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Identification of four novel MOR-1B splice variants of the mouse mu opioid receptor gene: Functional consequences of C-terminus splicing

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Abbreviations:

MOR-1	mu opioid receptor clone-1
DAMGO: `	[D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin
norBNI	norbinaltorphimine
[Dmt^1]DALDA	Dmt-D-Arg-Phe-Lys-NH_2
DSLET	[D-Ser^2,Leu^5]enkephalin-Thr^6
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
CTAP	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
M6G	morphine-6ß-glucuronide
NalBzoH	naloxone benzoylhydrazone
U50,488H	trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide
GppNHp	guanosine-5′-[(β,γ)-imido]triphosphate

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Abstract

The rat rMOR-1B, in which a novel exon 5 was found in the place of exon 4, was one of the first MOR-1 variants described. We now have identified the mouse homolog of the rat MOR-1B as well as four additional variants derived from splicing from exon 3 into different sites within exon 5. The sequences of all the variants were identical, except for the intracellular tip of the C-terminus encoded by exon 5 where each variant predicted a unique amino acid sequence ranging from 2 to 39 amino acids. All of the mMOR-1B variants were selective for mu opioids in receptor binding assays, as anticipated since they all have identical binding pockets defined by the transmembrane domains. However, the relative potency and efficacy of mu agonists to each other varied from variant to variant in [^{35}S]GTPγS binding studies, as shown by morphine-6ß-glucuronide which was the most efficacious agent against mMOR-1B1 and the least against mMOR-1B2. mMOR-1B4 was quite unusual. Although mMOR-1B4 was mu-selective in receptor binding studies and antagonists labeled mMOR-1B4 well, the binding affinities of most mu agonists were far lower than those seen with mMOR-1, suggesting that the 39 amino acids at the C-terminus of mMOR-1B4 influences the conformation of the receptor and its ligand recognition site itself either directly or through its interactions with other proteins. In conclusion, alterations in the amino acid sequence of the C-terminus do not alter the mu specificity of the receptor, but they can influence the binding characteristics, efficacy and potency of mu opioids.

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Introduction

Following the classification of the mu, kappa and delta opioid receptors (Martin *et al.*, 1976;Lord *et al.*, 1977;Kosterlitz and Leslie, 1978), drugs were classified by their selectivity profiles in receptor binding assays. Most clinical analgesics are mu-selective, having little affinity for either kappa or delta receptors. Yet, clinicians have long known that the patient responses to mu opioids can vary widely, both in terms of their relative analgesic activity and their side-effects (Payne and Pasternak, 1992). Clinicians also have utilized incomplete cross tolerance among mu opioids to regain analgesic activity in patients highly tolerant to one mu drug by switching them to a different mu opioid, a technique termed Opioid Rotation (Cherny *et al.*, 2001). These, and other clinical observations, raised the possibility that all the mu opioids might not be acting through identical receptor mechanisms.

The concept of multiple mu opioid receptors was formally proposed almost twenty five years ago (Wolozin and Pasternak, 1981), based upon identification of a novel receptor binding site (Pasternak and Snyder, 1975a;Lutz *et al.*, 1984) and the actions of the novel antagonists naloxonazone and naloxonazine, agents that dissociated opioid analgesia from other opioid actions, including respiratory depression, the inhibition of gastrointestinal transit and most signs of physical dependence (Pasternak *et al.*, 1980;Ling *et al.*, 1984;Ling *et al.*, 1985;Heyman *et al.*, 1988).

The cloning of the mu opioid receptor MOR-1 opened the study of mu opioid receptors at the molecular level (Chen *et al.*, 1993;Wang *et al.*, 1993;Eppler *et al.*, 1993;Thompson *et al.*, 1993). Soon after the initial reports came the identification of two MOR-1 splice variants, providing the first appreciation of the complexity of the mu opioid receptor gene, *Oprm* (Bare *et*

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al., 1994;Zimprich *et al.*, 1995). MOR-1B, initially isolated from the rat, differed from MOR-1 with the replacement of exon 4 by exon 5. At the protein level, the predicted 12 amino acids at the C-terminus of MOR-1 were replaced by a different sequence of 5 amino acids, which functionally made rMOR-1B was less sensitive to agonist-induced desensitization. MOR-1 splicing has now been observed in a variety of species. A number of additional variants involving splicing at the C-terminus have been identified in mice (Pan *et al.*, 1999;Pan *et al.*, 2000;Narita *et al.*, 2003;Kvam *et al.*, 2004), rats (Pasternak *et al.*, 2004) and humans (Pan *et al.*, 2003;Pan *et al.*, 2005). Like rMOR-1B, these have alternative exons in place of exon 4. Splicing in the mouse, however, is even more complex. A series of additional variants derived from splicing at the 5'-end of the mouse *Oprm* gene also have been described (Pan *et al.*, 2001). We now report the identification of the mouse homolog of rMOR-1B and a series of additional variants derived from alternative splicing within the mouse exon 5.

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Materials and Methods

Isolation of genomic BAC subclones containing exon 5.

In order to identify the mouse homolog of the rat exon 5 in mouse *Oprm* gene, the mouse BAC clone A (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001) containing exons 1, 2, 3 and 4 was digested with HindIII. The HindIII-digested fragments then were subcloned into the HindIII site of Bluescript SK vector. After being transformed into JM109 competent cells, the transformants were plated on LB plates containing 0.2 mM/ml IPTG, 0.008% X-gal and 100 μg/ml ampicillin for colony lifting. A 280 bp cDNA fragment of the rat exon 5 was obtained by RT-PCR with rat brain total RNA and a pair of primers from rat MOR-1B (Zimprich *et al.*, 1995) (sense primer: 5'-CCA CCA GAA AAT AGT ATT ATT TTG AAA AGG C-3' and antisense primer: 5'-GTT CAT TGA GAG AAG CTT GCC CAG AGT CTG-3'), were ^{32}P-labeled and used as a probe for colony hybridization. The colony lifting and hybridization procedures were performed using the protocols previously described (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001). Six positive clones with identical ~ 1 kb insertions were obtained. Sequence analysis of the fragment indicated that the clone contained the exon sequence, exon 5a, homologous to rat exon 5. The mouse exon 5a and rat exon 5 sequences shared 91% identity.

Reverse-transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from mouse brain by the guanidinium thiocyanate phenol-chloroform method (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001) and reverse-transcribed with random hexamers and Superscript II reverse transcriptase (Invitrogen). The first-strand cDNA was then used as template in PCR with a sense primer from exon 3 (5'-CCC AAC TTC CTC CAC AAT CGA A-3') and antisense primer from exon 5a (5'-GGT GTG CTT CTC CCA

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GTT CTG TGT ATA-3'). Two fragments were amplified. Sequence analysis of the fragments revealed that one fragment contained exons 3 and 5a sequences, and the other had a 699 bp new sequence inserted between exons 3 and 5a. The new exon sequence (exon 5b) was mapped upstream of exon 5a by PCR and by sequencing the BAC clone A.

To obtain full length cDNA clones of the potential exon 5 associated variants, two sense primers from the 5'-untranslated region of exon 1 (sense primer A: 5'-AGA GGA AGA GGC TGG GGC G-3' and sense primer B: 5'-GGA ACC CGA ACA CTC TTG AGT GCT-3'), two antisense primers from exon 5a (antisense A: 5'-CTT GCC CCA GAG ACG GAA TGA TGC AG-3' and antisense primer B: 5'-GGG GTT GGC ACC AGC ATT AGG TAC TC-3') and two antisense primers from exon 5b sequence (antisense primer C: 5'-GGT CTT CCT TTG GAG TTC CAC AGG AAG-3' and antisense primer D: 5'-TTG TAA TCT AAT CAC CAT GGA TTA GGT GCC-3') were used in nested PCRs with the first-strand cDNA from mouse brain total RNA as template. Five fragments arranging from ~1.3 to ~ 3.0 kb in length, respectively, were obtained, subcloned into pCRII-TOPO vector (Invitrogen) and sequenced. Sequence analysis of the fragments indicated that all the fragments contained the exons 1, 2, and 3 but different downstream exon sequences that all associated with exon 5a. Comparison these downstream exon sequences with the genomic sequences from the BAC clone A (see below) indicated that all the exon sequences were derived from a continuous genomic sequence, which was started from exon 5a and extended ~ 3.2 kb to its upstream. We named the whole sequences as exon 5 and subsets of exon 5, due to alternative splicing within exon 5, as exons 5a, 5b, 5c, 5d and 5e (Figs. 1 & 2). Based upon splicing pattern within exon 5, we named the five cloned full length cDNAs as mMOR-1B1, mMOR-1B2, mMOR-1B3, mMOR-1B4 and mMOR-1B5, respectively (Figs. 1 & 2). We have sequenced the clones in both orientations with appropriate

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primers. The sequences of exons 1, 2 and 3 were identical to those of mMOR-1 and the exon 5 sequences were 100% aligned to those of the mMOR-1 gene in the Mouse Genome Database (Ensembl and NCBI).

Identification and characterization of genomic BAC subclones containing the new exon sequence.

The BamHI- or KpnI- or SacI-digested BAC clone A fragments were subcloned into Bluescript SK vector. The resulting plasmids were screened through colony lifting and hybridization as mentioned above with two probes, one from the 108 bp sequence of exon 5c and the other from the 362 bp sequence of exon 5e, respectively. Four overlapping positive clones arranging from ~ 7 kb to ~ 10 kb in length were obtained and a 3.4 kb region of the overlapping clones that containing all the new exon 5s sequences was sequenced. The sequence was identical to that from public mouse genomic databases except one base mismatch in exon 5c and one more four-base GTTT repeat in exon 5b (Fig. 2), which may represent the difference between the strain mice.

Northern blot analysis

Northern blot analysis was performed as described previously (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001). In brief, 20 μg of total RNA extracted from mouse brain was separated on a 0.8% formaldehyde agarose gel and transferred to GenePlus membrane (NEN). The membranes were hybridized with a 125 bp ^{32}P-labeled exon 5a probe produced by PCR (sense primer, 5'-CAG AAA ATA GAT TTA TTT TGA AAA GGC A-3' and antisense primer, 5'-GGG GTT GGC ACC AGC ATT AGG TAC TC-3'), or a 273 bp ^{32}P-labeled exon 5b probe

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(sense primer, 5'-GCC TTG ATA ATT AGG GCA CCA AAG GGG-3' and antisense primer, 5'-CGG CTC AAT TCA CAG CTT TGG GCC-3') or a 300 bp ^{32}P-labeled exon 5c probe (sense primer, 5'-CAA GCC TCA CAC TTC AGT AAT GGA ATG-3' and antisense primer, 5'-CTT CTT CCA CCA AAG CCA GAC AGG C-3'), or a 243 bp ^{32}P-labeled exon 5d probe (sense primer, 5'-GCA CAC CAA AAA CCT CAA GAA TGC CTG-3' and antisense primer, 5'-CCC ATT TCA GCA CTA TGA GAA GTT ATC-3') or a 93 bp ^{32}P-labeled exon 5e probe (sense primer, 5'- GTG TAT GAG TGC TAT GCC CAC AGG G-3' and antisense primer, 5'- TTG CGG GGG GTG GGG GTG-3').

Expression of the variants

The cDNA fragments containing the full-length mMOR-1B1, mMOR-1B2, mMOR-1B3, mMOR-1B4 and mMOR-1B5 in pCRII-TOPO were subcloned into pcDNA3.1 or pcDNA5/FRT (Invitrogen) with appropriate restriction enzymes. The resulting plasmids, mMOR-1B1/pcDNA3, mMOR-1B2/pcDNA3, mMOR-1B3/pcDNA3, mMOR-1B4/pcDNA5/FRT and mMOR-1B5/pcDNA3, respectively, were transfected into Chinese hamster ovary (CHO) cells or CHO/Flip-In cells by LipofectAMINE reagent. Stable transformants were obtained 2 weeks after selection with G418 or hygromycin and screened in [^{3}H]DAMGO or [^{3}H]diprenorphine binding assays (New England Nuclear, Boston, MA).

Relative semi-quantitative RT-PCT

Total RNA obtained from mouse brain as described above was treated with TURBO DNA-free reagents (Ambion), and reverse-transcribed with random hexamers and Superscript II reverse transcriptase. Aliquots of the first-strand cDNA were used as templates in PCRs with sets

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of primers specific for each variant. The following PCR primers were used: mMOR-1B1, exon 3 sense primer A (5'-GCT GCC TGA ACC CAG TTC TTT ATG CG-3') and an exon 5a antisense primer (5'-GGG GTT GGC ACC AGC ATT AGG TAC TC-3'); mMOR-1B2, exon 3 sense primer B (5'-GCA TCC CAA CTT CCT CCA CAA TCG AAC-3') and an exon 5b antisense primer (5'-CCC CTT TGG TGC CCT AAT TAT CAA GGC-3'); mMOR-1B3, exon 3 sense primer A and an exon 5c antisense primer (5'-CTA GGT CTA GCT CAT GAA TGC TCT TTG GTT GG-3'); mMOR-1B4, exon 3 sense primer A and an exon 5d antisense primer (5'-CCC ATT TCA GCA CTA TGA GAA GTT ATC-3'); mMOR-1B5, exon 3 sense primer A and an exon 5e antisense primer (5'-TTG CGG GGG GTG GGG GTG GG-3'); and mMOR-1, exon 3 sense primer A and an exon 4 antisense primer (5'-GCA ACC TGA TTC CAA GTA GAT GGC AG-3'). PCRs were performed in a thermal cycler (MJ Research, PTC-200) using Platinum Taq DNA polymerase (Invitrogen) with an initial 2 min denaturing step at 94°C, followed by 48 cycles, each containing a 20 sec denaturing step at 94°C, a 20 sec annealing step at 65°C and a 45 sec of extension step at 72°C, and a final 3 min extension at 72°C. PCR products were separated on a 1.5% agarose gel. The gel then was stained with 0.02% ethidium bromide, and imagined with a FluorChem^{TM} 8000 (Alpha Innotech Corporation). Relative band intensities were quantified with the AlphaEase^{TM} FC software. All the PCR products showed bands with correct sizes predicted from the primers: 295 bp for mMOR-1B1; 187 bp for mMOR-1B2 ; 362 bp for mMOR-1B3; 417 bp for mMOR-1B4; 266 bp for mMOR-1B5 and 278 bp for mMOR-1. Each band was purified and the gel and its identity confirmed by DNA sequencing. PCR Controls without the template showed no visible bands (data not shown).

Receptor Binding assays

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Membranes were prepared from cells stably transfected with the pcDNA3 constructs as described above. [^{3}H]DAMGO or [^{3}H]diprenorphine saturation and competition binding assays were performed at 25°C for 60 min in 50mM potassium phosphate buffer, pH7.4, containing 5mM magnesium sulfate (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001). Specific binding was defined as the difference between total binding and nonspecific binding, determined in the presence of levallorphan (10 μM). Protein concentrations were determined as previously described using BSA as the standard (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001).

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding was performed on membranes prepared from transfected cells in the presence and absence of the indicated opioid for 60 min at 30°C in the assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.2 mM EGTA, 10 mM NaCl) containing 0.05 nM [^{35}S]GTPγS and 30 μM GDP, as previously reported (Pasternak *et al.*, 2004;Pan *et al.*, 2005). After the incubation, the reaction was filtered through glass-fiber filters (Schleicher & Schuell), and washed three times with 3 ml of ice-cold 50 mM Tris-HCl (pH 7.4) on a semiautomatic cell harvester. Filters were transferred into vials with 5 ml of Liquiscent (National Diagnostics, Atlanta, GA), and the radioactivity in vials were determined by scintillation spectroscopy in a Packard TRI-CAEB 2900TR counter. Basal binding was determined in the presence of GDP and absence of drug.

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Results

Cloning exon 5 splice variants of the mouse *Oprm* gene. In the rat, rMOR-1B contains exon 5 in place of exon 4 (Fig. 1) (Zimprich *et al.*, 1995). We obtained the mouse exon 5a sequence from the mouse genomic BAC clone A using a colony hybridization approach with a rat exon 5 probe. The mouse exon 5a sequence shared high homology (91% identity) with the rat exon 5. Subsequent RT-PCR with the primers from the mouse exons 3 and 5a revealed the anticipated splicing between exons 3 and 5a, but also demonstrated a novel pattern of splicing upstream of exon 5a (Fig. 2). To generate full length cDNAs, nested RT-PCR with primers from exons 1, 5a and 5b led to isolation of the full length mMOR-1B2, mMOR-1B3, mMOR-1B4 and mMOR-1B5 clones, as well as mMOR-1B1, which was homologous to the rat MOR-1B. Thus, exon 5 in the mouse contains five different 3' splice sites linked to exon 3.

All the mMOR-1B variants contained exons 1, 2 and 3, differing only in their downstream sequence as a result of alternative splicing within exon 5 (Figs. 1 & 2). All the splice junctions were consistent with the consensus splicing pattern. mMOR-1B1 had exon 5a as the fourth exon, which predicted the identical five amino acid sequence as reported in the rat rMOR-1B (Zimprich *et al.*, 1995). mMOR-1B2 contained a 699 bp insertion (exon 5b) between exons 3 and 5a, which encoded 23 amino acids. In mMOR-1B3, splicing from exon 3 to exon 5c (108 bp) predicted six amino acids. Only two amino acids were predicted from exon 5e (362 bp) of mMOR-1B5 due to early termination of translation. On the other hand, exon 5d (362 bp) in mMOR-1B4 predicted 39 amino acids. Each of the amino acid sequences predicted from exon 5b to exon 5e was unique and differed from all previously identified C-terminus variants. The predicted amino acid sequence from exon 5b of mMOR-1B2 contained a protein kinase C phosphorylation site.

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Northern blot analysis of the variant mRNA. We next performed Northern blot analysis with various exon probes to estimate the relative size of the variant mRNAs (Fig. 3). All the individual exon probes hybridized to a band of ~14 kb, although with different intensities. The individual exon probes also revealed additional bands. For example, the exon 5b probe hybridized to a sharp strong band at approximately 11 kb and a second diffuse band at 1.5 to 2.5 kb. A diffuse band in the 4-7 kb range was observed with all the probes, although it was far more prominent with the exon 5c, 5d and 5e probes. The different banding patterns illustrate the uniqueness of the various variant mRNAs and suggest that the individual exon probes associate with more than one transcript, as predicted from the structure.

Relative abundance of the variants: In order to estimate relative abundance of each variant mRNA, we performed semi-quantitative RT-PCR using mouse brain total RNA. The results showed that mRNAs of all the mMOR-1B variants were significantly lower than that of mMOR-1 ($p < 0.001$), ranging from 18% to 39% of mMOR-1 level (Fig. 5a & b). However, it should be pointed out that the band amplified by the exons 3 & 4 primers to estimate mMOR-1 levels might also have amplified six other splice variants (mMOR-1G – mMOR-1L) generated from the exon 11 promoter which are also present, although at lower expression levels than mMOR-1 itself (Pan *et al.*, 2001). Thus, all of these relative values must be considered only estimates of the expression of the variants.

Receptor binding of the variants. Earlier studies from our laboratory revealed that the mouse, rat and human C-terminus splice variants of MOR-1 retained their mu selectivity and affinity (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001;Pasternak and Pan, 2004;Pan *et al.*, 2005;Pasternak *et al.*, 2004). To assess the new variants, we cloned their full length cDNAs downstream of a CMV promoter in the mammalian expression vector pcDNA3 and established

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stably transfected CHO cell lines. In saturation studies, the agonist [^{3}H]DAMGO labeled all the variants with similar high affinities (Table 1), except for mMOR-1B4. The expression levels of the different variants also were comparable.

Competition studies using the agonist [^{3}H]DAMGO confirmed the mu selectivity of all the variants (Table 2). The mu ligands competed binding very potently while the kappa$_1$-selective opioid U50,488H and the delta-selective ligand DPDPE were ineffective. Several of the drugs showed differences in their K_i values among the variants, but these differences were modest. DADLE showed the greatest differences, which were due predominantly to mMOR-1B1. Within a variant, we also saw a modest range of affinities for the different drugs.

Binding determined with radiolabeled opioid agonists and antagonists differ (Pert *et al.*, 1973;Pasternak and Snyder, 1975b;Pasternak *et al.*, 1975;Snyder and Pasternak, 2003), presumably since agonists label only the agonist receptor conformation while antagonists bind to both agonist and antagonist conformations. This is clearly shown when looking at the B_{max} values for mMOR-1 using the agonist [^{3}H]DAMGO and the antagonist [^{3}H]diprenorphine. Here, the B_{max} value for [^{3}H]diprenorphine bound with high affinity (K_D 0.71 ± 0.18 nM; n=3) with a B_{max} that (1.64 ± 0.13 pmol/mg protein; n=3) was approximately 10-fold greater than the B_{max} seen with the agonist [^{3}H]DAMGO in membranes from the same cells (0.16 ± 0.04 pmol/mg protein) (Table 1). This implies that the vast majority of the sites expressed in the cell line are in an antagonist conformation. Although antagonist conformations predominate, even in brain (Pasternak and Snyder, 1975a), this may be even more pronounced in cells overexpressing the receptors. The differences also are evident when examining the K_i values of opioids for mMOR-1 (Table 3). K_i values with [^{3}H]DAMGO provide an indication of the affinity of the competitor for the agonist state while the values against [^{3}H]diprenorphine provide an estimate of the

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affinity for the antagonist state since the vast majority of sites labeled by the radiolabeled antagonist are in the antagonist conformation. The K_i value for morphine is shifted by almost 20-fold while M6G is shifted over 100-fold. The antagonist naloxone shows little difference between the two radioligands.

The binding profile of mMOR-1B4 was quite unique (Table 3). Attempts to screen stable CHO or HEK 293 clones transfected with mMOR-1B4 failed to identify any with specific [^{3}H]DAMGO binding. We then examined binding with the antagonist [^{3}H]diprenorphine, which labeled mMOR-1B4-transfected CHO cells with an affinity (K_D) of 1.4 ± 0.36 nM (n=3), which was comparable to its affinity for mMOR-1 sites. The expression of mMOR-1B4 in the cells, measured with [^{3}H]diprenorphine, was lower than that of mMOR-1, although it remained relatively robust, with a B_{max} value of 0.68 ± 0.03 pmol/mg protein (n=3). This level of binding is greater than that seen with the agonist [^{3}H]DAMGO in the other transfected cell lines (Table 1).

The overall binding profile of mMOR-1B4 differed from the other ten full length mMOR-1 variants. This was shown by the [^{3}H]diprenorphine competition studies in mMOR-1 and MOR-1B4-expressing cells (Table 3). The binding to the MOR-1B4 cells was mu-selective, as shown by the poor affinity of the kappa and delta antagonists norBNI and naltrindole. However, the affinity of many agonists in the mMOR-1B4 cells was far lower than in mMOR-1-expressing cells, with K_i values for most over 100 nM. The poor affinity of DAMGO in the competition studies explains why attempts to demonstrate [^{3}H]DAMGO binding directly were not successful. Several agonists stood out from the others. Etorphine, etonitazene, [DMT^1]DALDA and buprenorphine all retained high affinity in the mMOR-1B4 cells, with K_i values under 15 nM. Indeed, these ultra-potent analgesics competed binding to the mMOR-1B4

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cell membranes with higher affinity than some of the antagonists. Behaviorally, these drugs are notable for their extremely high analgesic potency. Yet, not all the highly potent mu opioids retained high affinity, as illustrated by fentanyl, with a K_i value over 100 nM.

To assess whether the mMOR-1B4 sites labeled by [^{3}H]diprenorphine represented antagonist conformations of the receptor, we explored the effects of the stabilized GTP analog, GppNHp on the binding in both mMOR-1 and mMOR-1B4 cells (Table 4). GTP and its analogs selectively diminish the affinity of agonists (Childers and Snyder, 1978). In the current study, we saw agonist shifts, but they remained relatively modest. The antagonists naloxone and CTAP showed no shift in the mMOR-1 cells. However, they did reveal a small shift with mMOR-1B4. Although this shift is very modest, it raises the possibility that they may be partial agonists at this variant. Overall, the small shifts for the agonists is consistent with the earlier studies implying that the vast majority of the sites in both the mMOR-1 and mMOR-1B4 cells are in an antagonist conformation.

Characterization of the variants by [^{35}S]GTPγS binding assay. Prior studies have documented functional differences among a number of MOR-1 variants (Bolan *et al.*, 2004;Pasternak *et al.*, 2004;Pan *et al.*, 2005). We therefore examined the ability of the mMOR-1B variants to activate G-proteins using a [^{35}S]GTPγS binding assay (Table 5). The range of the EC_{50} values differed from one variant to another. Some showed little difference among the drugs, while mMOR-1B1 revealed a greater range. For most of the drugs, their relative EC_{50} values in the [^{35}S]GTPγS stimulation assay did not correlate well with their relative receptor binding affinities. Although the K_i and EC_{50} values for M6G (r^2=0.72) and for endomorphin 2 (r^2=0.73) showed a modest correlation, the correlations for the remainder of the drugs were very poor, as was the correlation for all the drugs together (r^2=0.16).

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The EC_{50}/K_i ratio provides an indication of the ability of the drug to activate the receptor relative to its receptor occupancy (Table 5). Since the conditions used in the receptor binding and [^{35}S]GTPγS binding assays were not identical, this ratio should not be considered a direct indication of intrinsic activity, but it still is useful in evaluating the drugs. The ratios within a given variant ranged up to 10-fold and the rank order of the ratios of the drugs to each other varied among the splice variants. For example, endomorphin 2 had the lowest ratio for mMOR-1B5 and the highest ratio for mMOR-1B3. M6G was among the lowest for mMOR-1B5 and the highest against mMOR-1B1.

For many of the variants, the maximal stimulation did not vary very much among the drugs (Table 5). mMOR-1B1 had the greatest variation, with responses ranging from 117% of DAMGO to only 68% of endomorphin 1. DAMGO was the most efficacious among the all mMOR-1B variants, with the exception of mMOR-1B1. The most intriguing observation was that the relative efficacy of the drugs to each other varied from one variant to another. Among the mMOR-1B variants, M6G was the most efficacious with mMOR-1B1 and the least against mMOR-1B2. Conversely, endomorphin 1 was the lowest against mMOR-1B1 and among the highest with mMOR-1B3. Thus, the C-terminus differences of these variants were associated with varying effects on efficacy and potency of the mu drugs.

As noted earlier, mMOR-1B4 proved to be a very unique variant. Although mu opioid antagonists retained high affinity for this variant, the affinity of agonists in the binding was far lower (Table 3). Yet, a number of the compounds were active in [^{35}S]GTPγS binding assays, although their level of stimulation was lower than that seen with the other variants (Table 6). Of the active drugs, dynorphin A stimulated binding the most, followed by etonitazene, fentanyl and etorphine. As with the other variants, the maximal stimulation induced by the agonists did not

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correlate with their affinity for the receptors. Dynorphin A displayed very poor affinity in the binding assays, yet it was the most effective in the [^{35}S]GTPγS binding study. Similarly, fentanyl displayed an affinity 20-fold lower than either etorphine or etonitazene, but stimulated [^{35}S]GTPγS binding as well.

To determine whether or not the stimulation was opioid specific, we examined the sensitivity of both etorphine and etonitazene to several opioid antagonists (Fig. 4). The maximal stimulation of both agents at 1 μM was modest, although similar to values seen at 25 μM. Etorphine stimulated binding by only 27±3 % and etonitazene by only 35±6 %. The stimulated binding of both opioids was reversed by the opioid-selective antagonists naloxone, naltrexone and diprenorphine, confirming its specificity.

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Discussion

The rMOR-1B variant was the first variant identified in the rat (Zimprich *et al.*, 1995) and was recently reported in the mouse (Narita *et al.*, 2003). Other splice variants of the mouse *Oprm* gene have also been reported, many of which are alternatively spliced downstream of exon 3 (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001;Pasternak and Pan, 2004;Pan *et al.*, 2005;Kvam *et al.*, 2004). The present study describes five mouse variants generated from splicing within the mouse exon 5. The predicted amino acid sequences downstream from exon 3 for each of these variants differed in both composition and length, ranging from two to thirty-nine amino acids. Together with the variants previously isolated, there are now twelve full length and functional C-terminus variants of the mouse *Oprm* gene.

Like the other full length MOR-1 variants, all the full length MOR-1B variants were mu-selective in binding assays, which was anticipated since the binding pocket is defined by exons 1, 2 and 3, which are identical in all the variants. Functional differences, therefore. must be due to the amino acid sequence differences at the tip of the C-terminus. Like the other full length variants (Pasternak *et al.*, 2004;Bolan *et al.*, 2004;Pan *et al.*, 2005), the mMOR-1B splice variants differed in both the relative potency and efficacy of a number of mu opioids in stimulating [^{35}S]GTPγS binding, a measure of G-protein activation. Differences in the relative efficacy of the drugs to each other and in their relative potency from variant to variant raise interesting questions. Presumably, drugs administered in vivo will interact with all the variants. However, the relative activation of one splice variant to another will likely differ from one drug to the next, leading to differences in their overall activation profiles. This may help explain the subtle, but potentially significant, behavioral differences among these agents.

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Of the exon 5-containing variants, mMOR-1B4 clearly stands apart from the rest. Although its mu-selectivity was clearly demonstrated by the [^{3}H]diprenorphine competition studies, direct binding studies with [^{3}H]DAMGO were unsuccessful, presumably due to its low affinity. Most agonists displayed poor affinities against [^{3}H]diprenorphine binding in both mMOR-1 and mMOR-1B4 expressing cells, presumably due to the predominance of sites labeled by the radiolabeled antagonist in an antagonist conformation. The nearly 20-fold affinity shift of morphine, the 100-fold shift for M6G and the 25-fold shift for DAMGO in mMOR-1 cells were anticipated. Yet, the affinities of most of the agonists were even lower in the mMOR-1B4 cells. Interestingly, several agonists stood out from the others. Etorphine, etonitazene, [DMT^1]DALDA and buprenorphine all retained high affinity for both variants. Pharmacologically, all these drugs are very potent analgesics (Blane *et al.*, 1967;Schiller *et al.*, 2000;Neilan *et al.*, 2001), raising the interesting possibility that their enhanced analgesic activity might result from a unique ability to interact with receptors in both agonist and antagonist conformations.

It is still not clear why mMOR-1B4 displayed this unique binding profile. It has 39 amino acids at the tip of the C-terminus instead of the 12 amino acids encoded by exon 4. Yet, the increased length of the tail alone cannot be responsible for its unusual characteristics since several other variants with even longer sequences, mMOR-1C with 52 amino acids and mMOR-1F with 58, have typical binding affinities for the mu opioids (Pan *et al.*, 1999;Pan *et al.*, 2000). Thus, the distinct properties of mMOR-1B4 must reside in the sequence of the 39 amino acids. Since the binding pocket, which is defined by the transmembrane domains, is identical to that of the other full length mMOR-1 variants, the binding and pharmacological differences may reflect the presence or absence of interactions of the C-terminus with other membrane and/or cellular

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proteins. The presence of four cysteine residues within the 39 amino acid C-terminus of mMOR-1B4 raises additional possibilities regarding its structure and interactions with other proteins.

A number of proteins associated with G-protein coupled receptors can modulate binding and function, including G-proteins, RAMP proteins (Morfis *et al.*, 2003) or RGS proteins (Zachariou *et al.*, 2003;Sierra *et al.*, 2002;De Vries *et al.*, 2000). Receptor dimerization also influences binding and function (George *et al.*, 2000;Jordan and Devi, 1999;White *et al.*, 1998;Kaupmann *et al.*, 1998;Jones *et al.*, 1998;Pan *et al.*, 2002). Could the absence of a needed G-protein be responsible for the inability of mMOR-1B4 to show high affinity agonist binding? The repertoire of G-proteins varies among cell lines. CHO cells used in the current study reportedly lack $G_{i\alpha1}$, but transfection of $G_{i\alpha1}$ into the cells expressing mMOR-1B4 did not change any of the binding parameters (data not shown). We also expressed mMOR-1B4 in HEK293 cells to see if different cellular environments might restore agonist binding, without success (data not shown). Overexpression alone seems unlikely to explain our findings since the expression levels of mMOR-1 were even greater than mMOR-1B4. Other receptors also can modulate function. For example, some receptors lack function unless co-expressed with a second one, as seen with the $GABA_B$ receptors (White *et al.*, 1998;Kaupmann *et al.*, 1998;Jones *et al.*, 1998). In other situations, heterodimerization may change the pharmacology of the complexed receptors when compared to the individual receptors expressed alone, as shown for several pairs of opioid receptors (George *et al.*, 2000;Jordan and Devi, 1999;Pan *et al.*, 2002). All these possibilities must be considered.

MOR-1 splice variants also display regional distributions distinct from those of MOR-1 at the regional, cellular and ultracellular levels (Abbadie *et al.*, 2000b;Abbadie *et al.*, 2001;Abbadie *et al.*, 2000a). Similarly, the regional distribution of mMOR-1B4 was unlike any

others, including rMOR-1B (Y. Zhang, XY Pan and GW Pasternak, in preparation). Within the spinal cord, it weakly labeled the dorsal horn, robustly labeled Onuf's nucleus at the L5 and L6 levels and diffusely labeled the anterior horn. Within the brain, the most intense labeling was observed within the Purkinje cells of the cerebellum, with some labeling in the olfactory bulb. In the rat, rMOR-1B has an unusual immunohistochemical labeling pattern within the brain, with high levels of labeling restricted to the olfactory bulb and little labeling in areas typically associated with opioid modulation of pain (Schulz *et al.*, 1998).

These MOR-1 splice variants may help explain many of the clinical and behavioral observations seen mu opioids. Both clinical and preclinical studies reveal subtle differences among the mu opioids. With the exception of mMOR-1B4, mu opioids label all the full length variants with similar affinities. However, the overall pharmacological response of a drug reflects the summation of the activation of all the MOR-1 variants bound. Since both the efficacy and potency of each drug varies from variant to variant and does not correlate with binding affinity, the overall pharmacological activation profiles of each mu drug would be expected to differ. This ability to dissociate efficacy and potency from binding also opens the possibility of novel drugs. Selectivity among the MOR-1 variants is not likely to be achieved on the basis of binding affinities alone since their binding pockets are all structurally identical. However, it may be possible to obtain selectivity of action by independently modulating the efficacy and/or potency of the compounds at the different variants.

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Footnotes

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FIGURES

Fig. 1. Schematic of the mouse *Oprm* gene structure and alternative splicing.

The schematic of the gene structure. Exons and introns are shown by boxes and horizontal lines, respectively. The transcriptional start points are indicated by arrows. The genomic BAC clones are shown by heavy horizontal lines on the top. The complete cDNA and deduced amino acid sequences of mMOR-1B1, mMOR-1B2, mMOR-1B3, mMOR-1B4, and mMOR-1B5 have been deposited in the GenBank database (Accession number: AF167566, AF167567, AF346813, AF346814, and AF346812, respectively).

Fig. 2. Genomic sequence and predicted amino acid sequences of the exon 5 splice variants

The exon sequences are shown in capital letters, and partial intron sequences are indicated in lowercase letters. The predicted amino acids are shown in bold capital letters. Termination codons are indicated by ∗. The threonine, a potential protein kinase C phosphorylation site, from exon 5b is shown by italic and underlined. The one mismatch in exon 5c and one more GTTT repeat in exon 5b are shown by italic and bold. The sequence has been deposited in GenBank under accession number AY390763.

Fig. 3: Northern blot of exon 5 in mouse brain

Northern blot analysis was performed on mRNA isolated from whole brain as described previously (Pan *et al.*, 1999;Pan *et al.*, 2000;Pan *et al.*, 2001). In brief, 20 μg of total RNA extracted from mouse brain was separated on a 0.8% formaldehyde agarose gel and transferred to GenePlus membrane (NEN). The membranes were hybridized with a 125 bp ^{32}P-labeled exon 5a probe, a 273 bp ^{32}P-labeled exon 5b probe, a 300 bp ^{32}P-labeled exon 5c probe, a 243 bp ^{32}P-labeled exon 5d probe or a 93 bp ^{32}P-labeled exon 5e probe, as described in Methods.

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Fig. 4: Opioid-induced stimulation of [^{35}S]GTPγS binding in mMOR-1B4 and its sensitivity to antagonists.

[^{35}S]GTPγS binding was determined in membranes from CHO cells stably transfected with mMOR-B4 that were treated with either etorphine (1μM) or etonitazine (1 μM) alone or in the presence of naloxone (10 μM), naltrexone (10 μM) or diprenorphine (10 μM). The increase in stimulation is expressed as the percentage increase over basal levels. Results are the means ± s.e.m. of at least three independent determinations. ANOVA reveals differences between the agonist alone and the antagonists for both etorphine ($p < 0.033$) and for etonitazine ($p < 0.031$).

Fig. 5. Semi-quantitative RT-PCT of the mMOR-1B variants.

A. RT-PCR was performed as described in Materials and Methods. Agarose gel stained with ethidium bromide was imagined with FluorChem™ 8000 system, as described in the Methods. B. Relative band intensities from the gel were quantified with AlphaEase™ FC software. Bars represent the mean ± S.E. of the relative band intensity from three independent experiments. Relative intensity values were as follows: mMOR-1: 1042 ± 65; mMOR-1B1: 403 ± 31; mMOR-1B2: 236 ± 15; mMOR-1B3: 189 ± 7; mMOR-1B4: 255 ± 31; mMOR-1B5: 205 ± 17. Significance was assessed using ANOVA. Overall, the values were significant different ($p<0.001$). Tukey Posthoc analysis revealed that mMOR-1 differed from all the individual variants ($p<0.001$); mMOR-1B1 also differed from mMOR-1B3 ($p<0.01$) and mMOR-1B5 ($p<0.05$); mMOR-1B2 also differed from mMOR-1 ($p<0.001$)..

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Table 1. [^{3}H] DAMGO Saturation studies in mMOR-1 variants

Clone	K_D (nM)	B_{max} (pmol/mg protein)
mMOR-1	1.75 ± 0.44	0.16 ± 0.04
mMOR-1B1	1.20 ± 0.33	0.08 ± 0.01
mMOR-1B2	1.99 ± 0.29	0.12 ± 0.01
mMOR-1B3	0.77 ± 0.11	0.11 ± 0.02
mMOR-1B5	1.60 ± 0.41	0.11 ± 0.02

[^{3}H] DAMGO saturation studies were performed in membranes of CHO cells stably expressing the indicated variant as described in Methods. K_D values were determined by nonlinear regression analysis using Prism (Graphpad). Results are the mean ± S.E. of at least three independent determinations.

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Table 2: Competition of [^{3}H]DAMGO binding in cells stabling expressing mMOR-1 variants

Ligand	K_i value (nM)					ANOVA
	mMOR-1	mMOR-1B1	mMOR-1B2	mMOR-1B3	mMOR-1B5	
Morphine	**5.3 ± 2.0**	**5.3 ± 1.0**	**3.9 ± 0.4**	**1.5 ± 0.5**	**1.4 ±0.6**	**N.S.**
M6G	**5.2 ± 1.8**	**10.1 ± 1.6**	**8.4 ± 1.3**	**3.9 ± 1.3**	**5.2 ± 0.1**	**N.S.**
DAMGO	**1.8 ± 0.5**	**1.4 ± 0.2**	**1.3 ± 0.1**	**1.8 ± 0.9**	**1.0 ± 0.3**	**N.S.**
DADLE	**2.1 ± 0.3**	**9.0 ± 1.1**	**4.9 ± 0.7**	**1.9 ± 0.4**	**2.4 ± 0.4**	**P<0.001**
DSLET	**12.5 ± 3.6**	**8.9 ± 1.1**	**22.1 ± 1.5**	**8.7 ± 1.8**	**11.5 ± 5.2**	**P<0.04**
Naloxone	**4.3 ± 0.9**	**0.5 ± 0.3**	**1.3 ± 0.1**	**1.4 ± 0.5**	**0.6 ± 0.3**	**N.S.**
Dynorphin A	**10.9 ± 0.5**	**14.6 ± 7.1**	**34.3 ±18.4**	**8.7 ± 1.8**	**8.9 ± 2.3**	**N.S.**
β-Endorphin	**10.8 ± 2.9**	**6.8 ± 3.2**	**4.9 ± 1.7**	**3.1 ± 1.4**	**5.7 ± 1.2**	**N.S.**
Endomorphin 1	**2.1 ± 0.8**	**10.8 ± 5.6**	**5.0 ± 1.8**	**3.2 ± 0.6**	**4.3 ± 0.8**	**N.S.**
Endomorphin 2	**4.2 ± 1.8**	**12.4 ± 1.5**	**8.4 ± 1.1**	**3.2 ± 0.8**	**10.6 ± 1.8**	**P<0.01**
U50,488H	**> 500**	**> 500**	**> 500**	**> 500**	**> 500**	
DPDPE	**> 500**	**> 500**	**> 500**	**> 500**	**> 500**	

[^{3}H] DAMGO binding was performed in membranes from CHO cells stably transfected with the indicated variant constructs. K_i values were determined from at least three independent determinations of IC_{50} values (Cheng and Prusoff, 1973). Comparisons of the K_i values for each drug were then compared among the variants using ANOVA (N.S., not significant). Of the compounds, only DADLE ($p<0.0001$), DSLET ($p<0.04$) and endomorphine 2 ($p<0.01$) showed significant differences. Tukey post-hoc analysis for DADLE revealed that the K_i value for mMOR-1B1 differed significantly from mMOR-1, mMOR-1B3 and mMOR-1B5 ($p <0.001$), as well as mMOR-1B2 ($p<0.05$). With DSLET, mMOR-1B2 differed significantly from both mMOR-1B1 and mMOR-1B3 ($p<0.05$) while with endomorphin mMOR-1B1 differed from both mMOR-1 and mMOR-1B3.

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Table 3: Receptor binding of [^{3}H]DAMGO and [^{3}H]diprenorphine binding in mMOR-1 and mMOR-1B4

Ligand	K_i value (nM)		
	mMOR-1		mMOR-1B4
	[^{3}H]DAMGO	[^{3}H]Diprenorphine	[^{3}H]Diprenorphine
Morphine	5.3 ± 2.0	94.9 ± 18.3	349 ± 79
M6G	5.2 ± 1.8	609.3 ± 110	> 1000
DAMGO	1.8 ± 0.5	50.2 ± 2.5	208 ± 23
DADLE	2.1 ± 0.3	137 ± 13	600 ± 48
Endomorphin 1	2.1 ± 0.8	56.4 ± 7.4	> 1000
Etorphine		0.7 ± 0.2	6.7 ± 0.4
Fentanyl		48.5 ± 8.0	137 ± 22
Etonitazene		3.0 ± 0.7	6.6 ± 0.5
β-Endorphin	10.8 ± 2.9	34.6 ± 5.5	873 ± 116
Dynorphin A	10.9 ± 0.5	127 ±29	> 1000
NalBzoH		1.9 ± 0.3	5.4 ± 2.0
[Dmt^1]DALDA		2.1 ± 0.4	13.6 ± 5.3
Nalorphine		27.4 ± 6.0	80 ± 31
Buprenorphine		0.4 ± 0.1	0.8 ± 0.2
Naloxone	4.3 ± 0.9	8.2 ± 1.0	17.6 ± 1.8
Diprenorphine		1.0 ± 0.3	2.4 ± 0.2
Naltrexone		1.4 ± 0.1	6.1 ± 0.4
CTAP		1.9 ± 0.2	8.0 ± 1.3
CTOP		3.5 ± 0.3	18.8 ± 4.3
norBNI		188 ± 28	267 ± 34
Naltrindole		66.7 ± 3.3	198 ± 33
U50,488H	> 500	> 1000	> 1000
DPDPE	> 500	> 1000	> 1000

[^{3}H] Diprenorphine binding was performed in membranes from CHO cells stably transfected with the indicated variant constructs. K_i values were determined from at least three independent determinations of IC_{50} values (Cheng and Prusoff, 1973). K_i values for [^{3}H]DAMGO are from Table 2.

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Table 4. Effect of 5'-Guanylylimidodi-phosphate (GppNHp) on [^{3}H]diprenorphine binding in mMOR-1 and mMOR-1B4.

	K_i (nM)					
	mMOR-1			mMOR-1B4		
	Control	with GppNHp	Shift	Control	with GppNHp	Shift
Morphine	104.3 ± 2.2	147.8 ± 8.1*	1.4	296 ± 21	949 ± 29**	3.2
DAMGO	55.5 ± 1.3	82.8 ±10.8	1.5	343 ± 161	908 ± 304	2.6
Etorphine	0.49 ± 0.06	0.55 ± 0.04	1.1	2.1 ± 0.4	4.6 ± 0.6	2.2
Fentanyl	53.6 ± 4.0	89.3 ± 7.4	1.7	446 ± 130	659 ± 56	1.5
Naloxone	15.4 ± 1.6	14.1 ± 0.7	0.9	20.9 ± 1.1	37.3 ± 10.9	1.8
CTAP	3.7 ± 0.5	2.1 ± 0.4**	0.6	8.9 ± 3.1	10.8 ± 5.8	1.2

[^{3}H] Diprenorphine binding was performed in membranes from the stably transfected CHO cells with or without 100 µM of GppNHp. K_i values were determined from at least three independent determinations. Student's test was used to compare the K_i values between groups with and without GppNHp for each drug. * $p < 0.05$; ** $p < 0.01$.

Table 5: Stimulation of [^{35}S]GTPγS binding in mMOR-1 splice variants

Ligand	mMOR-1			mMOR-1B1			mMOR-1B2			mMOR-1B3			mMOR-1B5		
	EC_{50}	EC_{50}/Ki	% max	EC_{50}	EC_{50}/Ki	% max	EC_{50}	EC_{50}/Ki	% max	EC_{50}	EC_{50}/Ki	% max	EC_{50}	EC_{50}/Ki	% max
DAMGO	68±4	38	100	39±8	28	100	85±18	65	100	100±14	56	100	89±13	89	100
Morphine	23±2	4.3	102±5	100±38	18.9	104±38	76±13	19.5	82±8	51± 6	34	91±3	53± 4	37.9	87±7
M6G	75±18	11.7	122±9	527±245	52.2	117±15	150±6	17.9	71±5	83±19	21.2	82±7	85±18	16.3	88±9
Dynorphin A	34±9	3.2	109±7	137±69	9.4	83±23	210±25	6.1	81±6	147±56	16.9	90±6	197±32	22.4	75±3
β-Endorphin	64±7	7.6	97±2	113±47	16.6	69±21	163±22	33.3	84±5	75±19	24.1	93±2	83±27	14.6	80±4
Endomorphin 1	26±4	12.3	98±8	57±23	5.3	68±19	126±29	25.2	82±8	99±1	30.9	97±2	89±13	20.7	86±7
Endomorphin 2	72±11	17.1	124±8	197±95	15.9	90±0	187±23	22.3	92±4	110±6	34.4	80±3	155±8	14.6	82±4

Basal [^{35}S]GTPγS binding was assessed in membranes from cells stably transfected with the indicated variants followed by the stimulation of [^{35}S]GTPγS binding by the indicated drug. The maximal stimulation, defined as the percent increase over basal binding, and the dose of drug needed to elicit 50% of the maximal response (EC_{50}) were calculated as described in Methods. Results are the means ± S.E.M. of at least three independent determinations.

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Table 6: Stimulation of [^{35}S]GTPγS binding in stably mMOR-1B4 transfected CHO cells.

Ligand	**Stimulation of [^{35}S]γGTP binding**
Morphine	16.7 ± 2.8 %
DAMGO	25.9 ± 2.6 %
Etorphine	27.6 ± 7.0 %
Fentanyl	31.7 ± 12.2 %
Etonitazene	34.6 ± 5.8 %
β-Endorphin	17.5 ± 2.6 %
Dynorphin A	38.5 ± 10.4 %

Basal [^{35}S]GTPγS binding was assessed in membranes from cells stably transfected with the indicated variants followed by the stimulation of [^{35}S]GTPγS binding by the indicated drug at 25 μM. The stimulation was defined as the percent increase over basal binding. Results are the means ± S.E.M. of at least three independent determinations.

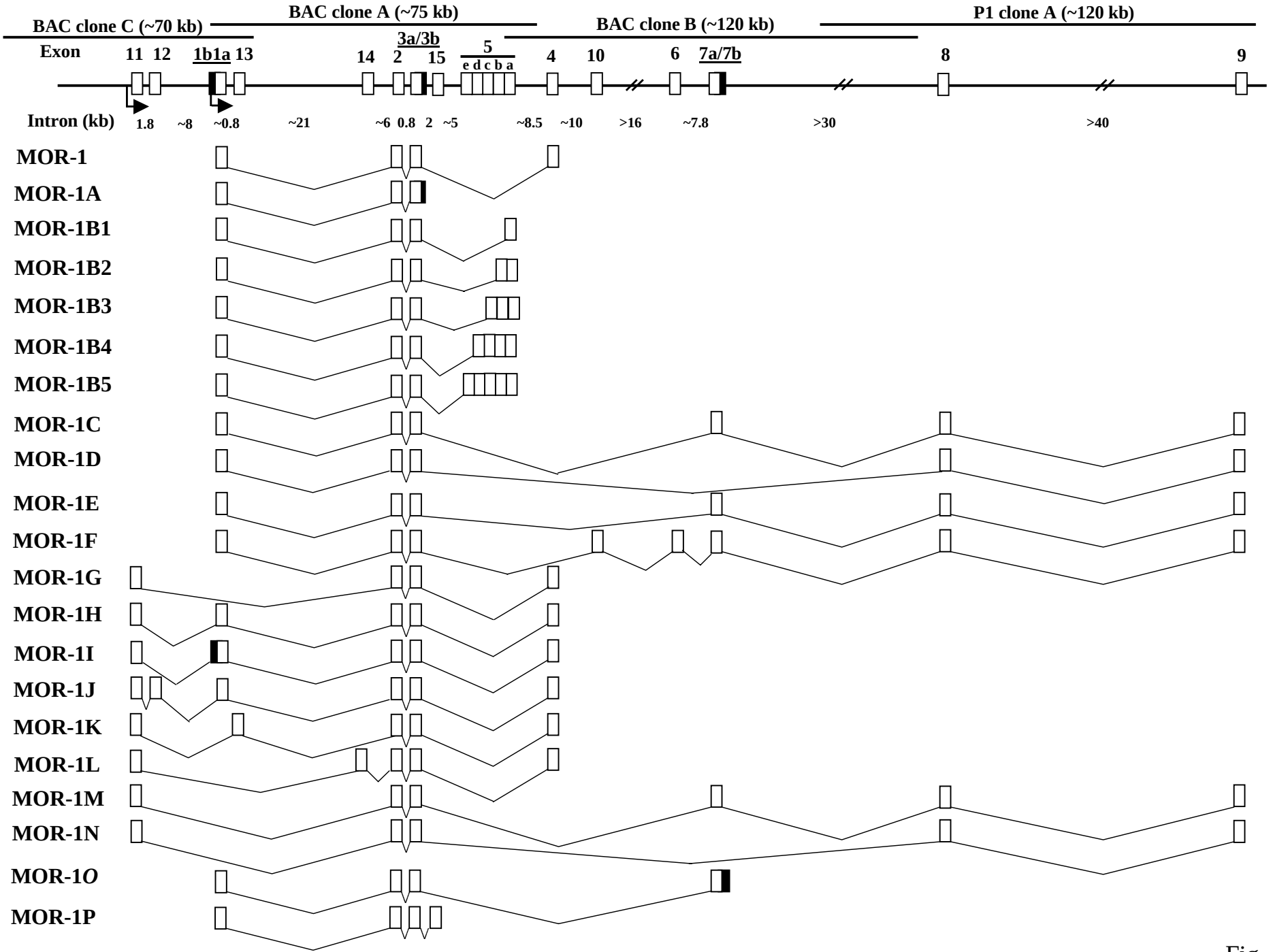

Fig. 1

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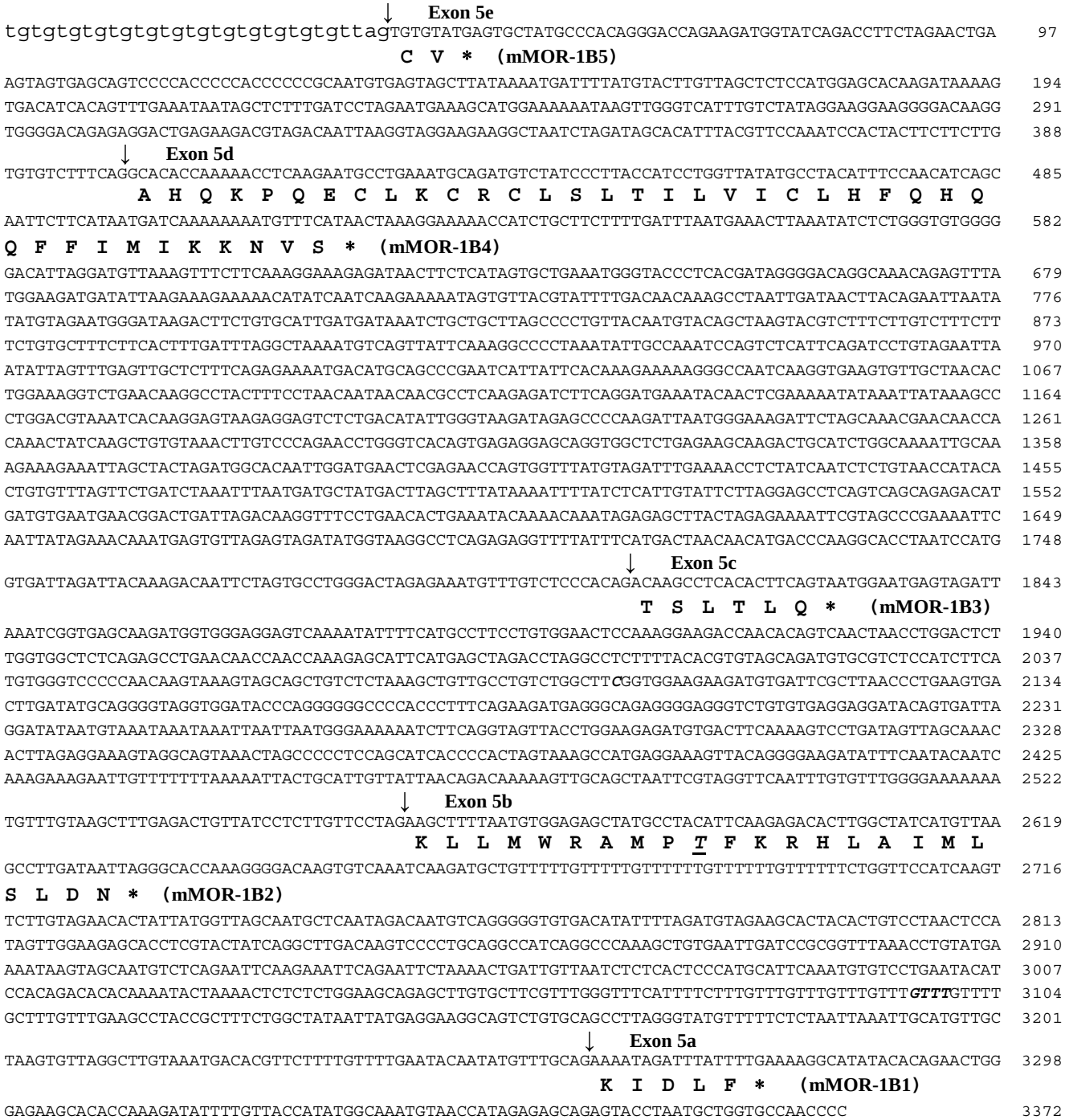


FIG. 2

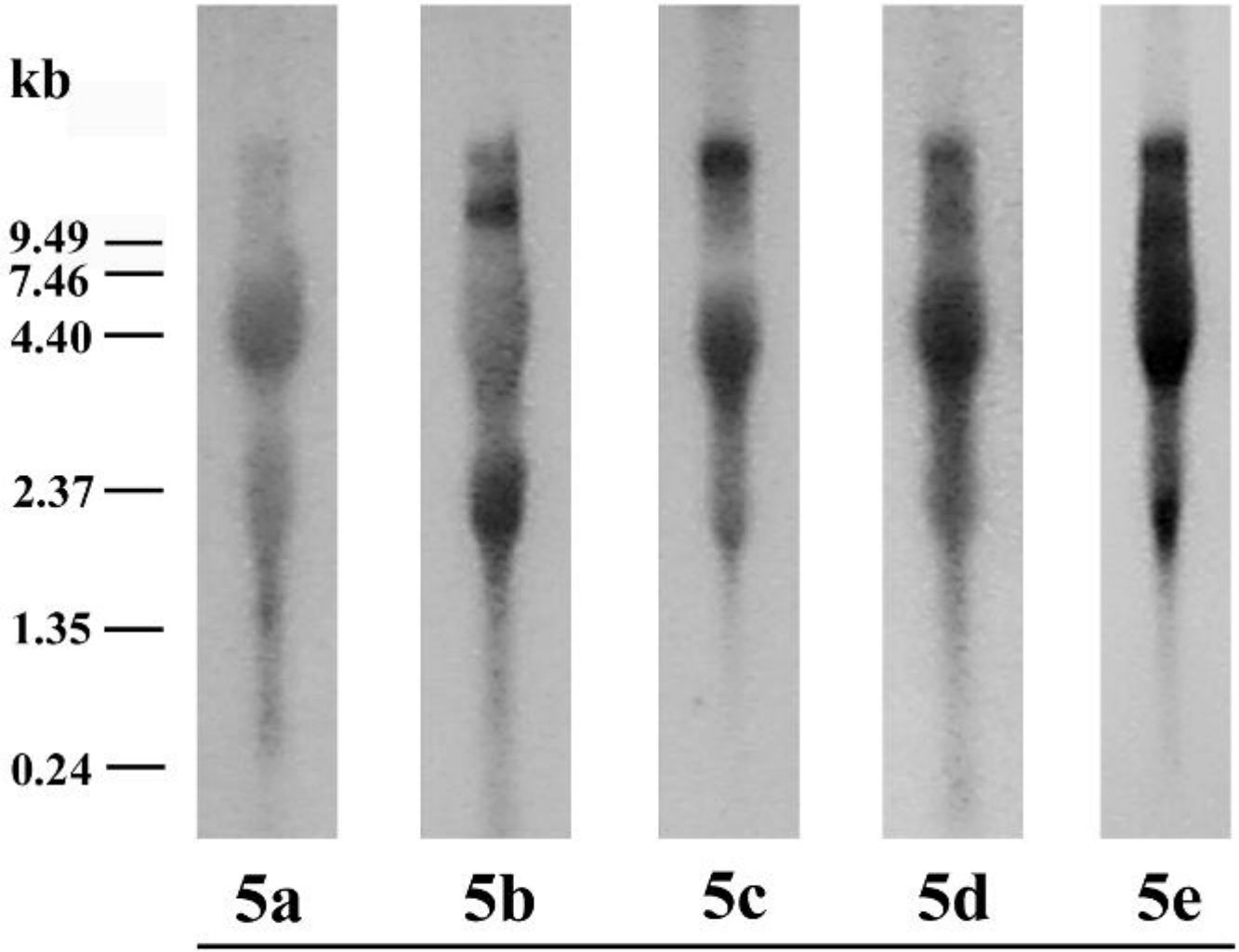


Fig 3

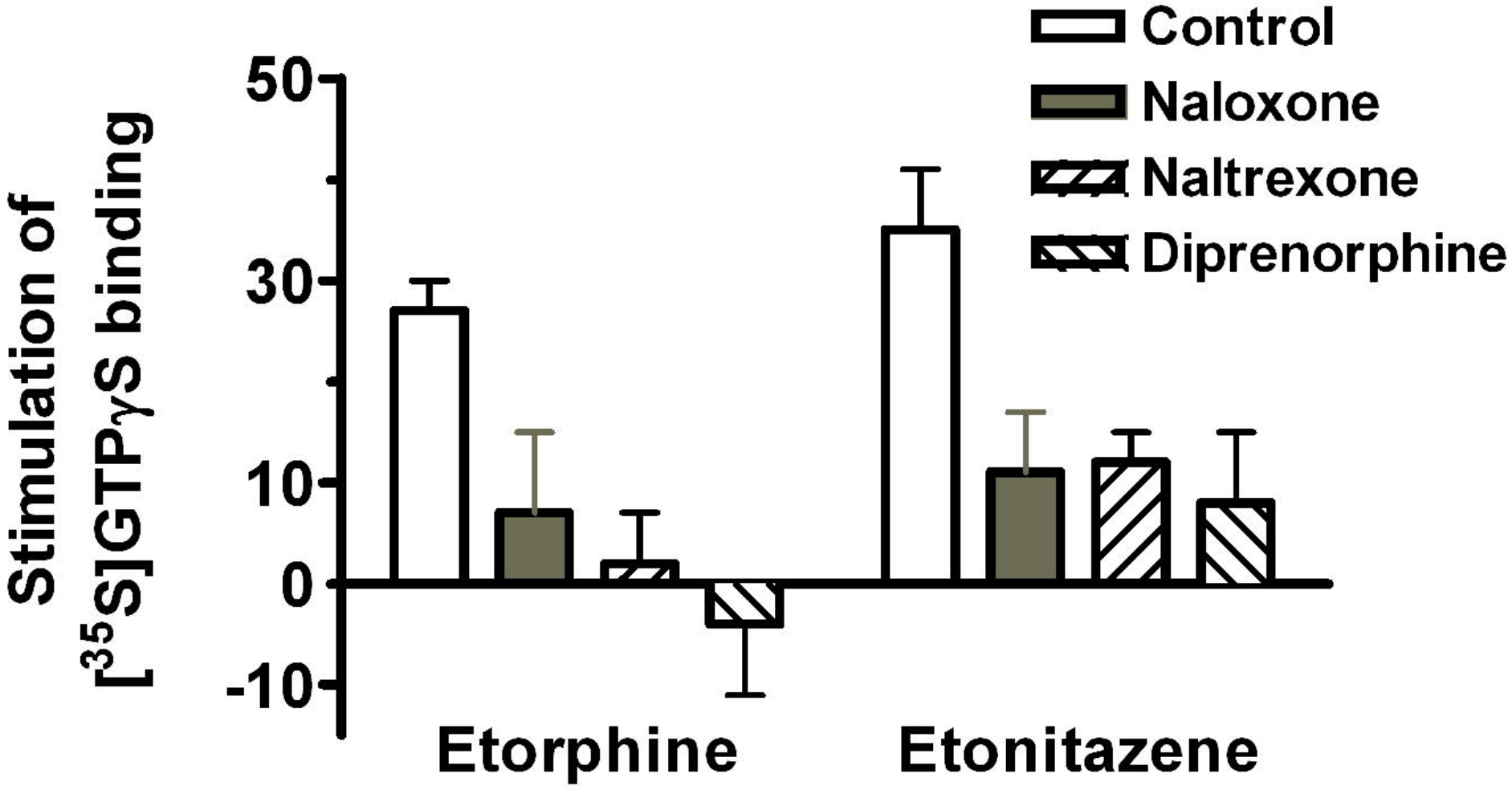

Control
Naloxone
Naltrexone
Diprenorphine
Stimulation of
[35S]GTPγS binding
50
30
10
0
-10
Etorphine
Etonitazene

Fig. 4

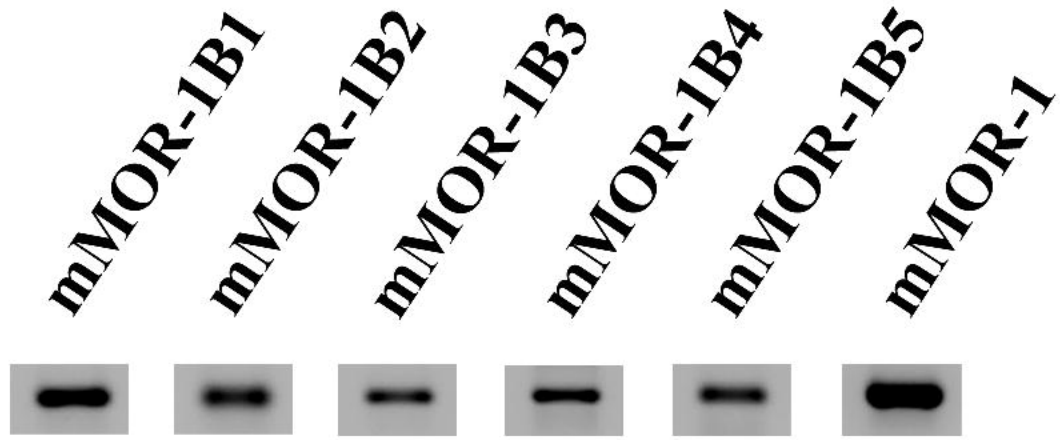
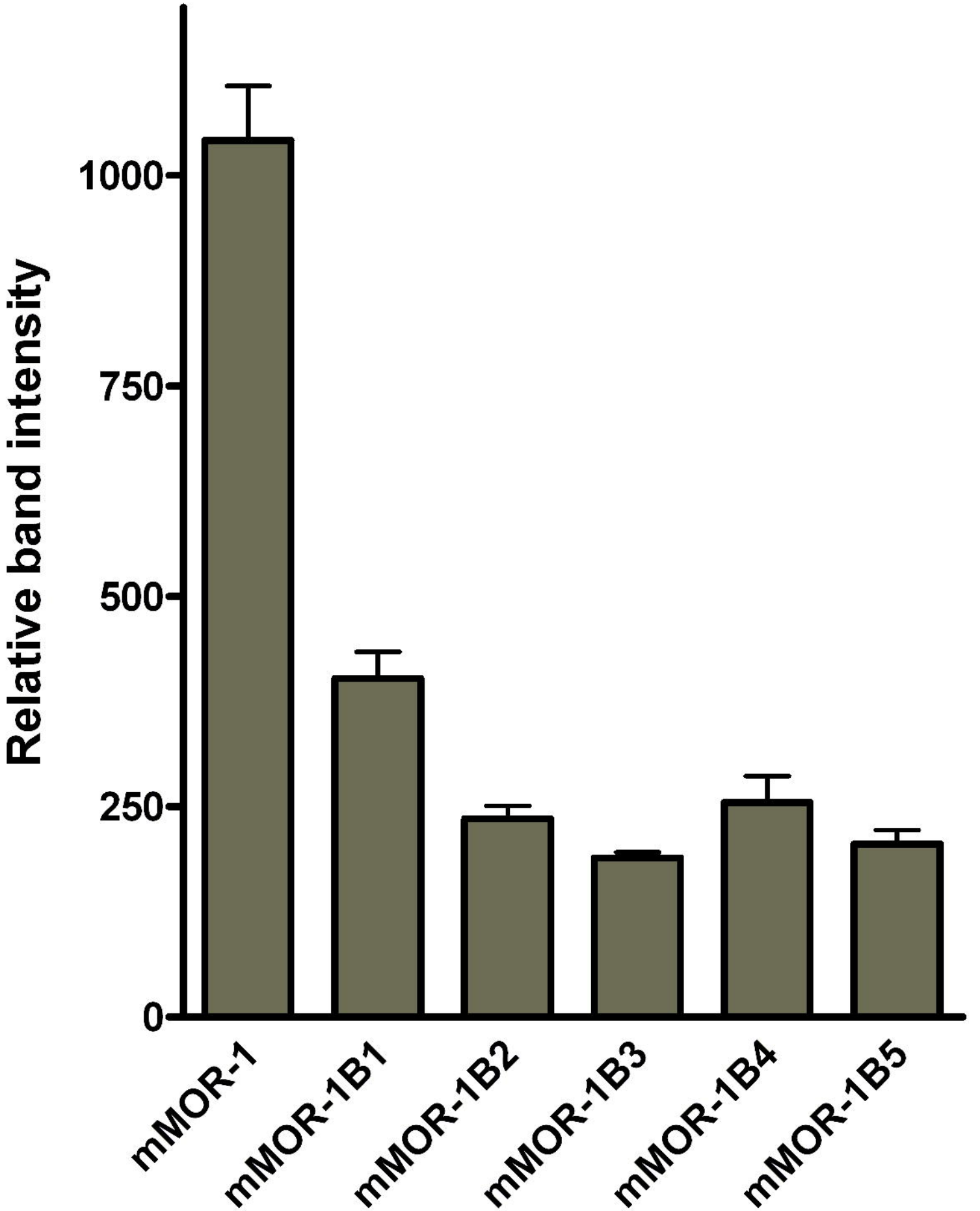


Fig 5a

Fig. 5b


Relative band intensity
1000
750
500
250
0
mMOR-1
mMOR-1B1
mMOR-1B2
mMOR-1B3
mMOR-1B4
mMOR-1B5