiable inserts were cultured in Luria Broth, and plasmid DNA was isolated by a modified alkaline lysis/PEG precipitation method (Applied Biosystems protocol, Norwalk, CT). DNA was then sequenced in a modified Sanger ddNTP chain terminator reaction with the use of fluorescence-tagged ddNTPs. Reactions were run on 6% polyacrylamide gels and read on ABI 373A automated fluorescent sequencers (Applied Biosystems). One novel PCR product, designated as LCR 59 (locus ceruleus receptor fragment, clone No. 59), was selected for further study.

Receptor cloning. cDNA library screening and 5′ RACE were carried out simultaneously. LCR 59 was [α-^{32}P]dCTP random prime labeled and used to screen a rat brainstem/spinal cord cDNA library (Stratagene) under standard conditions (30). A 3-kb clone containing an open reading frame and a stop codon but lacking an initiation codon as well as several hundred base pairs of 5′ sequence was isolated. Based on the sequence of LCR 59, a 691-bp 5′ RACE clone was generated using cDNA reverse-transcribed from rat pituitary RNA with an LCR 59-specific primer (5′-CTGGCGCTAGGAGAATTACAC-3′). First- and second-round 5′ RACE was conducted using MC4-R-specific primers (5′-CTCAGACCGGTACTCCTTGTAC-3′ and 5′-CACCTGTATCTCTCTGCGGTAC-3′, respectively), according to kit instructions (GIBCO BRL 5′ RACE kit, Gaithersburg, MD). The RACE product had an open reading frame as well as the initiation codon and overlapped with the coding region of the library clone. Ligation of these two clones resulted in a 1.6-kb cDNA, both strands of which were sequenced to show that this clone contained a fully coding MC4-R with flanking 5′ and 3′ untranslated regions.

Animals and treatment paradigm. Male Sprague-Dawley rats (150–200 g) (CAMM, Wayne, NJ) were housed in groups and maintained on a 12-hr light/dark cycle with food and water freely available. While the animals were under halothane anesthesia, 75-mg morphine pellets (National Institute on Drug Abuse, Rockville, MD) were implanted subcutaneously once daily for 1, 3, or 5 days. Morphine administration for 5 days has been shown to produce opiate tolerance and dependence (31). Control animals were exposed to the same anesthesia but to sham surgery. The number of rats per group for the time course study in the neostriatum and nucleus accumbens/olfactory tubercle was 17 for sham surgery, 12 for 1-day morphine, 5 for 3-day morphine, and 12 for 5-day morphine. The total number of rats for the morphine studies in the frontal cortex, periaqueductal gray, hypothalamus, ventral tegmentum/substantia nigra, and olfactory bulb was 16 for sham surgery and 16 for 5-day morphine. For smaller regions, tissue samples from several rats were pooled before RNA was extracted. The number of rats for the *in vitro* binding assays was 4 for sham surgery and 4 for 5-day morphine. All rats were used ~24 hr after the final administration of morphine. The results were subjected to Student's *t* test or analysis of variance, with significance determined at the $p < 0.05$ level. In the regional distribution studies of the MC-R subtypes, a total of six male Sprague-Dawley rats were used for all regions except for skin and muscle, in which a single male Dark Agouti rat (300 g) (Bantin-Kingman, Fremont, CA) was used. All animal use procedures were in strict accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Yale Animal Care Committee.

Receptor expression in *Xenopus laevis* fibroblasts and cAMP measurement. The 1.6-kb MC4-R cDNA was subcloned into pcDNAI/Neo (InVitrogen, San Diego, CA) and transfected by electroporation into *X. laevis* fibroblast cells. Transfection conditions were as follows: 5×10^6 cells in 70% phosphate-buffered saline plus 10 μg of plasmid cDNA were added to 0.2-cm cuvettes and electroporated in a BTX ECM-600 (BTX, San Diego, CA) (475 V, 720 Ω, 400 μF). Cells were then plated to confluency in 12-well culture plates in 70% L-15 medium (Sigma Chemical, St. Louis, MO) plus 0.5% bovine serum albumin (Sigma). At 48 hr after transfection, cells were rinsed in fresh L-15/bovine serum albumin for 1 hr and again for 5 min with added 0.5 mM isobutylmethylxanthine (Aldrich Chemical, Milwaukee, WI). Intracellular cAMP accumulation in response to varying concentrations of ACTH(1–39), α-melanocyte-stimulating hormone, and γ-melanocyte-stimulating hormone (Calbiochem, San Diego, CA) was measured using a [^{3}H]cAMP assay kit (TRK 432; Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Northern blot analysis. RNA from rat brainstem and neostriatum was extracted by homogenization in guanidine isothiocyanate followed by centrifugation through cesium chloride gradients. Poly(A)$^+$ RNA, enriched through oligo(dT) selection (Promega PolyATtract Kit, Madison, WI), was fractionated on a formaldehyde denaturing agarose gel and transferred by capillary blotting to a nitrocellulose membrane. After fixation by UV cross-linking (Stratagene), the membrane was then hybridized at 65° for 17 hr in 50 mM Tris·HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 5× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50% deionized formamide, 150 μg/ml denatured salmon sperm DNA, and 10^6 cpm/ml [α-^{32}P]CTP-labeled antisense MC4-R riboprobe. The riboprobe corresponded to a 184-bp region of the MC4-R cDNA clone (from bp −6 to +178), which had been amplified by PCR and subcloned into pBluescript SK^-. The membrane was washed at 65° in 0.1× standard saline citrate/0.1% SDS and exposed to X-ray film without an intensifier screen for 2 days.

RPA. Total RNA was incubated with 10^5 cpm of [α-^{32}P]CTP-labeled antisense riboprobes for mouse MC1-R, rat MC3-R, rat MC4-R, or rat MC5-R at 63° for 16–18 hr in 80% formamide, 40 mM piperazine-N,N′-bis(2-ethanesulfonic acid), pH 6.4, 0.4 M NaCl, and 1 mM EDTA. The MC1-R riboprobe corresponded to a 253-bp fragment of the cDNA clone (from bp +694 to +947; generously supplied by Linda Roselli-Rehfuss, IRCM, Montreal, Canada). The MC5-R riboprobe corresponded to a 198-bp fragment of the cDNA clone (from bp +402 to +600) (32) and was generously supplied by Roger Adan (Utrecht University, Utrecht, The Netherlands). The MC3-R riboprobe was amplified by PCR and subcloned into pCRII (InVitrogen) and corresponded to a 140-bp fragment of the cDNA clone (from bp +22 to +162). Samples were RNase treated for 45 min at 37° in 400 μl of RNase digestion buffer (10 mM Tris·HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 20 μg/ml RNase A, and 100 units/ml RNase T1 (Boehringer-Mannheim Biochemicals, Indianapolis, IN)). On the addition of 20 μl of 10% SDS and 50 units of Proteinase K (Boehringer-Mannheim), samples were again incubated at 37° for 15 min to destroy RNases. After phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation with 10 μg of tRNA carrier, samples were resuspended in 80% formamide plus 1 mM EDTA and electrophoresed in 1× buffer (50 mM Tris base, 50 mM boric acid, 1 mM EDTA) on an 8% polyacrylamide/8 M urea denaturing gel. Gels were dried and exposed to X-ray film with an intensifier screen, and autoradiograms were quantified by laser densitometry.

***In vitro* binding and autoradiography.** Specific melanocortin binding to serial 8-μm cryostat sections of rat brain was measured by *in vitro* binding and autoradiography using the synthetic α-melanocyte-stimulating hormone analog ^{125}I-NDP-MSH (33), as described in detail (34, 35). NDP-MSH (Bachem California, Torrance, CA) was radioiodinated and purified as described previously (33). Briefly,

slide-mounted brain tissue sections were subjected sequentially to the following steps: 15-min preincubation wash, 2-hr incubation in binding buffer containing ^{125}I-NDP-MSH (2.5×10^5 cpm/ml, ~0.15 nM), a series of washes to remove unbound tracer, air drying; and fixation in hot formaldehyde vapors. Autoradiograms were prepared by exposing sections directly to X-ray film (Ultrofilm-^{3}H, Leica, Deerfield, IL) for 2 weeks before development. Sections were counterstained with cresyl violet before dehydration and mounting of coverslips.

To assess melanocortin binding, serial coronal sections prepared from several rostrocaudal levels ranging between the nucleus accumbens and midbrain were studied. Each slide contained anatomically matched sections from two sham-treated and two morphine-treated rats to control for potential slide and position effects, and a total of four rats were studied per treatment group. Consecutive serial sections were exposed to ^{125}I-NDP-MSH in the presence and absence of 1 μM α-melanocyte-stimulating hormone to determine the specificity of tracer binding. The mean value for right and left measurements in the paired brain structures was determined for each section, and the mean values of each of two replicate sections per rat were used to calculate group mean and variance. The concentration-response relationship for inhibition of tracer binding by NDP-MSH was determined in a second study using additional sections from the same series in each of the same eight rats. In that study, binding was measured in the ventrolateral striatum in six consecutive sections from each rat, each of which was incubated with either ^{125}I-NDP-MSH alone (two sections) or ^{125}I-NDP-MSH plus one of four different concentrations of NDP-MSH. Autoradiograms were digitized and analyzed with NIH IMAGE 1.54 as described previously (34). For each brain structure analyzed, an area of an appropriate size and shape within the corresponding region of the autoradiogram image was sampled. Specific binding was calculated by subtracting the tissue background. Film absorbance values were converted into radioactivity concentrations (expressed in terms of dpm/mg of tissue) using a calibration curve prepared using cryosections of rat brain paste containing known amounts of ^{125}I-NDP-MSH.

Results

Cloning and expression of the rat MC4-R. Degenerate oligonucleotide primers derived from the third and sixth transmembrane domains of related G protein-coupled receptors (29) were used in the PCR to amplify double-stranded bovine locus ceruleus cDNA. A novel receptor fragment, encoded LCR 59, was identified and used to screen a rat brainstem/spinal cord cDNA library. A single 3-kb library clone was isolated, and partial sequence analysis revealed that although an open reading frame and stop codon were present, the initiation codon and several hundred base pairs of 5′ sequence were missing. 5′ RACE of rat pituitary cDNA yielded a 691-bp product containing the start codon and sequence overlap with the library isolate. The RACE clone and library clone were ligated at a unique restriction site within this region of overlap to generate a 1.6-kb full-length receptor cDNA.

The cDNA and predicted protein sequences of the clone are shown in Fig. 1. The 332-amino acid open reading frame is 95% identical to that of the human MC4-R, the sequence of which was published while the rat clone was being characterized (21, 24). Based on this high degree of homology, the full-length LCR 59 clone can be identified as the rat MC4-R. Fig. 2 shows a comparison between this receptor and the other members of the melanocortin receptor family. The rat MC4-R is most similar to the MC5-R and MC3-R (77.4% and 76.1% amino acid similarity, respectively) but is also highly homologous to the MC1-R and MC2-R (70.1% and 70.3%, respectively).

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CCC GGG GGA GCC ACT AGT TCT AGA TGC ATG CTC GAG CGG CCG CCA GTG -99
TGA TGG ATA TCT GCA GAA TTC GGC TTC TAC TAC TAC TAG ACC ACG CGT -51
CGA CTA GTA CGG GGG GGG GGA GGA TTC GAA TCC AGC TGC TGC AGG -4

AAG ATG AAC TCC ACC CAC CAC CAT GGC ATG TAT ACT TCC CTC CAC CTC 45
    Met Asn Ser Thr His His His Gly Met Tyr Thr Ser Leu His Leu

TGG AAC CGC AGC AGC CAC GGG CTG CAC GGC AAT GCC AGC GAG TCT CTG 93
Trp Asn Arg Ser Ser His Gly Leu His Gly Asn Ala Ser Glu Ser Leu

GGG AAG GGG CAC TCA GAC GGA GGA TGC TAT GAG CAA CTT TTT GTC TCC 141
Gly Lys Gly His Ser Asp Gly Gly Cys Tyr Glu Gln Leu Phe Val Ser

CCC GAG GTG TTT GTG ACT CTG GGT GTC ATA AGC CTG TTG GAG AAC ATT 189
Pro Glu Val Phe Val Thr Leu Gly Val Ile Ser Leu Leu Glu Asn Ile

CTA GTG ATC GTG GCG ATA GCC AAG AAC AAG AAC CTG CAC TCA CCC ATG 237
Leu Val Ile Val Ala Ile Ala Lys Asn Lys Asn Leu His Ser Pro Met

TAC TTT TTC ATC TGT AGT CTG GCT GTG GCG GAC ATG CTG GTG AGC GTT 285
Tyr Phe Phe Ile Cys Ser Leu Ala Val Ala Asp Met Leu Val Ser Val

TCG AAC GGG TCA GAA ACC ATC GTC ATC ACC CTG CTA AAC AGT ACG GAC 333
Ser Asn Gly Ser Glu Thr Ile Val Ile Thr Leu Leu Asn Ser Thr Asp

ACG GAC GCC CAG AGC TTC ACC GTG AAT ATT GAT AAT GTC ATT GAC TCT 381
Thr Asp Ala Gln Ser Phe Thr Val Asn Ile Asp Asn Val Ile Asp Ser

GTG ATC TGT AGC TCC TTG CTC GCA TCC ATT TGC AGC CTG CTT TCC ATT 429
Val Ile Cys Ser Ser Leu Leu Ala Ser Ile Cys Ser Leu Leu Ser Ile

GCA GTG GAC AGG TAT TTC ACT ATC TTT TAC GCG CTC CAG TAC CAT AAC 477
Ala Val Asp Arg Tyr Phe Thr Ile Phe Tyr Ala Leu Gln Tyr His Asn

ATT ATG ACG GTT AGG CGG GTC GGG ATC ATC ATC AGT TGT ATC TGG GCA 525
Ile Met Thr Val Arg Arg Val Gly Ile Ile Ile Ser Cys Ile Trp Ala

GCT TGC ACA GTA TCG GGC GTT CTT TTT ATC ATT TAC TCG GAC AGC AGC 573
Ala Cys Thr Val Ser Gly Val Leu Phe Ile Ile Tyr Ser Asp Ser Ser

GCT GTC ATC ATC TGC CTC ATT ACC ATG TTC TTC ACC ATG CTG GTT CTC 621
Ala Val Ile Ile Cys Leu Ile Thr Met Phe Phe Thr Met Leu Val Leu

ATG GCC TCT CTC TAT GTC CAC ATG TTC CTG ATG GCG AGG CTT CAC ATT 669
Met Ala Ser Leu Tyr Val His Met Phe Leu Met Ala Arg Leu His Ile

AAG AGG ATC GCT GTC CTC CGG GGC ACG GGT ACC ATC CGA CAG GGT GCC 717
Lys Arg Ile Ala Val Leu Pro Gly Thr Gly Thr Ile Arg Gln Gly Ala

AAC ATG AAG GGC GCA ATT ACC TTG ACC ATT CTG ATT GGA GTG TTT GTT 765
Asn Met Lys Gly Ala Ile Thr Leu Thr Ile Leu Ile Gly Val Phe Val

GTC TGC TGG GCC CCG TTT TTC CTC CAT TTA CTG TTC TAC ATC TCT TGT 813
Val Cys Trp Ala Pro Phe Phe Leu His Leu Leu Phe Tyr Ile Ser Cys

CCT CAG AAT CCA TAC TGC GTG TGC TTC ATG TCT CAT TTT AAC TTG TAT 861
Pro Gln Asn Pro Tyr Cys Val Cys Phe Met Ser His Phe Asn Leu Tyr

CTC ATA CTG ATC ATG TGT AAC GCT GTC ATC GAC CCT CTC ATT TAT GCC 909
Leu Ile Leu Ile Met Cys Asn Ala Val Ile Asp Pro Leu Ile Tyr Ala

CTG CGG AGT CAA GAA CTG AGG AAA ACC TTC AAA GAG ATC ATC TGT TTC 957
Leu Arg Ser Gln Glu Leu Arg Lys Thr Phe Lys Glu Ile Ile Cys Phe

TAC CCC CTG GGA GGC ATC TGT GAG TTA CCT GGC AGG TAT TAA GTG GGG 1005
Tyr Pro Leu Gly Gly Ile Cys Glu Leu Pro Gly Arg Tyr

ACA GAG TGC ATA CTA GGT AGA GAC CTG CAG AAT TTG TCA CTC AGG CAC 1053
AAC CTG AGC AGT GTA CTT CCC AAC AGC TGC CTC TAC TGT ATA GTG CTT 1101
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GTG TGT AAA CTG CTT ATT TAT GTT CTA CAG CGT GGG CGC TAT GGA GTT 1245
CCA TAA AAG AAA AAG ACA CCC TTA TTA AAA CTT TGA CAG TGT TTC TTT 1293
CCA TGT TAT TTA TCA AGA GTC AAC CCT TGT TCT TTC TGT GGT AGC AGA 1341
AAT CAG AGC CTT CTG AAA AGC TGT TTC CAT TGC ATC ACC CCC ACA GCA 1389
CAG CAG AAG CCT GAT TCC ACT GTT TAT GGG GAA ATA TTT AAA CAC TGG 1437
ATG CTC GAT CAT TTA ATG AGT CAG CTC TAC TCG TGA AAT TTC ACT CGT 1485
GCC GAA TT                                                     1493
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Fig. 1. Sequence of the rat MC4-R. The entire 1640-bp sequence of the rat MC4-R cDNA clone is aligned with the predicted amino acid sequence of the receptor. The coding region spans bp 1–996 and is flanked by both 5′ and 3′ untranslated sequence.

The entire 1.6-kb cDNA encoding the full-length rat MC4-R was subcloned into pcDNAI/Neo and transiently transfected by electroporation into *X. laevis* fibroblast cells. cAMP production in response to several concentrations of α-melanocyte-stimulating hormone, γ-MSH, and ACTH was then measured (Fig. 3). Incubation with α-melanocyte-stimulating hormone and ACTH resulted in dose-dependent stim-

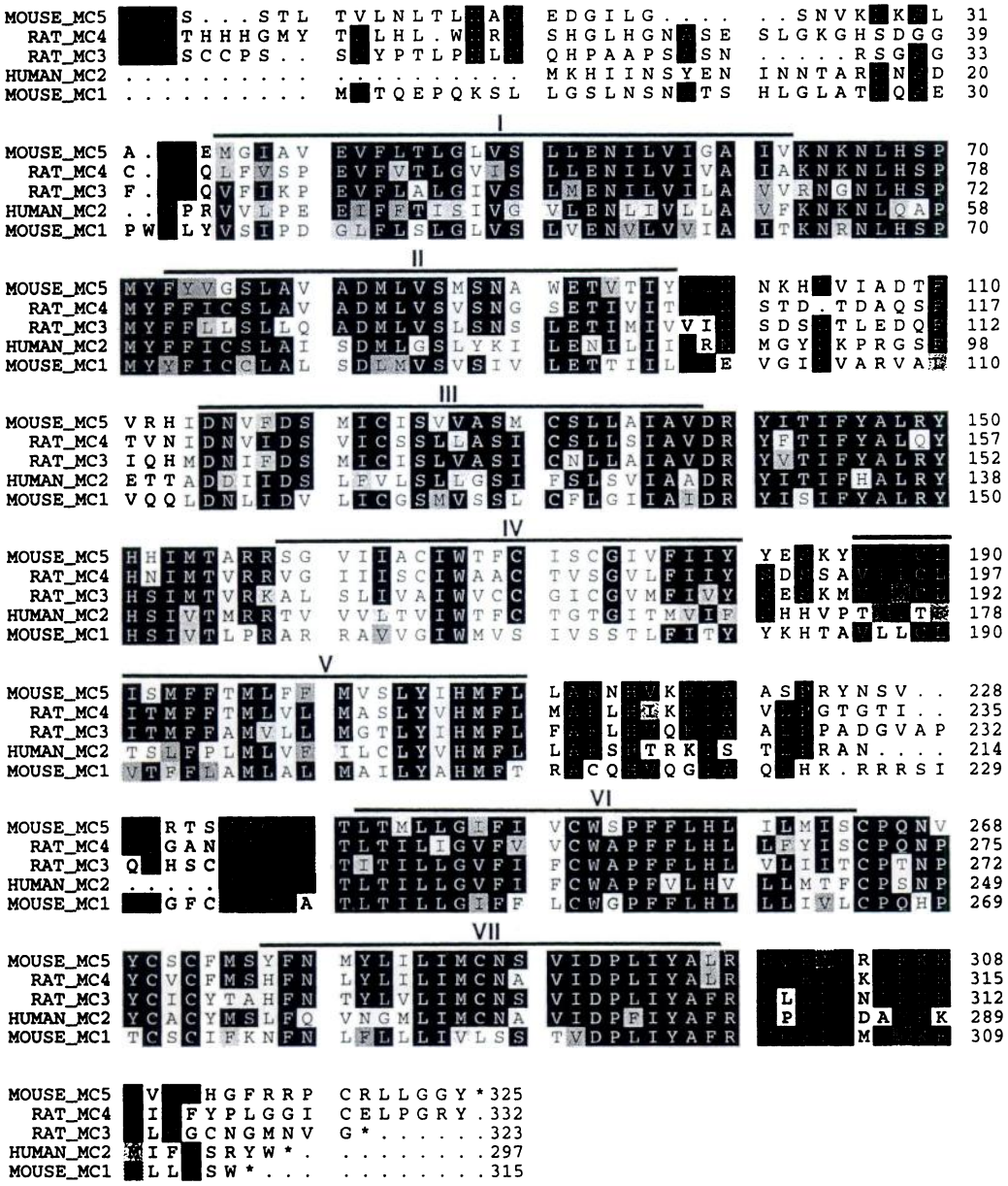


Fig. 2. Alignment of the rat MC4-R with the other members of the MC-R family. Amino acid sequences of the five receptor subtypes were aligned using the Pileup program (Genetics Computer Group, Madison, WI). *Black boxes*, amino acid identities among the receptors; *gray boxes*, conservative substitutions between sequences; *solid lines*, predicted transmembrane domains numbered with Roman numerals.

ulation of cAMP production, with the two peptides being approximately equipotent. In contrast, γ-MSH was significantly less potent in its ability to stimulate cAMP production. This agonist profile is similar to that of the human MC4-R (21).

Regional distribution of MC4-R mRNA. A 184-bp radiolabeled riboprobe was generated from the 5′ end of the rat MC4-R (from −6 to +178 bp), a region in which DNA sequence identity among members of the melanocortin receptor family is only 35–45%. By Northern blot analysis, this probe detected a predominant transcript of ~2 kb in the brain (Fig. 4). Several minor mRNA species, just below and above the 28*S* ribosomal RNA, were also detected and may represent either splice variants of the MC4-R or related members of the G protein-coupled receptor family. As expected in the more-sensitive and highly specific RPA, the same probe protected a single 184-bp mRNA fragment corresponding to the MC4-R. As seen in Fig. 5, MC4-R mRNA is most abundant in the septum and nucleus accumbens; is moderately expressed in the periaqueductal gray, hypothalamus, neostriatum, ventral tegmentum, and olfactory bulb; and is least abundant in the cerebellum, substantia nigra, frontal cortex, and hippocampus.

Morphine regulation of MC4-R mRNA. Rats were administered morphine under conditions known to produce opiate tolerance and dependence and opiate withdrawal on removal of drug (31). At ~24 hr after the final treatment, MC4-R mRNA levels were measured by RPA. Morphine treatment resulted in a time-dependent down-regulation of MC4-R mRNA in the neostriatum (Fig. 6A). Five days, but not 1 day, of drug treatment significantly decreased levels of MC4-R mRNA in this region. After 3 days, a small but nonsignificant decrease was noted. Morphine also decreased MC4-R mRNA in a nucleus accumbens/olfactory tubercle dissection, with a time course different from that observed in the neostriatum. Levels of MC4-R mRNA were significantly decreased 24 hr after the first morphine treatment (Fig. 6B). This down-regulation persisted after 3 days and then re-

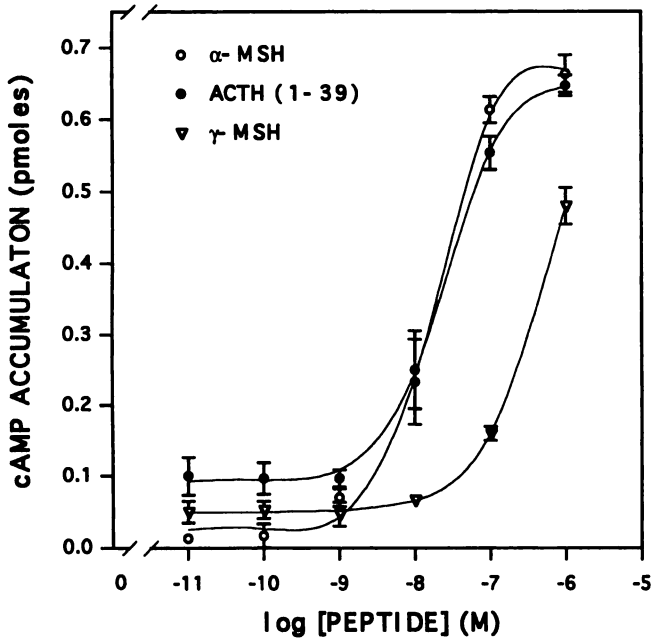


Fig. 3. cAMP accumulation in transfected *X. laevis* fibroblasts. MC4-R in pcDNAI/Neo was electroporated into *X. laevis* fibroblasts, and the ability of ACTH, α-melanocyte-stimulating hormone, and γ-MSH to stimulate cAMP production was measured. ACTH and α-melanocyte-stimulating hormone were approximately equipotent in their ability to increase cAMP in a dose-dependent manner (EC_{50} = 24 nM ± 2.0 nM and 18 nM ± 3.5 nM, respectively), whereas γ-MSH was much less potent ($EC_{50} \gg 100$ nM). *Points*, mean ± standard error of triplicate samples in which each sample had equivalent numbers of cells. *Curves* were generated in SigmaPlot using nonlinear regression of a logistic function (50).

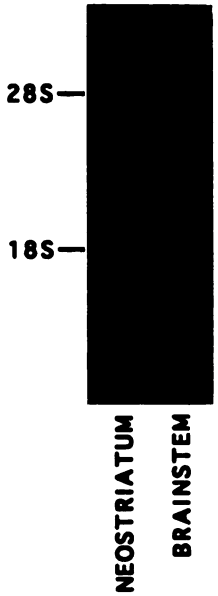


Fig. 4. Northern blot analysis of MC4-R mRNA. Rat brainstem and neostriatum poly(A)$^+$-enriched mRNA (5 μg/lane) was electrophoresed, transferred to nitrocellulose, hybridized with a radiolabeled MC4-R riboprobe, and exposed to X-ray film for 2 days without an intensifier screen. The migrations of the 18S and 28S ribosomal RNAs are indicated.

turned to control levels. Several additional brain regions were examined for comparison. As shown in Fig. 7, a 30% decrease in MC4-R mRNA was seen in the periaqueductal

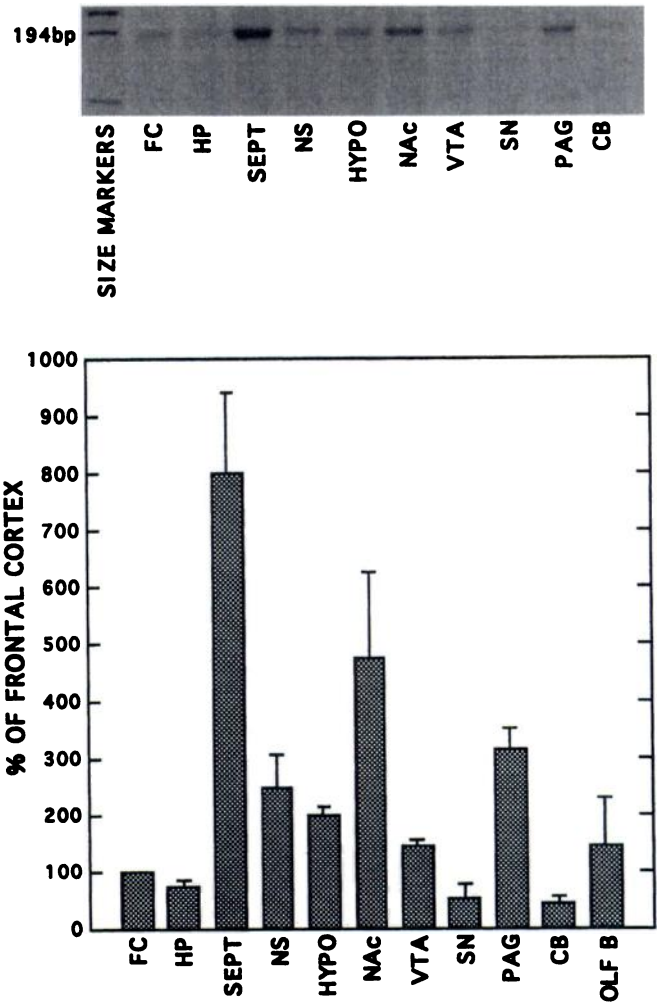
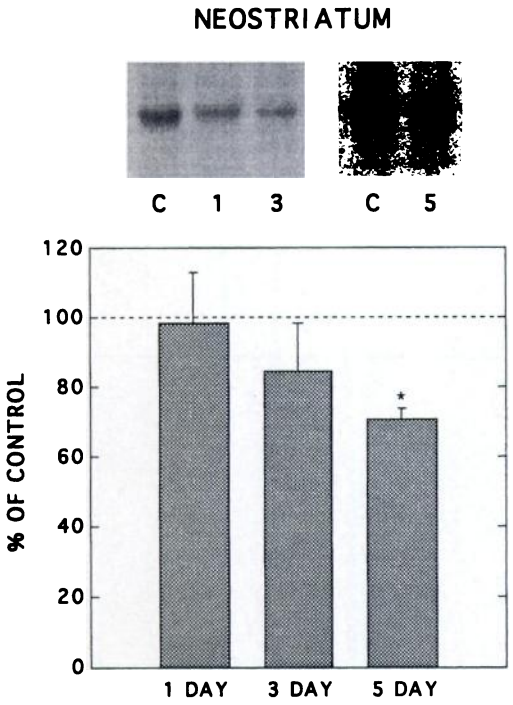


Fig. 5. Regional distribution of the MC4-R by the RPA. A 300-bp radiolabeled MC4-R riboprobe was hybridized overnight with 30 μg of total RNA for each brain region. Samples were RNase treated, and the 184-bp protected RNA fragments were run on polyacrylamide gels. *Top*, autoradiogram of one experiment. The 194-bp DNA molecular mass marker is labeled. Levels of MC4-R mRNA in each brain region were determined three times using three independent RNA samples, and autoradiograms were quantified by laser densitometry. The mean ± standard error for each region is shown and expressed as a percentage of MC4-R mRNA in frontal cortex (defined as 100%). *FC*, frontal cortex; *HP*, hippocampus; *SEPT*, septum; *NS*, neostriatum; *HYPO*, hypothalamus; *NAc*, nucleus accumbens; *VTA*, ventral tegmental area; *SN*, substantia nigra; *PAG*, periaqueductal gray; *CB*, cerebellum; *OLF B*, olfactory bulb.

gray after 5 days of morphine administration. In contrast, no changes were detected in the hypothalamus, ventral tegmentum/substantia nigra, olfactory bulb, or frontal cortex.

To assess whether the effects of morphine were specific to MC4-R, we determined which of the other melanocortin receptor subtypes were enriched in the periaqueductal gray, neostriatum, and nucleus accumbens. Both MC1-R and MC5-R were undetectable in these regions. The sensitivity of the RPAs for MC1-R and MC5-R was validated by demonstration of MC1-R mRNA in the skin of the Dark Agouti rat and of MC5-R mRNA in Dark Agouti muscle and Sprague-Dawley adrenal (Fig. 8), which is consistent with previous reports (17, 23, 26, 27). Fig. 8 also demonstrates that although MC3-R mRNA was expressed in abundance in the hypothalamus, it was expressed only at very low levels in the



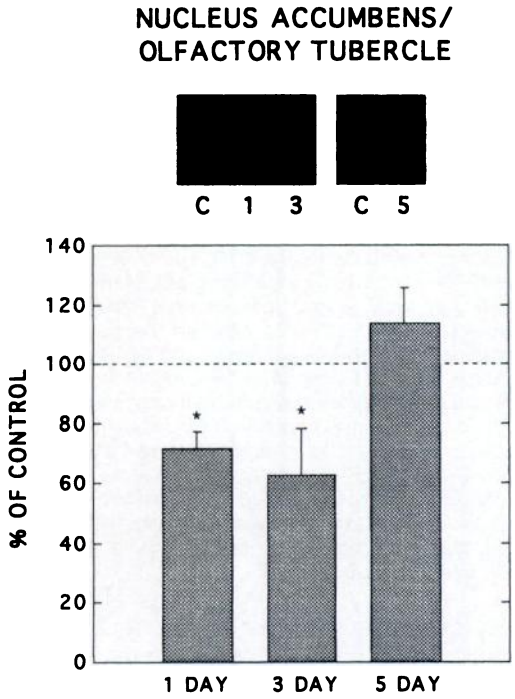


Fig. 6. Time course of MC4-R mRNA regulation by morphine in the neostriatum and nucleus accumbens/olfactory tubercle. Rats were administered morphine or sham treatment for 1 day (*1*), 3 days (*3*), or 5 days (*5*), and levels of MC4-R mRNA in sections of neostriatum and nucleus accumbens/olfactory tubercle were determined by RPA using 30 μg of total RNA. Autoradiograms show representative gel results. Levels of MC4-R mRNA were quantified by laser densitometry of autoradiograms. The results are expressed as percentage of sham-treated controls (*C*) and are mean ± standard error. *Dotted lines*, 100% control value. Standard error of the controls for each region is < ± 10%. The number of rats per group was 15 for controls, 10 for 1 day, 5 for 3 day, and 10 for 5 day for the striatum and 16 for controls, 12 for 1 day, 4 for 3 day, and 10 for 5 day for the nucleus accumbens/olfactory tubercle. *, $p < 0.05$ by analysis of variance.

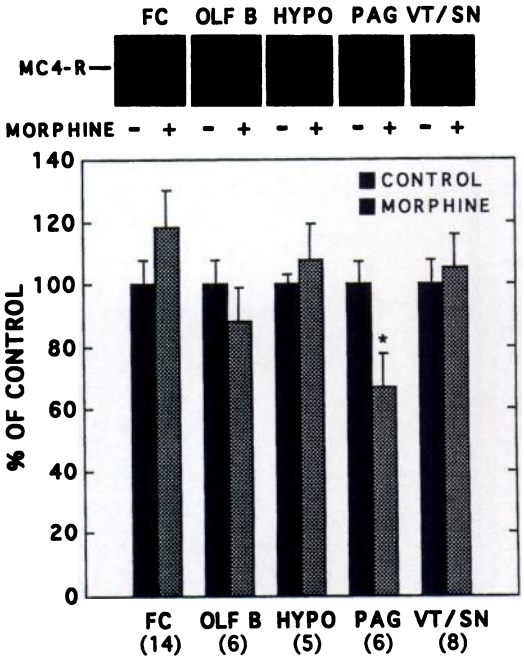


Fig. 7. Region-specific MC4-R mRNA regulation by morphine. RPAs were run on 25–35 μg of total RNA isolated from different brain regions of rats administered morphine or sham treatment for 5 days. Autoradiograms show representative gel results. Autoradiograms were quantified by laser densitometry. The results are expressed as percentage of sham-treated controls and are mean ± standard error. *Numbers in parentheses*, sample size for each region. *FC*, frontal cortex; *OLF B*, olfactory bulb; *HYPO*, hypothalamus; *PAG*, periaqueductal gray; *VT/SN*, ventral tegmentum/substantia nigra. *, $p < 0.05$ by Student's *t* test.

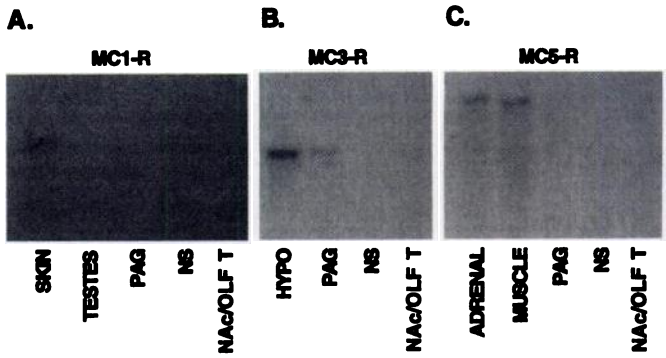


Fig. 8. Regional expression of MC-R subtypes. RPAs were run on 40 μg of total RNA from various rat tissues using receptor-specific radiolabeled riboprobes. RPA gels were exposed to X-ray film without an intensifier screen for 3 days. A, A mouse MC1-R riboprobe detected receptor mRNA in Dark Agouti rat skin but not in periaqueductal gray (*PAG*), neostriatum (*NS*), or nucleus accumbens/olfactory tubercle (*NAc/OLF T*). B, A rat MC3-R riboprobe detected receptor mRNA in hypothalamus but not in the other brain regions indicated. C, A rat MC5-R riboprobe detected receptor mRNA in adrenal tissue and Dark Agouti muscle but not in brain. *HYPO*, hypothalamus.

periaqueductal gray and was at the limits of detection in the neostriatum and nucleus accumbens. The regulation of this subtype by morphine could not be determined in the nucleus accumbens and striatum because of the extremely low level of mRNA expression; in the periaqueductal gray, chronic morphine did not regulate MC3-R mRNA levels (control, 100 ± 17.7%; treated, 108 ± 10%; mean ± standard error, $n = 3$ samples per group, 2 rats per sample).

Morphine regulation of receptor binding. To determine whether morphine regulation of MC4-R mRNA is associated with alterations in melanocortin receptor expression at the protein level, *in situ* binding assays using ^{125}I-NDP-MSH were conducted on coronal brain sections from 5-day morphine-treated rats. Consistent with earlier results (35), a distinct subset of brain structures showed ^{125}I-NDP-MSH binding, which was specific as shown by its complete blockade in the presence of 1 μM α-melanocyte-stimulating hormone or 30 nM NDP-MSH (not shown). Chronic morphine treatment decreased ^{125}I-NDP-MSH binding levels by 50% in the ventrolateral striatum (Fig. 9, A and B). This decrease was probably due to a decrease in the density of receptors rather than to a decrease in ligand binding affinity because the potency of unlabeled NDP-MSH to inhibit ^{125}I-NDP-MSH binding to tissue sections was similar in sham- and morphine-treated rats (IC_{50} = 0.13 ± 0.052 and 0.074 ± 0.051 nM, respectively; n = 4 rats per group). In contrast, morphine treatment did not significantly affect ^{125}I-NDP-MSH binding in neighboring structures, including the dorsomedial striatum and bed nucleus of the stria terminalis (Fig. 9B). In addition, no changes in ^{125}I-NDP-MSH binding were found in the nucleus accumbens (control, 1135.1 ± 130.2 dpm/mg of tissue; morphine, 1369 ± 120.4 dpm/mg of tissue; n = 4 rats per group), ventral tegmental area (control, 904 ± 90.6 dpm/mg of tissue; morphine, 994.7 ± 41.2 dpm/mg of tissue), and periaqueductal gray (control, 898.2 ± 9.3 dpm/mg of tissue; morphine, 996.8 ± 75.8 dpm/mg of tissue).

Discussion

The results of the current study characterize the rat MC4-R and demonstrate that morphine administration regulates the expression of this receptor in brain regions implicated in opiate tolerance and dependence. The rat MC4-R displays a high degree of sequence identity to its human homolog and has a similar affinity for melanocortin peptides (ACTH = α-melanocyte-stimulating hormone > γ-MSH) (21, 24). This is in contrast to the potency of these peptide analogues for the other two melanocortin receptors expressed in brain. The rat MC3-R is as potently activated by γ-MSH as by α-melanocyte-stimulating hormone and ACTH (19), and the rat MC5-R is much more potently activated by α-melanocyte-stimulating hormone than by ACTH (26).

Expression of MC4-R mRNA was highest in the septum, nucleus accumbens, striatum, and periaqueductal gray, with lower levels expressed in several other brain regions examined. The relative enrichment of MC4-R mRNA in certain brain regions is similar but differs to some extent with reports based on *in situ* hybridization studies (21, 24). This could result from expression of MC4-R mRNA in discrete neuronal populations in some regions, which could conceivably yield more or less intense *in situ* hybridization signals than might be predicted from RPA results. Together with previous studies of other melanocortin receptor subtypes, the results indicate that MC4-R is the predominant melanocortin receptor subtype expressed in brain (19, 22, 24, 26). Furthermore, the relative potencies of melanocortin peptides for binding in brain (36) are consistent with the agonist profile of the MC4-R subtype.

Administration of morphine was found to down-regulate the expression of MC4-R mRNA in the striatum, nucleus

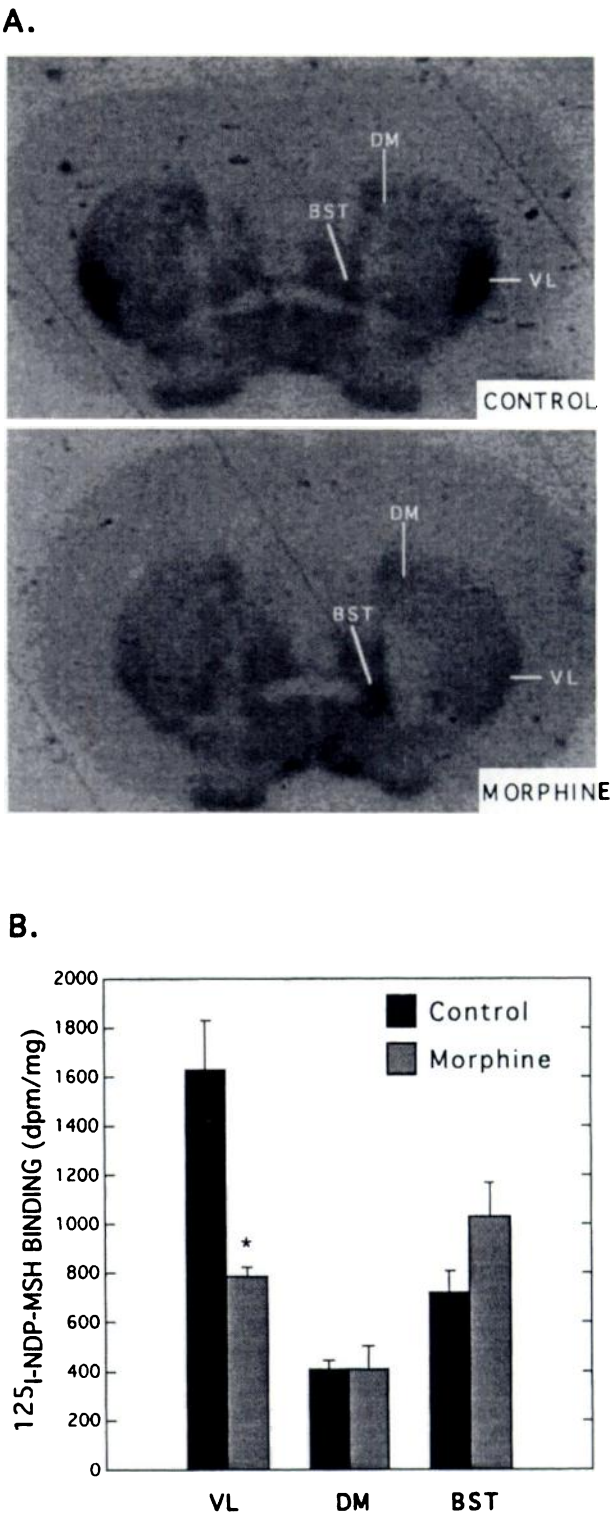


Fig. 9. Down-regulation of striatal receptor ligand binding by morphine treatment. A, Digitized images of X-ray film autoradiograms of ^{125}I-NDP-MSH binding to coronal brain sections of control and morphine-treated rats at the level of the anterior commissure. The relatively intense level of tracer binding in the ventrolateral striatum (*VL*) of control rats was suppressed after morphine treatment, whereas binding in the dorsomedial striatum (*DM*) and bed nucleus of the stria terminalis (*BST*) was unchanged. Images are representative of the results for four rats per treatment group. B, Comparison of ^{125}I-NDP-MSH binding levels in several striatal areas of control and morphine-treated rats. *Error bars*, mean ± standard error for four rats per treatment group. *, $p < 0.05$ by Student's t test.

accumbens, and periaqueductal gray, three brain regions that have been implicated in various behavioral actions of opiates. In contrast, MC4-R mRNA levels were unaffected by morphine in several other brain areas. Analysis of the regional distribution and regulation of MC1-R, MC3-R, and MC5-R indicates that the effects of morphine are specific to MC4-R. MC1-R has been reported to be present in a few neurons in the periaqueductal gray (37), but mRNA for this receptor could not be detected in the current study by RPA. Similarly, MC5-R has been detected in rat striatum and nucleus accumbens but only by PCR (26). We and others were not able to detect this subtype in the brain by RPA (Fig. 8).[1] Finally, our results show that although MC3-R is expressed at very low levels in the striatum, nucleus accumbens, and periaqueductal gray, it is not regulated by morphine.

Regions of the striatum, including the ventrolateral area, are believed to play a role in mediating the psychomotor activating (38), and perhaps even the reinforcing (39), properties of opiates. We detected a decrease in MC4-R mRNA in this region and a corresponding decrease in ^{125}I-NDP-MSH binding in the ventrolateral striatum after 5 days of morphine administration. Although ^{125}I-NDP-MSH binds to other melanocortin receptor subtypes, it is highly likely that down-regulation of MC4-R accounts for most of the measured decrease in receptor binding. Two lines of evidence support this conclusion. First, the pharmacological profile of melanocortin receptor binding in the ventrolateral striatum is similar to that of MC4-R but is clearly distinct from that of MC3-R (36), the only other MC-R subtype detected in brain in the current study. Second, only MC4-R mRNA is expressed appreciably in the striatum.

In the nucleus accumbens, which is a major neural substrate for the reinforcing properties of opiates and other drugs of abuse (40–42), MC4-R mRNA was more rapidly decreased by morphine treatment. In the periaqueductal gray, an important anatomic site for the expression of physical dependence to morphine (43), a decrease in MC4-R mRNA was detected after 5 days of drug administration. Unlike the results seen in the striatum, levels of ligand binding in the nucleus accumbens and periaqueductal gray were not influenced at the time points examined, suggesting that the time course for regulation of receptor protein differs from that of mRNA. Nevertheless, down-regulation of MC4-R mRNA in the striatum, nucleus accumbens, and periaqueductal gray suggests that decreased melanocortin receptor function is involved in the development of long term opiate effects in brain.

A role for regulation of MC4-R expression in long term opiate actions is further supported by previous behavioral studies demonstrating that melanocortins antagonize various functional effects of opiate treatments. First, melanocortins can reduce opiate self-administration (44). Second, they have the ability to decrease opiate physical dependence (14). Third, they can attenuate tolerance to the analgesic effects of opiates (13, 14). Furthermore, the relative potency of various melanocortin peptides to block tolerance correlates with their ability to activate the MC4-R *in vitro* (45, 46). Finally, melanocortins have been shown to induce an opiate withdrawal-like effect in drug-naive animals (15, 44).

Based on these previous studies and on the results of the present study, we hypothesize that down-regulation of MC4-R expression may be an important adaptation contributing to chronic opiate effects in the brain. Decreased melanocortin function is also suggested by reports that chronic morphine treatment decreases expression of the melanocortin precursor pro-opiomelanocortin (47–49). Given the reports that melanocortins antagonize opiate self-administration, tolerance, and dependence, down-regulation of melanocortins and MC4-R expression would be expected to have a permissive effect on the development of these long term behavioral actions of opiates. A direct test of this hypothesis will involve disrupting MC4-R function *in vivo* and examining the effects of this interference on opiate-induced behaviors. In summary, the findings of the current study indicate that exogenous opiates, in an animal model of opiate addiction, modulate endogenous central melanocortin pathways.

[1] R. Adan, personal communication.

Acknowledgments

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