Human μ opiate receptor

cDNA and genomic clones, pharmacologic characterization and chromosomal assignment

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Abstract

A human μ opiate receptor cDNA has been identified from a cerebral cortical cDNA library using sequences from the rat μ opiate receptor cDNA. The human μ opiate receptor (hμOR1) shares 95% amino acid identity with the rat sequence. The expressed μOR1 recognizes tested opiate drugs and opioid peptides in a sodium- and GTP-sensitive fashion with affinities virtually identical to those displayed by the rat μ opiate receptor. Effects on cyclic AMP are similar to those noted for the rat μ opiate receptor. An 18 kb genomic clone hybridizing with the hμOR1 cDNA contains 63 and 489 bp exonic sequences flanked by splice donor/acceptor sequences. Analysis of hybridization to DNA prepared from human rodent hybrid cell lines and chromosomal in situ hybridization studies indicate localization to 6q24–25. An MspI polymorphism, producing a 3.7 kb band, may prove useful in assessing this gene's involvement in neuropsychiatric disorders involving opiatergic systems.

Key words: Morphine; Heroin; Pain; Addiction; Enkephalin; G-linked receptor

1. Introduction

Opiate receptors [1–5], sites recognizing exogenous opiate drugs and endogenous opiate peptides, include the morphine-preferring μ opiate receptor first defined by Martin and colleagues [2]. μ receptor distributions and pharmacologic properties place them among the receptors most identified with the analgesic and addicting properties of opiate drugs [3–7]. These receptors are G-linked members of the seven transmembrane domain neuropeptide receptor subfamily [8–14].

Recent studies have identified the cDNAs encoding rodent μ [15–17], δ [17–19] and κ opiate receptors [20–22], thus defining at least one member of each of the other major opiate receptor subclasses postulated by Martin, Kosterlitz, Hughes, and associates [1–5]. The μ opiate receptor has the structure of a G-protein coupled receptor. G-protein receptor coupling was confirmed for rμOR1 [15–17]; morphine effects adenyl cyclase in expressing cells [5,23].

Because of interest in μ receptors as targets for development of selective analgesic and anti-addictive therapies [24–28], and because of interest in identifying μ receptor gene markers that could detect individuals possessing allelic variants of this gene that could confer differential susceptibility to abused drugs, we have used the rat μ receptor cDNA identified in this laboratory [15] to identify its human homolog. In the present study, we describe the sequence of the human μ opiate receptor, identify the sodium- and GTP analog-sensitive high-affinity binding that its' expression confers on COS cells, document the changes in adenyl cyclase that opiate drugs can induce in expressing COS cells, assign it to a human chromosomal region, identify a hμOR1 genomic clone and describe a polymorphic genetic marker at the hμOR1 locus. These data document the biochemical and genetic nature of the principal human receptor for analgesic and addicting opiate ligands.

2. Materials and methods

Candidate human μ opiate receptor cDNAs were obtained using several cDNA libraries screened with fragments of the rat opiate receptor rμOR1 radiolabeled by random priming to specific activities of 10^9 dpm/μg. phμOR1 was a 2.1 kb cDNA obtained from a human cerebral

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cortical cDNA library prepared by random and oligo-dT priming in λ-Zap II (Stratagene). Filters were hybridized at 30°C in 29% formaldehyde, 6 × SSPE, washed at 42°C in 0.4 × SSPE/0.1% SDS, and exposed overnight to X-ray film. Plasmids were autoexcised from lambda-Zap II phage DNA grown from positive plaques as described and analyzed by restriction mapping and cDNA sequencing. phμOR1 was subjected to complete sequencing using automated and manual methods as described [29], with sequence analyses using GCG software [30], and the insert was subcloned into the expression plasmid pcDNA1 to yield pcDNA1hμOR1. pHG4 was an 18 kb hμOR1 genomic clone isolated from a human genomic library prepared in lambda-Zap II phage (Stratagene) using hybridization with the phμOR1 cDNA and analysis by sequencing as described [30].

COS cells were transfected by electroporation with 20 μg/10⁷ cells of plasmid pcDNA1hμOR1, grown for two to three days and tested for opiate receptor expression by radioligand binding as described [15], except that whole cell suspensions were used. Adenyl cyclase activities were assessed by radioimmunoassay as described [15]. Cells transfected with the pcDNA1 vector alone served as negative controls.

A 1.2 kb 3′ fragment of hμOR1 contained 526 bp of coding sequence beginning in the protein's predicted second extracellular loop and displayed nucleotide sequence 87% identical to the rat rμOR1 nucleotide sequence. This 1.2 kb fragment, and the genomic clone HG4, were radiolabeled by random priming, and used as hybridization probes in southern analyses of the somatic cell hybrid panels #1 and #2 (BIOS Labs, New Haven, CT 06511). These panels contain different complements of *Hind*III digests of human chromosomes in 26 independent cell lines in 36 panels. The panels were incubated separately with each radiolabelled hybridization probe in 29% formamide/6 × SSPE at 30°C overnight, washed with 0.1 × SSPE/0.1% SDS for 30 min at 37°C then twice for 30 min at 60°C. Radioactive patterns were detected by phosphorimaging following overnight exposure. Probes were then removed from panels by incubation in water at 90°C for 20 min, with removal assessed by overnight autoradiographic phosphorimager exposure.

Higher resolution mapping of the hμOR1 gene was accomplished with chromosomal in situ hybridization. A genomic clone, pHG4, was nick-translated with biotin-14 dATP (BRL, Gaithersburg, MD), with 81% incorporation as determined by tritium tracer incorporation. Slides with chromosome spreads were made from normal male lymphocytes cultured with BrdU [31]. Fluorescence in situ hybridization was performed as described [32] with modifications. 12.5 ng/μl biotinylated probe in 2 × SSCP, 50% formamide, 10% dextran sulfate, 0.5 μg/μl Cot-1 DNA and 0.5 μg/μl salmon sperm DNA were denatured at 70°C for 5 min, preannealed at 37°C for 30 min, placed on slides and hybridized at 37°C overnight. Slides were washed in 50% formamide/2×SSC at 43°C for 20 min, then twice for 5 min in 2 × SSC at 37°C. Biotinylated probe was detected with FITC-avidin and biotinylated anti-avidin [33]; (Oncor, Inc. Gaithersburg, MD), following manufacturer's instructions.

DNA isolated from the leukocytes of 49 unrelated individuals subjected to experimental protocols at the Addiction Research Center, NIDA [34,35] was digested with *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, *Hinf*I, *Msp*I, *Pst*I, *Rsa*I, and *Taq*I and subjected to Southern analyses using the 1.2 kb 3′ radiolabeled hybridization probe. Only *Msp*I digestion produced polymorphisms; no reproducible polymorphisms were present in lanes digested with other enzymes.

3. Results

Although several partial length cDNAs were obtained from other libraries, the 2.1 kb phμOR1 appeared to contain the entire μOR1 open reading frame as well as 3′ and 5′ untranslated sequences. The human cDNA displayed an overall nucleotide identity of 79% with the rat cDNA; 87% nucleotide identity was noted in coding regions. This cDNA displayed an open reading frame with 95% indentity to amino acids encoded by the rat μ opiate receptor cDNA [15–17], 59% amino acid and 50% nucle-

Fig. 1. Predicted amino acid sequence of the human brain μ opiate receptor (hMOR1). The sequence from the human cDNA clone hMOR1 is compared to the rat homologs of the μ (rMOR1)[15–17], δ (rDOR1)[17], and κ (rKOR1)[21–22] opiate receptor amino acid sequences by using the program PILEUP. Boldface type and shading, transmembrane domain candidates; *, consensus sites for N-linked glycosylation; italics, amino acid residues different between rat and human μ opiate receptor; @, indicates intron/exon boundary. The nucleotide sequence has been submitted to GenBank (#L25119)

otide identity with the rat κ receptor [21–22] and 62% amino acid and 59% nucleotide identity with the rat δ receptor [17] (Fig. 1). Amino acid identities with the rat somatostatin receptor [36], 40%, and β₂ adrenergic receptor [37], 27%, were higher in transmembrane domains. This sequence manifests five N-terminal sites for potential N-linked glycosylation, seven hydrophobic potential transmembrane domains, and sites for possible phosphorylation characteristic of members of the G-protein linked receptor family (Fig. 1). An 18 kb human genomic subclone, pHG4, displayed sequence beginning in the middle of transmembrane domain 4 at its 5′ end, an intron between sequences encoding its' second extracellular loop, another exon containing sequences encoding transmembrane regions 5–7 and the first 47 amino acids of the C-terminal domain, and a second intron (Fig. 1). The genomic clone failed to hybridize with an oligonucle-

otide complementary to a sequence located ca. 400 bp into the 3' untranslated region of the cDNA (data not shown). Partial sequence analysis of this genomic clone thus identified 552 bp of sequence identical to those of the hμOR1 cDNA.

Expression of the hμOR1 in COS cells revealed high affinity recognition of the μ opiate specific ligand [^{3}H]DAMGO (D-ala2, N-methyl-phe4, glyol5) enkephalin with K_d 1.2 ± 0.13 nM (Fig. 2). This binding was
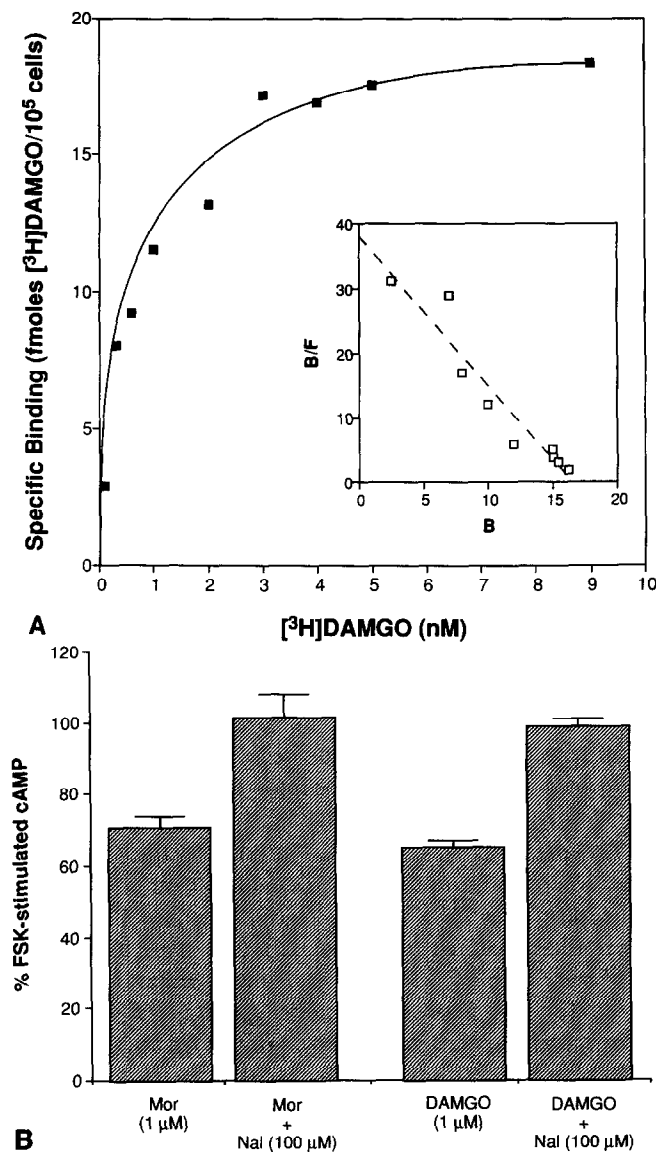

A

B

Fig. 2. (A) Saturation analysis of [^{3}H]DAMGO binding to COS cells transfected with pcDNA1.hμOR1. Results of a representative experiments (4 replicas) are shown. The non-specific binding is less than 15% of total radioactivity bound. The inset shows scatchard plots of the data. No significant [^{3}H]DAMGO binding could be detected in COS cells transfected with pcDNA1. (B) Functional coupling of hMOR1 to adenylate cyclase. COS-7 cells expressing the hMOR1 were initially treated with 1 mM IBMX for 15 min prior to being stimulated with 10 μM forskolin to elevate adenylate cyclase activity above basal levels. A μ-selective drug, peptide or naloxone were included in the medium at the concentrations indicated. Cellular cAMP levels were determined as described in the Section 2. Data are the mean ± standard error from three experiments.

displaced by a number of compounds recognizing μ receptors with high affinity. Morphine (4.1 ± 1.4 nM), CTOP (D-phe-cys-tyr-D-trp-orn-thr-pen-thr-NH2; Peninsula Labs; 16 ± 6 nM), levorphanol and DADLE (D-ala2, D-leu5 enkephalin; 16 ± 1.3 nM) displayed nanomolar affinities. U-50,488, DPDPE (D-pen2, D-pen5 enkephalin), dextrorphan and dynorphin A 1–17 (284 ± 110 nM) each displayed low affinities of less than 100 nM. Each compound's binding could be blocked by 1 μM (−)naloxone. The addition of 50 μM Gpp(NH)p reduced the affinity of DAMGO more than three-fold. Addition of concentrations of sodium chloride as low as 5 mM reduced binding affinity by two-fold. As noted for the rat μ receptor, the human receptor can couple to adenylate cyclase. Addition of morphine or DAMGO to forskolin-stimulated COS cells expressing the human μOR1 transiently resulted in greater than 30% decreases in adenyl cyclase activity that were naloxone-reversible (Fig. 2B).

Southern analyses revealed that both the human cDNA and genomic hybridization probes hybridized to total genomic DNA extracted from human but not from hamster. Southern analyses of DNA from 36 panels derived from 26 independent hamster/human somatic cell hybrid lines revealed that both the human cDNA and genomic hybridization probes hybridized to DNA from each of the six panels derived from four independent cell lines that contained human chromosome six. In studies using the cDNA hybridization probe, none of the 30 panels derived from 22 independent cell lines containing material from human chromosomes other than six produced a positive hybridization signal. No other human chromosome was uniformly present in cell lines producing positive hybridization signals. Hybridization patterns with the genomic clone HG4, however, also revealed signals in four panels containing chromosome 3 and two panels containing chromosome 5 (data not shown).

Analysis of 42 metaphase cells by fluorescent in situ hybridization demonstrated 20 cells (48%) that had at least one pair of hybridization signals that involved both chromatids of a single chromosome. Thirty-two paired signals were seen; 23 (72%) were located near the terminal end of the long arm of a large C-group (chr. 6,7 or X) chromosome. To determine the specific chromosome and band, cells were G-banded by fluorescence plus Giemsa [31] techniques, and photographs of banding patterns aligned with photographs of the fluorescence in situ hybridization signals to determine sub-band location. Eighteen signals of 27 analyzable signals (67%) were on chromosome 6, bands q24–25 (Fig. 3A). Of the remaining 9 signals, seven were located on chromosome 3, band q26, while two were on other chromosomes.

Digestion with AluI, BamHI, EcoRI, HaeII, HindIII, HinfI, MspI, PstI, RsaI, and TaqI produced constant bands detectable with the 1.2 kb hμOR1 cDNA. How-

Fig. 3. (A) Ideogram of human chromosome 6, displaying localization of hybridization with human μOR1 genomic sequences to 6q24–25. Each dot represents a paired hybridization signal noted on a G-banded metaphase chromosome. Signals clearly assigned to a single band are depicted to the right, those assigned to less precise regions indicated by brackets are depicted on the left side. (B) MspI RFLP patterns of DNAs extracted from leukocytes of 8 unrelated individuals probed with radiolabeled 1.2 kb 3′ fragment of hμOR1 (Lanes 1–2, 3–9). Lane 3, no DNA. Fragment size estimates based on the mobilities of λ phage/HindIII DNA size markers were: 15.0 kb (upper band present in all DNAs except individual in lane 2); 7.5 and 6.4 kb (second and third bands present in all DNAs); 3.7 kb (present in individuals represented in lanes 2,4,7–9).
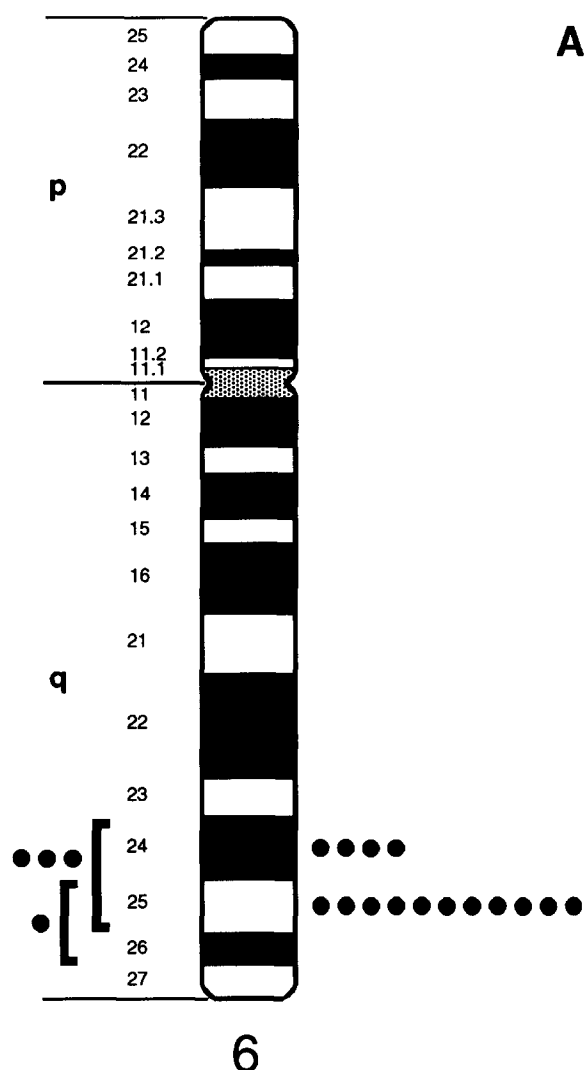
Fig. 3A

ever, MspI digestion produced hybridizing fragments of 15.0, 7.5, 6.4, and a 3.7 kb that showed variability from individual to individual, creating a distinct polymorphic pattern (Fig. 3B). The 3.7 kb band was present in DNA from 31 of 49 (63%) of the caucasian individuals studied. An apparently rare variant generated by the absence of the 15.0 kb band (Fig. 3B) was detected in only 2 (4.1%) of our 49 subjects. No band allelic with the 3.7 kb band could be identified in MspI digests. Conceivably, the other allele could comigrate with one of the constant bands in MspI digests, or be unrecognized by the 1.2 kb 3′ hybridization probe used here.

4. Discussion

These results document the nature and function of the human μ opiate receptor. The high conservation of the receptor with the rat sequence, more than 95%, may reflect its important bi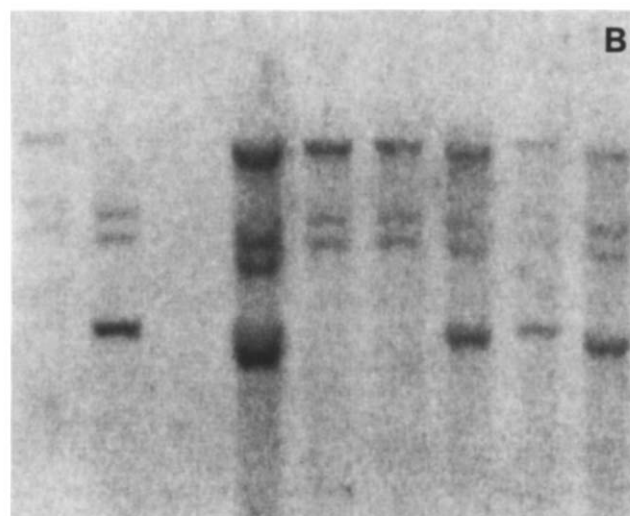ological roles. The conservation also extends to function. Each of the measures of opioid peptide and drug affinities and second messenger activities noted for the human receptor nicely parallels that noted for the rat.

The previously best-studied model of the human μ receptor may be the receptor expressed on the human neuroblastoma SH-SY 5Y cells [38]. In this cell line, the expressed receptor displays affinities for morphine, DAMGO and CTOP that are quite similar to values noted for our expressed cloned hμOR1 [39–40]. This pharmacologic parallel is also manifest as the ability of a 500-base pair fragment of phμOR1 to protect RNA prepared from the human SH-SY 5Y cells in preliminary RNase protection experiments (A. Moriwaki, Y. Imai, and GRU, unpublished observations). Each of these results is consistent with the notion that SH-SY5Y cells may express the same μOR1 cDNA expressed by the plasmid whose cloning is reported here. SH-SY5Y cells also display robust μ receptor coupling to adenylyl cyclase [38,41] (P.S.J., data not shown). The current description of μ opiate receptor binding from the expressed human cDNA fits well with previous work describing the properties of the expressed rat μ opiate receptor [15–17]. Each of the studies of cloned μ receptors to date has identified a receptor with high affinity for DADLE and

morphine, features imputed to a 'μ_1' subtype of μ opiate receptor defined by Pasternak and co-workers [3,42].

Both somatic cell hybrid panels probed with each of two hμOR1 probes and chromosomal in situ hybridization studies using the longer genomic hybridization probe produce concordant hybridzation signals associated with chromosome 6. Recognition of sequences on chromosome 3 and, in somatic cell hybrid studies, 5 by the HG4 genomic probe, but not by the cDNA, could be accounted for by hybridization to apparent repetitive sequences identified in the HG4 clone, but not in the cDNA (J.B.W. and X.-D. Yang, in preparation).

Opiate receptor-mediated effects on several different second messenger systems, including adenylyl cyclase, have been suggested by neuropharmacologic studies [8–14]. The human μOR1 receptor appears to be able to couple well to adenylyl cyclase, as noted for the rat receptor. The exact G-protein mechanisms, direct or indirect, whereby these effects are manifest remain to be elucidated. Interestingly, the rat μ opiate receptor peptide purified by Eppler and colleagues co-purifies with a $G_{i\alpha3}$ immunoreactive G-protein species [43].

Classical genetic studies, including family, twin and adoption approaches, suggest that individual differences in vulnerability to substance abuse are likely to be, at least in part, genetically determined [44–46]. μ opiate receptor systems are plausible candidate genes that might display allelic variants contributing to these individual differences. Conceivably, such allelic variation could also contribute to individual differences in potency and power of opiate mediated analgesia, or to development of tolerance during chronic treatments. Information on the chromosomal assignment and polymorphic human genetic markers noted in this report could contribute to our abilities to assess possible human clinically-significant allelic variants in this most-analgesia-and-addiction-associated opiate receptor subtype.

References

[1] Lord, J.A.H., Waterfield, A.A., Hughes, J. and Kosterlitz, H.W. (1977) Nature 267, 495–499.

[2] Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E. and Gilbert P.E. (1976) J. Pharmacol. Exp. Ther. 197, 517–532.

[3] Wolozin, B.L. and Pasternak, G.W. (1981) Proc. Natl. Acad. Sci. USA 78, 6181–6185.

[4] Su, T.-P. (1985) J. Pharmacol. Exp. Ther. 232, 144–148.

[5] Stefano, G.B., Melchiorri, P., Negri, L., Hughes, T.K. and Scharrer, B. (1992) Proc. Natl. Acad. Sci. USA 89, 9316–9320.

[6] Clark, J.A., Liu, L., Price, M., Hersh, B., Edelson, M. and Pasternak, G.W. (1989) J. Pharmacol. Exp. Ther. 251, 461–468.

[7] Rothman, R.B., Bykov, V., DeCosta, B.R., Jacobson, A.E., Rice, K.C. and Brady, L.S. (1990) Peptides 11, 311–331.

[8] Koski, G. and Klee, W.A. (1981) Proc. Natl. Acad. Sci. USA 78, 4185–4189.

[9] Koski, G., Streaty, R.A. and Klee, W.A. (1982) J. Biol. Chem. 257, 14035–14040.

[10] Blume, A.J. (1978) Proc. Natl. Acad. Sci. USA 75, 1713–1717.

[11] Frances, B., Moisand, C. and Meunier, J.-C. (1985) Eur. J. Pharmacol. 117, 223–232.

[12] Demoliou-Mason, C.D. and Barnard, E.A. (1986) J. Neurochem. 46, 1118–1128.

[13] Makman, M.H., Dvorkin, B. and Crain, S.M. (1988) Brain Res. 400, 185–190.

[14] Miyake, M., MacDonald, J.C. and North, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 3419–3422.

[15] Wang, J.B., Imai, Y., Eppler, C.M., Gregor, P., Spivak, C., and Uhl, G.R. (1993) Proc. Natl. Acad. Sci. USA. 90, 10230–10234.

[16] Chen, Y., Mestek, A., Liu, J., Hurley, J.A. and Yu, L. (1993) Mol. Pharmacol. 44, 8–12.

[17] Fukuda, K., Kato, S., Mori, K., Nishi, M. and Takeshima, H. (1993) FEBS Lett. 327, 311–314.

[18] Evans, C.J., Keith Jr., D.E., Morrison, H., Magendzo, K. and Edwards, R.H. (1992) Science 258, 1952–1955.

[19] Kieffer, B.L., Befort, K., Gaveriaux-Ruff, C. and Hirth, C.G. (1992) Proc. Natl. Acad. Sci. USA 89, 12048–12052.

[20] Yasuda, K., Raynor, K., Kong, H., Breder, C., Takeda, J., Reisine, T. and Bell G.I. (1993) Proc. Natl. Acad. Sci. USA 90, 6736–6740.

[21] Minami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S. and Satoh, M. (1993) FEBS Lett. 329, 291–295.

[22] Nishi, M., Takeshima, H., Fukuda, K., Kato, S. and Mori, K. (1993) FEBS Lett. 330, 77–80.

[23] Johnson, P.S., Wang, J.B., Wang, W.F. and Uhl, G.R. (1994) NeuroReports 5, 507–509.

[24] Ward, S.J. and Takemori, A.E. (1983) J. Pharmacol. Exp. Ther. 224, 525–530.

[25] Goodman, R.R., Snyder, S.H., Kuhar, M.J. and Young, W.S. (1980) Proc. Natl. Acad. Sci. USA 77, 6239–6243.

[26] Goodman, R.R. and Pasternak, G.W. (1985) Proc. Natl. Acad. Sci. USA 82, 6667–6671.

[27] Tempel, A. and Zukin, S. (1987) Proc. Natl. Acad. Sci. USA 84, 4308–4312.

[28] Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H. and Watson, S.J. (1987) J. Neurosci. 7, 2445–2464.

[29] Kitayama, S., Shimada, S. and Uhl G.R. (1992) Ann. Neurol. 32, 109–111.

[30] Gregor, P., Yang, X., Mano, I., Takemura, M., Teichberg, V. and Uhl, G.R. (1992) Mol. Brain Res. 16, 179–186.

[31] Bhatt, B., Burns, J., Flannery, D. and McGee, J. (1988) Nucleic Acids Res. 16, 3951–3961.

[32] Lichter, P., Tang, C., Call, K., Hermanson, G., Evans, G., Housman, D. and Ward, D. (1990) Science 247, 64–69.

[33] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.

[34] Smith, S.S., O'Hara, B.F., Persico, A.M., Gorelick, D.A., Newlin, D.B., Vlahov, D., Solomon, L., Pickens, R. and Uhl, G.R. (1992) Arch. Gen. Psych. 49, 723–727.

[35] Persico, A.M., Vandenbergh, D.J., Smith, S.S. and Uhl, G.R. (1993) Biol. Psych. 34, 265–267.

[36] Li, X.J., Forte, M., North, R.A., Ross, C.A. and Snyder, S.H. (1992) J. Biol. Chem. 267, 21307–21312.

[37] Buckland, P.R., Hill, R.M., Tidmarsh, S.F. and McGuffin, P. (1990) Nucleic Acids Res. 18, 682–682.

[38] Kazmi, S.M. and Mishra, R.K. (1987) Mol. Pharmacol. 32, 109–118.

[39] Toll, L. (1990) Eur. J. Pharmacol. 176, 213–217.

[40] Costa, E.M., Hoffman, B.B. and Loew, G.H. (1992) Life Sci. 50, 73–81.

[41] Yu, V.C., Hochhaus, G., Chang, F.-H., Richards, M., Bourne, H. and Sadee, W. (1988) J. Neurochem. 51, 1892–1899.

[42] Pasternak, G.W. and Wood, P.J. (1986) Life Sci. 38, 1889–1898.

[43] Eppler, C.M., Hulmes, J.D., Wang, J.B., Johnson, B., Corhbett, M., Luthin, D.R., Uhl, G.R. and Linden, J. (1993) J. Biol. Chem. 268, 26447–26450.

[44] Goldberg, J., Lyons, M.J., Eisen, S.A., True, W.R. and Tsuang, M. (1993) Behav. Genet. Soc. Abstr.

[45] Pickens, R.W., Svikis, D.S., McGue, M., Lykken, D.T., Heston, L.L. and Clayton, P.J. (1991) Arch. Gen. Psych. 48, 19–28.

[46] Uhl, G.R., LaBuda, M., Elmer, G. and Pickens, R. (1994) in Meltzer, H. (ed.) Psychopharmacology: A Fourth Generation of Progress, in press.