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Isolating and characterizing three alternatively spliced mu opioid receptor variants: mMOR-1A, mMOR-1O and mMOR-1P

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Abstract

Extensive alternative pre-mRNA splicing of the mu opioid receptor gene, *OPRM1*, has demonstrated an array of splice variants in mouse, rat and human. Three classes of splice variants have been identified: full length 7 transmembrane (TM) domain variants with C-terminal splicing, truncated 6TM variants and single TM variants. The current studies isolates and characterizes an additional three full length C-terminal splice variants generated from the mouse *OPRM1* gene: mMOR-1A, mMOR-1O and mMOR-1P. Using RT-qPCR, we demonstrated differential expression of these variants' mRNAs among selected brain regions, supporting region-specific alternative splicing. When expressed in Chinese Hamster Ovary cells, all the variants displayed high mu binding affinity and selectivity with subtle differences in the affinities toward some agonists. [³⁵S]γGTP binding assays revealed marked differences in agonist-induced G protein activation in both potency and efficacy among the variants. Together with the previous studies of mu agonist-induced phosphorylation and internalization in several carboxyl terminal splice variants, the current studies further suggest the existence of biased signaling of various agonists within each individual variant and/or among different variants.

Keywords

alternative splicing; G-protein coupled receptor; GPCR; biased agonism; biased signaling; morphine; opioid peptide; *OPRM1*

Introduction

Mu opioid receptors mediate the actions of morphine and most clinical analgesic agents, as well as drugs of abuse such as heroin. Pharmacological studies have defined several mu receptor subtypes, including mu₁, mu₂ and morphine-6β-glucuronide (M6G) receptors (Moebius et al., 1997; Pasternak, 1993; Reisine and Pasternak, 1996; Rossi et al., 1995; Rossi et al., 1996; Wolozin and Pasternak, 1981). Our understanding of the mu opioid receptor was markedly advanced with its cloning (Chen et al., 1993; Thompson et al., 1993; Wang et al., 1993) and identification of a gene (*OPRM1*) (Giros et al., 1995; Liang et al., 1995; Min et al., 1994). Antisense mapping studies targeting individual exons differentiated between morphine and M6G analgesia, clearly separating their molecular mechanisms of action

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(Pasternak and Standifer, 1995; Rossi et al., 1995; Rossi et al., 1997). Similarly, morphine analgesia was lost in an exon 1-knockout mouse with a disruption of exon 1 while morphine-6 β -glucuronide (M6G) and heroin analgesia was retained (Schuller et al., 1999). Reconciling these observations with a single gene was difficult, leading to the proposal of splice variants.

The first two splice variants, hMOR-1A and rMOR-1B, were isolated from human and rat, respectively (Bare et al., 1994; Zimprich et al., 1995). Over the years, our group and others have identified multiple additional alternatively spliced variants from the mouse, rat and human *OPRM1* genes which have been reviewed in detail (Pan, 2005; Pasternak and Pan Y-X., 2013). These splice variants can be categorized into three classes based upon their predicted protein structures. The first class comprises the full-length variants with 7 transmembrane (TM) domains, differing only in at the tip of the C-terminus through 3' alternative splicing. They all share identical transmembrane domains and binding pockets, but the 12 amino acids encoded by exon 4 are replaced with unique sequences ranging from 1 to over 80 amino acids. Several features of these variants suggest that they are relevant. They differ in their regional distributions (Abbadie et al., 2000b; Abbadie et al., 2000c; Abbadie et al., 2001a; Pan et al., 1999; Pan et al., 2001), agonist-induced G-protein coupling, as measured by GTP γ S binding (Bolan et al., 2004; Pan et al., 2005a; Pan et al., 2005b; Pasternak et al., 2004), receptor phosphorylation (Koch et al., 2001), internalization (Koch et al., 1998; Koch et al., 2001), and post endocytic sorting (Tanowitz et al., 2008), as well as in morphine-induced itch (Liu et al., 2011).

The second class of variants are truncated, containing only 6 transmembrane domains due to absence of exon 1 that encodes the TM1. Instead of exon 1, these variants contain exon 11, which does not traverse the membrane and remains intracellularly. Expression of these truncated 6TM variants is under control of the exon 11 promoter (Pan, 2002; Xu et al., 2006). Disrupting these 6-TM variants in an exon 11 knockout (KO) mouse model significantly diminishes the analgesic responses to heroin, M6G and fentanyl with no effect on morphine and methadone analgesia (Pan et al., 2009), complementing the opposite observations with the exon 1 KO mouse model (Schuller et al., 1999). These data suggested that different classes of splice variants may mediate analgesic actions of various mu agonists. The truncated 6TM variants also mediate the actions of a novel class of opioid analgesics lacking many of the side-effects of traditional opiates (Majumdar et al., 2011; Majumdar et al., 2012; Pasternak and Pan, 2013). The third class of variants also are truncated, containing only a single TM encoded by exon 1. Although these variants do not bind opioids, they modulate opioid action through a chaperone-like function to reduce endoplasmic reticulum-associated degradation of the full-length MOR-1 variants, thereby increasing MOR-1 expression at the protein level (Xu et al., 2013).

Three human C-terminal splice variants, hMOR-1A (Bare et al., 1994), hMOR-1O and hMOR-1X (Pan et al., 2003), were previously isolated, but it was not clear whether they exist in the mouse. The current studies isolate and characterize the three C-terminal mouse homologs in the mouse.

Materials and Methods

Isolation of *mMOR-1A*, *mMOR-1O* and *mMOR-1P*

To isolate the mouse homologs of human MOR-1A (hMOR-1A) and hMOR-1O, we first determined the partial sequences of the mouse exon 3b (human exon 3b homolog) and exon 7b (human exon O homolog) by directly sequencing the BAC clones A and B (Fig. 1), respectively, with appropriate primers. To identify the mouse homolog of hMOR-1X, we searched the Ensembl mouse genome database with the human exon X sequence, and found

a homologous region that was located at approximately 2 kb downstream of exon 3, a location similar to that of the human exon X. We confirmed the exon 15 sequences by sequencing BAC clone A with appropriate primers. To isolate the full-length cDNA clones, we then designed one antisense primer from the mouse exon 3b) (mE3b-A: 5'-CAG AAT TTG GTC CCC TAC TCC CTC TCT-3'), two antisense primers from the mouse exon 7b (E7b-A: 5'-CAC TTT GCA TGA ATG AAA ACG TCT AAT C-3' and E7b-B: 5'-GCC GTG CAA AAA GCT AAA ACT TGC C-3'), and two antisense primers from the mouse exon 15 (E15A, 5'-GCT TTT CTG TTA TCT GAT GCT GAC GCT G-3' and E15B, 5'-GCA GAG GCA GTG ATA ATG ATA AGT GCT AAT TG-3'). These antisense primers were used in combination with two sense primers from the 5' untranslated region of the exon 11 (E1A: 5'-AGA GGA AGA GGC TGG GGC G-3' and E1B: 5'-GGA ACC CGA ACA CTC TTG AGT GCT-3') in nested PCRs with the first-strand cDNA reverse-transcribed from mouse brain total RNA as template. The PCR fragments were subcloned into pcDNA3.1/TOPO or pCRII-TOPO vector (Life Technologies, Grand Island, NY) and sequenced in both directions with appropriate primers. Sequence analysis of the fragments revealed that all the clones contained identical exons 1, 2 and 3, but had different sequences downstream of exon 3 that were derived from corresponding exons 3b, 7a/7b and 15, respectively. We named these cDNA clones as mMOR-1A, mMOR-1O and mMOR-1P, respectively.

Reverse-transcription-quantitative PCR (RT-qPCR)

Total RNAs from different mouse brain regions were obtained from Zyagen (San Diego, CA), treated with DNase I using Turbo DNA-free reagents (Life Technologies) and reverse-transcribed with Superscript II and random hexamers. The first-strand cDNAs were used as template in SYBR green qPCRs using HotStart SYBR green Master Mix (Affymetrix, Santa Clara, CA) in an MJ Opticon 2 qPCR machine (Bio-Rad, Hercules, CA). The mouse succinate dehydrogenase subunit A (mSDHA), TATA box binding protein (mTBP) and beta-2 microglobulin (mB2M) were used as reference genes to determine normalization factors with $(C(t)_{mSDHA} \times C(t)_{mTBP} \times C(t)_{mB2M})^{1/3}$ format. All variant C(t) values were normalized with the normalization factors to obtain $\delta C(t)$ ($\delta C(t) = C(t)_{variant} - \text{normalization factor}$). All PCR primers and conditions are listed in Table S1. Expression levels of the splice variants were calculated through $2^{-\delta \delta C(t)}$ format.

Expression of mMOR-1A, mMOR-1O and mMOR-1P

The mMOR-1A cDNA was initially subcloned into pcDNA3.1/TOPO that has a cytomegalovirus (CMV) promoter to drive expression of the inserted cDNAs in mammalian cells. The cDNA fragments containing the full-length mMOR-1O and mMOR-1P in pCRII-TOPO were subcloned into pcDNA5/FRT and pcDNA3.1 (Life Technologies), respectively, with appropriate restriction enzymes. The resulting plasmids, mMOR-1O /pcDNA5 and mMOR-1P/pcDNA3, as well as mMOR-1A/pcDNA3TOPO, were transfected into Chinese hamster ovary (CHO) or CHO/Flp-In cells with LipofectAMINE (Life Technologies). Stable transformants were obtained 2 weeks after selection with G418 or hygromycin and screened in a [³H] DAMGO binding assay.

Receptor Binding assays

Membranes were prepared from cells stably transfected with the pcDNA3 constructs as previously described (Pan et al., 1999). Briefly, [³H]DAMGO saturation and competition binding assays were performed at 25°C for 60 min in 50mM potassium phosphate buffer, pH7.4, containing 5 mM magnesium sulfate. Specific binding was defined as the difference between total binding and nonspecific binding, determined in the presence of levallorphan (10 μ M). K_D and K_i values were calculated by nonlinear regression analysis (GraphPad

Prism, Carlsbad, CA). Protein concentrations were determined by as previously described (Lowry et al., 1951) using BSA as the standard. K_i values were determined as previously described (Cheng and Prusoff, 1973).

[³⁵S]GTPγS binding assay

[³⁵S]GTPγS binding assay was performed using membranes isolated from the stably transfected cells as previously described (Bolan et al., 2004; Pasternak et al., 2004). Briefly, membranes were incubated with or without the indicated opioids 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 [³⁵S]GTPγS and 30 μM GDP. After the incubation, the reaction was filtered through glass-fiber filters and washed three times with 3 ml of ice-cold 50 mM Tris-HCl (pH 7.4) on a Brandel 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-CARB 2900TR counter. Basal binding was determined in the presence of GDP and absence of drug.

Results

Cloning mMOR-1O

The human MOR-1O (hMOR-1O) was initially identified through homologous cloning with the mouse exon 7 sequences (Pan et al., 2003). However, unlike the mouse exon 7-associated variants including mMOR-1C, mMOR-1E, mMOR-1F, mMOR-1M, mMOR-1U and mMOR-1V in which exon 7 was further spliced into other downstream exons such as exons 8, 9 and 19 (Doyle et al., 2007a; Doyle et al., 2007b; Pan et al., 1999; Pan et al., 2000; Pan et al., 2001), hMOR-1O had exon O (homologous to the mouse exon 7) as the last exon since no exons downstream of exon O were identified (Pan et al., 2003). To identify mouse splice variants homologous to hMOR-1O, we determined the sequence corresponding to exon 7b by sequencing the BAC clones containing exon 7a directly. We then used nested RT-PCR's with primers designed from exon 1 and exon 7b to isolate full length cDNA encoding mMOR-1O. Similar to hMOR-1O, mMOR-1O contained the same exons 1, 2 and 3a as the original mMOR-1, differing only in their downstream sequences (Figs. 1 & 2). There is only one nucleotide (G) in exon 7b as the extended coding sequence from exon 7a was immediately followed by a TGA stop codon. Therefore, the mouse exons 7a/7b in mMOR-1O contain thirty amino acids that are identical to the thirty amino acids encoded by exon 7a in mMOR-1C and mMOR-1M, and to the twenty-nine amino acids encoded by exon 7a in mMOR-1U (Figs. 2 & 3). But there are additional twenty-two amino acids encoded by exons 8 and 9 in mMOR-1C and mMOR-1M, and fifty-nine amino acids encoded by exons 7a/19 in mMOR-1U, all of which are absent in mMOR-1O. The thirty amino acids encoded by exons 7a/7b in mMOR-1O shared 70% homology to those predicted from the human exon O in hMOR-1O (Fig. 3). Several potential phosphorylation sites including Ca²⁺/calmodulin-dependent protein kinase II (CaM-II), protein kinase C (PKC), insulin receptor tyrosine kinase (INSR) and casein kinase 2 (CKII) were conserved in the thirty amino acids of both mMOR-1O and hMOR-1O, while an additional CaM-II site and a cGMP-dependent protein kinase (PKG) site were found in mMOR-1O and hMOR-1O, respectively (Fig. 3). mMOR-1O can be defined as an intron-retention variant in which exon 7b, originally considered an intron in mMOR-1C, mMOR-1M and mMOR-1U, was retained by skipping the 5' splice donor sites downstream of exons 7a, in addition to alternative 3' splicing from exon 3a to exon 7a.

Cloning mMOR-1P

Using the human exon X sequence, we uncovered a putative homologous sequence by searching the mouse genome database and sequentially sequencing a mouse BAC clone. We

named the sequence exon 15 based upon the order in which the exons were uncovered. The genomic location of exon 15 is approximately 2 kb downstream of exon 3, a location similar to that of the human exon X. The nested-PCR strategy was again used to isolate the full-length mMOR-1P with the primers designed from exons 1 and 15. Sequence analysis of the PCR fragment revealed that mMOR-1P was composed of exons 1/2/3/15 (Figs. 1 & 2). Similar to all other C-terminal splice variants, exon 15 in mMOR-1P generates a unique C-terminal tail containing seventy amino acids that have 40% homology to that from the human exon X in hMOR-1X (Fig. 3). Five CaM-II sites were predicted from the exon 15 of mMOR-1P, whereas eight CaM-II sites were found in exon X of hMOR-1X. In addition, the coding sequence of exon 15 contained additional two potential PKC sites.

Cloning mMOR-1A

To identify the mouse homologue of the human MOR-1A (hMOR-1A), we first obtained exon 3b sequence by sequencing a BAC clone (BAC clone A, Fig. 1). We then used primers designed from exon 3b and exon 1 in nested RT-PCRs to isolate a full length cDNA encoding mMOR-1A. Similar to hMOR-1A and rMOR-1A, loss of the 5' splice donor site in the end of exon 3a led to retention of exon 3b that is a part of an intron in other splice variants (Fig. 1). mMOR-1A differs from mMOR-1 only in the replacement of the 12 amino acids encoded by exon 4 with the four amino acids, VRSL, predicted from exon 3b, which were identical to those in hMOR-1A (Bare et al., 1994), but less homologous to rMOR-1A (VCAF) (Pasternak et al., 2004). The serine residue in both mMOR-1A and hMOR-1A are a potential phosphorylation site for PKC.

Region-specific expression of mMOR-1A, mMOR-1O and mMOR-1P mRNAs

We observed marked differences in the regional distribution in the brain of the variants' mRNAs using RT-qPCR (Fig. 4). The qPCR for mE3-4 mainly amplifies the original mMOR-1 mRNA, although it detects several exon 11-associated variants such as mMOR-1G and mMOR-1H that are expressed at lower levels. The mE3-4 transcripts were highly expressed in most regions, as compared to the variant transcripts. For example, the expression levels of mMOR-1A, mMOR-1O and mMOR-1P in the striatum were approximately 22%, 8% and 0.5% of mE3-4, respectively, when converted from $2^{-\Delta\Delta C(t)}$ values using the $2^{-\Delta\Delta C(t)}$ format. However, the expression levels of mMOR-1P and mMOR-1O in the hippocampus were 125% and 347% higher than that of mE3-4, respectively. Each variant also displayed differential expression patterns among the regions. For example, mMOR-1P had the highest level in the hippocampus and a lower level in the striatum, contrasting to mE3-4 with its greatest abundance in the striatum and lower level in the hippocampus (Fig. 4). On the other hand, mMOR-1A had higher expression in the both striatum and hippocampus. Interestingly, mMOR-1O was highly expressed in the cerebellum (Fig. 4). Consistent with our previous observations using regular RT-PCR (Pan et al., 1999; Pan et al., 2000; Pan et al., 2001), these data further suggest region-specific alternative splicing of the *OPRM1* gene.

Characterization of opioid binding in the expressed variants

To explore opioid binding properties of these variants, we first established CHO cell lines stably expressing the cDNA clones, and then performed [3 H]DAMGO binding on membranes isolated from the stable cells. In saturation studies, [3 H]DAMGO labeled all the variants with high affinity (Table 1), although the K_D value for mMOR-1O was slightly higher than mMOR-1A and mMOR-1C. Competition studies with various opioids established the mu selectivity of the variants (Table 2). Mu agonists such as morphine, M6G and DAMGO competed the binding with high affinity, while the kappa $_1$ opioid U50,488H and the delta opioid DPDPE were inactive. However, significant differences did exist among

the variants for M6G, DADL, DSLET and dynorphin A. Of these, dynorphin A revealed the greatest differences, with far lower affinities for mMOR-1O and mMOR-1P.

Functional characterization of the expressed variants with [³⁵S]γGTP binding

To determine whether different C-terminal tails influence G-protein activation, we determined the stimulation [³⁵S]GTPγS binding. We observed significant differences among the variants and the ligands in both potency, as determined by the EC_{50} values, and efficacy, as indicated by maximal stimulation (% Max) (Tables 3). For example, the EC_{50} values for β-endorphin among the variants ranged up to 20-fold despite a similar binding affinity for them all (Table 2).

The ratio of EC_{50} to K_i (EC_{50}/K_i) is a useful parameter for comparing the drugs since it normalizes their EC_{50} based upon their receptor binding affinity. It provides an indication of the intrinsic activity of the compound, although it is not a formal measurement. These ratios differed among the variants. For example, the morphine EC_{50}/K_i ratio for mMOR-1P was over 11-fold greater than for mMOR-1, while the β-endorphin ratio varied by approximately 6-fold between mMOR-1A and mMOR-1O. Thus, the spliced amino acid differences at the C-terminus influence the ability of opioids to activate the receptor independently of their receptor binding affinities. Furthermore, the EC_{50}/K_i values among different drugs within the same variant. For example, the EC_{50}/K_i value for morphine was 7.5-fold higher than that for β-endorphin in mMOR-1O. In contrast the EC_{50}/K_i value for morphine was approximately 4-fold lower than that for β-endorphin in mMOR-1A.

We also observed marked differences in efficacies of the opioids as indicated by the maximal stimulation of [³⁵S]GTPγS binding (Tables 3). The relative efficacies for each opioid differed among the variants and among themselves. For example, morphine was a partial agonist at mMOR-1O (66%) and a full agonist at mMOR-1P (115%). Conversely, β-endorphin was a full agonist at mMOR-1O (141%) and a partial agonist at mMOR-1P (55%). There also was little correlation between the ability of the drugs to activate the receptor, as shown by the EC_{50}/K_i ratio, and the efficacy of the opioids.

Discussion

The original cloned mu receptor, mMOR-1, is composed of four exons (exons 1, 2, 3 and 4). Since then, a number of exons have been uncovered that undergo 3'splicing in which exon 4 is replaced by combinations of these other exons, yielding full length variants with unique C-terminal amino acid sequences for each (Doyle et al., 2007a; Doyle et al., 2007b; Kvam et al., 2004; Pan et al., 1999; Pan et al., 2000; Pan et al., 2005b). While the N-termini and extracellular portions of the receptors and their binding pockets of these variants encoded by exon 1/2/3 remain identical, the C-terminal 12 amino acids encoded by exon 4 in mMOR-1 are replaced by different amino acid sequences ranging from only two in mMOR-1B5 (Pan et al., 2005b) to 88 in mMOR-1U (Doyle et al., 2007b). These C-terminal splice variants are conserved from rodent to human. The current study further isolates and characterizes an additional three C-terminal variants in mouse that are homologous to those in human, further extending the repertoire of the mouse *OPRM1* alternative splicing.

Earlier studies revealed the differential expression in brain regions of the mRNA of several C-terminal variants using regular RT-PCR (Pan et al., 1999; Pan et al., 2000; Pan et al., 2001), as well as immunohistochemically (Abbadie et al., 2000a; Abbadie et al., 2000d; Abbadie et al., 2000e; Abbadie et al., 2001b; Abbadie et al., 2004). The current study also observed regional differences using RT-qPCR, which provides a more quantitative approach. Of particular interest, the hippocampus had much higher expression of both mMOR-1O and mMOR-1P than other regions, with the levels even higher than that of the

original mMOR-1 represented by mE3-4. These results raise questions regarding not only the underlying mechanisms of region-specific alternative splicing, but also the role of mMOR-1O and mMOR-1P in the hippocampus. A more general question is whether the diverse distribution of the variants may help explain various aspects of opioid pharmacology. Unfortunately, we not yet able to answer this question.

Similar to other C-terminal splice variants, mMOR-1O and mMOR-1P displayed high affinity toward [³H]DAMGO in saturation studies, and mu selectivity in competition studies. This is not surprising since the transmembrane domains making up binding pockets are all identical. Nevertheless, they had subtle binding differences for some agonists, such as M6G, DADLE, DSLET and dynorphin A. This would suggest that alternatively spliced regions of the C-terminus can slightly modulate receptor agonist conformation and therefore binding affinity.

The influence of the spliced regions of the C-terminal tails is even more evident functionally in assays examining activation of G protein, as determined by [³⁵S]γGTP binding. mMOR-1O and mMOR-1P markedly differed from each other and from other variants in both potency and efficacy of G protein stimulation toward several agonists. However, this is not unique in that earlier work from our laboratory showed similar types of differences for other 3' spliced C-terminal tails (Bolan et al., 2004;Pan et al., 2005a;Pan et al., 2005b;Pasternak et al., 2004).

Comparing the [³⁵S]GTPγS binding profiles between mMOR-1C and mMOR-1O is quite interesting. Structurally, mMOR-1O can be considered as a truncated version of mMOR-1C. The absence of exon 8 and 9 in mMOR-1O eliminates the last 22 amino acids in the C-terminus of mMOR-1C. Yet, β-endorphin was 20-fold more potent and 3-fold more efficacious in mMOR-1O expressing cells than in mMOR-1C. These differences are independent of binding affinity, which were not significantly different between the two. (If anything, β-endorphin binds to mMOR-1C with greater affinity.) This raises the intriguing question of the role of these 22 amino acids in their ability to activate G-proteins. One difference is the presence of several putative phosphorylation sites, including PKC, CaM-II and INSR, within the 22 amino acids missing in mMOR-1O.

The comparison of mMOR-1C and mMOR-1O illustrate how small, subtle changes in the C-terminus can have rather prominent effects on function. How this occurs is not known. One possibility is that the overall conformation of the receptor may subtly change, leading to changes in the binding pocket. This seems unlikely since the changes in G-protein activation were independent of binding affinity. Alternatively, the C-terminus itself may influence the transduction mechanisms. It may influence which G-protein associates with the receptor, supported by preliminary co-purification studies indicating an association of mMOR-1 with Giα1 and mMOR-1C with Gq. Signaling also may be impacted by phosphorylation, which has been suggested to provide the “bar code” for transduction (Reiter et al., 2012a). The various C-terminus sequences have a wide range of putative phosphorylation sites that may provide their unique barcode for transduction systems. This is supported by the observation that morphine induces lower levels of receptor phosphorylation in mMOR-1 and mMOR-1C than DAMGO, the two agonists stimulated mMOR-1D and mMOR-1E phosphorylation equally well (Koch et al., 2001).

Biased signaling of different mu agonists has been demonstrated for MOR-1 with receptor phosphorylation, β-arrestin recruitment and trafficking (DeWire et al., 2013;Groer et al., 2011;Raehal et al., 2011;Reiter et al., 2012b). Our current results, together with earlier findings examining other MOR-1 splice variants, suggest the presence of biased signaling for the C-terminal variants as well. Exploring biased signaling among these C-terminal

splice variants will provide new perspectives on the complex pharmacology of mu opioid actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic of the mouse *OPRM1* gene structure and alternative splicing

Exons and introns are shown by colored boxes and black horizontal lines, respectively. Promoters are indicated by red arrows. Exons are numbered in the order in which they were identified. Splicing is indicated by tilted lines connecting exons. Translation start and stop codons are shown on the bottom and top of the exon boxes, respectively. The complete list of the mouse *OPRM1* alternative splicing is described in reviews (Pan, 2005; Pasternak and Pan Y-X., 2013). The genomic BAC and P1 clones are shown by blue horizontal lines on the top.

mMOR-1A

Exon 3a ↓ Exon 3b
 ---ACTAACCACAGGTATGTGCTTTCTAGAATTATGTATAACATATAAAAAACACAGCACCTGATACCAGTCTAAGATTTAGATCCTTAAGGAGGTC
 T N H Q V C A F *
 GGTACTGAGAAATCCAGCCAAGCCTAAAAATAGAGAGGGAGTAGGGGACCAAATTCCTG

mMOR-1O

Exon 3a ↓ Exon 7a
 ---ACTAACCACAGCCAACCCTGGCAGTCAGCGTGGCCAGATCTTTACAGGATATCCTTCTCCGACTCATGTTGAAAAACCCTGCAAGAGTTGCA
 T N H Q P T L A V **S** V A Q I F **T** G **Y** P S P **T** H V E K P C K **S** C
 ↓ Exon 7b
 TGGACAGGTGAGTGTGACCCGGACTCAGGTGACAAAATAAAAGGCAAGTTTTAGCTTTTGCACGGCGAGGATTAGACGTTTTTCATTCATGCAAAGT
 M D R *
 GAATATTTTTTTAAAAATCATGTATCTGTTCTATTTTTAAACCTCCATTCCGTGCCTAGAAAACCCGGGCAAAGACACTTATATGCAAGT

mMOR-1P

Exon 3a ↓ Exon 15
 ---ACTAACCACAGATAATGAAATTTGAAGCTATCTACCCTAAACTGAGCTTCAAATCTTGGGCATTGAAATATTTTACTTTTCATTAGAGAAAAGA
 T N H Q I M K F E A I Y P K L **S** F K **S** W A L K **Y** F **T** F I R E K
 AAAGGAACACAAAAGCTGGGGCTCTTCCCTCCCCTCCCTACGTGTCTGCTGGATCTCCCTCCCAGGCTCACAGGGGCGTGGCTGCTTGGTTGCTTCC
 K R N **T** K A G A L P P L P T C H A G **S** P **S** Q A H R G V A A E L L P
 TCTAAGACACATGGGGCCTTCTTATCCTTCTGACCCACCTGACCTTCTCTAATGGAAGCAGAGCCCCAAGCTCTCTATTCCAGCACATCCCTGTT
 L R H M G P **S** Y P **S** *
 TTAAACATAGCTGCCCTTCAGATTCTCTAACGCTGCCTTCGACTCCCTTCCACACCCAGTGTTCATGTCAGTGTGGATCTTGCACAGCCAGAGAG
 TAGAAGGGAAGATAATTGGAGAAGCTCTGTCTAAGTAACATAAAAGGTTTGCTTTAAAAATACATCCAATTAGCACTTATCATTATCACTGCCTCTG
 CGGAGCAGCGTCAGCATCAGATAACAGAAAAGCACATTAGCCTGGAAGTGGTGGCCTCCCAAAGGCCAGCTGGGAGAGCTAGACCAAGAAGAGAAG
 CAGAAAGAAGCAGAAAAGATGCCCTGACATAACTGAAATGAACAGAAATCAAAGATCCTATTACCCATCT

Fig. 2. Partial cDNA sequences with predicted amino acid sequences of mMOR-1A, mMOR-1O and mMOR-1P

Exon-exon junctions are shown by arrows. The stop codons are indicated by asterisks. The complete cDNA and deduced amino acid sequences were deposited in GenBank as AF167565 (mMOR-1A), AY036621 (mMOR-1O) and AY160190 (mMOR-1P), respectively. Colored/italic letters are potential phosphorylation sites for Ca²⁺/calmodulin dependent protein kinase II (CaM-II, red letter), casein kinase II (CKII, light blue letter), protein kinase C (PKC, green letter), insulin receptor tyrosine kinase (INSR, brown letter) and cGMP dependent protein kinase (PKG, dark blue letter), predicted by using NetPhosK Serve (Blom et al., 2004).

[illegible]

Fig. 3. Comparison of the predicted amino acid sequences between mouse and human variants
Identical residues are aligned with red colons, and homologous residues, with green colons. Colored/bold/italic letters are potential phosphorylation sites for CaM-II (red letter), CKII (light blue letter), PKC (green letter), INSR (brown letter) and PKG (dark blue letter), predicted by using NetPhosK Serve(Blom et al., 2004).

Fig. 4. Expression levels of the variant mRNAs by RT-qPCR

RT with total RNAs from various brain regions and SYBR green qPCRs were performed as described in the methods. Expression levels of the variants were calculated by $2^{-\delta C(t)}$, where $\delta C(t)$ value was the difference of $C(t)$ values between the variant and the normalization factor that was determined by using three reference genes, mSDHA, mTBP and mB2M. mE3-4 qPCR mainly determined expression level of the original MOR-1 mRNAs. Bars represent the mean $\pm$ S.E.M. from three independent experiments. Str: striatum; Tha: thalamus; Hip: hippocampus; Hyp: hypothalamus; BS: brainstem; Med: medulla; Cb: cerebellum; SP: spinal cord; WB: whole brain. Significant difference was calculated by One-way ANOVA with Tukey post hoc analysis (Prism 5.0). Red star: compared to Str; Blue star: compared to Tha; Black star: compared to Cb; Green star: compared to Hip. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Table 1
Saturation studies with [³H]DAMGO binding in membranes from stably transfected cells

Clone	K_D (nM)	B_{max} (pmol/mg protein)
mMOR-1	1.75 ± 0.44	0.16 ± 0.04
mMOR-1A	0.62 ± 0.09	0.45 ± 0.12
mMOR-1C	0.57 ± 0.14	0.41 ± 0.07
mMOR-1O	2.19 ± 0.43	0.79 ± 0.15
mMOR-1P	1.45 ± 0.32	0.29 ± 0.06

[³H]DAMGO binding was performed in membranes isolated from CHO cells stably transfected with the indicated cDNA clones, as described in Experimental Procedure. The binding parameters were established by non-linear regression analysis. Results are the mean $\pm$ SEM of at least three independent determinations. Significant differences of K_D and B_{max} among the variants using ANOVA were $p < 0.05$ and $p < 0.01$, respectively. For K_D , Tukey post hoc analysis indicated that mMOR-1O differed from mMOR-1A and mMOR-1C ($p < 0.05$). For B_{max} , Tukey post hoc analysis determined that mMOR-1O differed from mMOR-1 and mMOR-1P ($p < 0.05$). The data of mMOR-1 and mMOR-1C are from literature (Pan et al., 1999).

Table 2
Competition studies using [³H]DAMGO binding in membranes from stably transfected variants

Ligand	<i>K_i</i> value (nM)					ANOVA	Tukey	
	mMOR-1	mMOR-1A	mMOR-1C	mMOR-1O	mMOR-1P		MOR	<i>p</i>
Morphine	5.3 ± 2.0	3.1 ± 0.5	2.4 ± 0.6	2.7 ± 0.6	1.2 ± 0.8	N.S.		
M6G	5.2 ± 1.8	5.0 ± 1.5	4.1 ± 1.2	17 ± 1.0	11 ± 3.4	< 0.05	1 vs IO	< 0.05
							1A vs IO	
							IC vs IO	
DAMGO	1.8 ± 0.5	1.0 ± 0.3	0.9 ± 0.2	3.3 ± 1.2	0.8 ± 0.3	N.S.		
DADLE	2.1 ± 0.3	2.2 ± 0.2	3.2 ± 1.9	7.7 ± 1.4	3.5 ± 0.5	< 0.05	1 vs IO	< 0.05
							1A vs IO	
							IC vs IO	
DSLET	13 ± 3.6	5.1 ± 1.1	8.1 ± 1.2	28 ± 7.9	20 ± 2.4	< 0.05	1A vs IO	< 0.05
							IC vs IO	< 0.05
Naloxone	4.3 ± 0.9	1.1 ± 0.2	2.8 ± 0.8	4.0 ± 1.0	1.0 ± 0.3	N.S.		
β-Endorphin	11 ± 2.9	4.3 ± 1.0	5.8 ± 0.5	16 ± 5.3	5.9 ± 2.4	N.S.		
Dynorphin A	11 ± 0.5	8.2 ± 2.8	5.6 ± 0.8	58 ± 26	103 ± 23	< 0.01	1 vs IP	< 0.05
							1A vs IP	< 0.05
							IC vs IP	< 0.01
U50,488H	> 500	> 500	> 500	> 500	> 500			
DPDPE	> 500	> 500	> 500	> 500	> 500			

Competition studies were performed against [³H]DAMGO (~ 1 nM) using various concentrations of the indicated ligands with the same cell membranes. IC₅₀ values were calculated by non-linear regression analysis (Prism) and converted to *K_i* values as previously described ((Cheng and Prusoff, 1973). Most *K_i* values of mMOR-1, mMOR-1A and mMOR-1C are from the literature (Bolan et al., 2004; Pan et al., 1999). Results are the mean ± S.E.M. of at least three independent determinations.

Table 3
Stimulation of [³⁵S]GTPγS binding in membranes from stably transfected variants

Ligand		mMOR-1	mMOR-1A	mMOR-1C	mMOR-1O	mMOR-1P
Morphine	EC_{50}	23 ± 2	19 ± 4	23 ± 5	85 ± 31	58 ± 9
	EC_{50}/K_i	4.3	6.1	9.6	31	48
	% Max	102 ± 5	91 ± 2	75 ± 4	66 ± 23	115 ± 23
M6G	EC_{50}	75 ± 18	114 ± 78	51 ± 9	104 ± 17	65 ± 4
	EC_{50}/K_i	14	23	12	6.1	5.9
	% Max	122 ± 9	85 ± 9	63 ± 7	71 ± 17	69 ± 3
DAMGO	EC_{50}	68 ± 4	70 ± 3	62 ± 4	60 ± 19	133 ± 23
	EC_{50}/K_i	38	70	69	18	166
	% Max	100	100	100	100	100
β-Endorphin	EC_{50}	64 ± 7	111 ± 27	123 ± 19	6 ± 1	24 ± 5
	EC_{50}/K_i	5.9	26	21	3.9	8.3
	% Max	97 ± 2	83 ± 3	44 ± 3	141 ± 8	55 ± 3

The maximal stimulation (% Max), defined as the percent increase over basal binding and normalized with % Max values of DAMGO, and the dose of drug needed to elicit 50% of the maximal response (EC_{50}) were determined by nonlinear regression analysis (Prism). Results are the means ± S.E.M. of at least three independent determinations. The data of mMOR-1, mMOR-1A and mMOR-1C are from Bolan et al.2004. Significant differences of the EC_{50} among the variants analyzed by ANOVA were morphine and M6G ($p > 0.05$), DAMGO ($p < 0.05$), and β-endorphin ($p < 0.001$). Tukey post hoc analysis revealed that for DAMGO, mMOR-1P differed from mMOR-1 and mMOR-1C ($p < 0.05$), and for β-endorphin, mMOR-1A differed from mMOR-1P ($p < 0.01$), and mMOR-1C differed from mMOR-1O ($p < 0.001$) and from mMOR-IP ($p < 0.01$). Significant differences of the Maximal stimulation (% Max) among the variants analyzed by ANOVA were morphine ($p > 0.05$), M6G ($p < 0.01$) and β-endorphin ($p < 0.001$). Tukey post hoc analysis revealed that for M6G, mMOR-1 differed from mMOR-1O and mMOR-1P ($p < 0.05$) and from mMOR-1C ($p < 0.01$), and for β-endorphin, mMOR-1 differed from mMOR-1C, mMOR-1O and mMOR-IP ($p < 0.001$); mMOR-1A differed from mMOR-1C and mMOR-IP ($p < 0.01$); and mMOR-1O differed from mMOR-1C and mMOR-IP ($p < 0.001$).