Stabilization of the \( \mu \)-Opioid Receptor by Truncated Single Transmembrane Splice Variants through a Chaperone-like Action

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Background: The \( \mu \)-opioid receptor gene undergoes extensive alternative splicing. The single transmembrane (TM) splice variants function as a chaperone to stabilize 7-TM MOR-1, enhancing morphine analgesia.

Results: Single TM variants play an important role in expression and function of 7-TM MOR-1.

Significance: The function of truncated variants is significant for understanding the regulation of \( \mu \)-opioid receptors or other GPCR families.

The \( \mu \)-opioid receptor gene, OPRM1, undergoes extensive alternative pre-mRNA splicing, as illustrated by the identification of an array of splice variants generated by both 5′ and 3′ alternative splicing. The current study reports the identification of another set of splice variants conserved across species that are generated through exon skipping or insertion that encodes proteins containing only a single transmembrane (TM) domain.

Using a Tet-Off system, we demonstrated that the truncated single TM variants can dimerize with the full-length 7-TM MOR-1, using a Tet-Off system, we demonstrated that the truncated single TM variants can dimerize with the full-length 7-TM MOR-1, enhancing morphine analgesia.

Conclusions: Single TM variants play an important role in morphine analgesia, presumably through modulation of receptor expression levels. Our studies suggest the functional roles of truncated receptors in other G protein-coupled receptor families.

Pharmacological studies have defined three families of opioid receptors, \( \mu \), \( \delta \), and \( \kappa \). The \( \mu \)-opioid receptor (MOR) has a special place within the opioid receptor family because it mediates the actions of most clinical analgesic agents, including morphine, codeine, and methadone, as well as drugs of abuse, such as heroin. The first proposal of \( \mu \) receptor subtypes came from a combination of binding and behavioral studies (1). These investigations derived from clinical observations in which the responses differed from patient to patient for various \( \mu \)-opioids (2–4), with similar differences in animal models (1, 5–11). Perhaps the most dramatic example involved the CXBK mouse, which is insensitive to morphine but still responds normally to other \( \mu \)-opioids, including methadone, heroin, and fenanyl (6). These pharmacological studies strongly implied that \( \mu \)-opioids were not acting through a single mechanism.

The cloning of MOR-1 (13–16) provided opportunities to explore the hypothesis of multiple \( \mu \)-opioid receptors at a molecular level. Only a single \( \mu \)-opioid receptor (OPRM1) gene has been identified in different species, raising the possibility that alternative pre-mRNA splicing might be responsible for multiple \( \mu \)-opioid receptor subtypes. The first examples were MOR-1A, which was reported from human neuroblastoma cells (17), and MOR-1B, which was isolated from rats (18) and involved 3′-splicing.

The first suggestion that alternative splicing may be important in explaining the variability of responses to \( \mu \)-opioids came from antisense mapping studies in mice and rats (19–21). Studies in an exon 1 knock-out (KO) mouse model generated by Pintar further supported a molecular mechanism for the differences in \( \mu \)-opioid pharmacology (22). Disrupting exon 1 in this mouse completely eliminated all of the full-length MOR-1 splice variants due to the deletion of exon 1. However, alterna-

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§ The abbreviations used are: MOR, \( \mu \)-opioid receptor; TM, transmembrane; DAMGO, \( \delta \)-Ala\(^2\),MePhe\(^4\),Gly(ol)\(^2\))-enkephalin; BFA, brefeldin A; I.C.V., intracerebroventricularly; I.T., intrathecal; PDI, protein-disulfide isomerase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERQC, ER quality control; MEM\(\alpha\), minimum Eagle’s medium; GPCR, G protein-coupled receptor; GTP\(\gamma\)S, guanosine 5′-3-O-(thiotriophosphate); PAG, periqueductal gray; qPCR, quantitative PCR; E1–2, exons 1 and 2; ANOVA, analysis of variance; DME, Dulbecco’s modified Eagle’s medium.
ative variants lacking exon 1 were still expressed. Behaviorally, morphine analgesia was completely lost in the knock-out mice, but morphine-6β-glucuronide and heroin retained full activity, raising the possibility that the residual activity of morphine-6β-glucuronide and heroin actions are mediated through alternatively spliced variants without exon 1 that were still expressed.

Over the last 15 years, our group and others have extensively investigated alternative splicing of the OPRM1 gene, as demonstrated by the identification of multiple 3′- and 5′-splice variants of the OPRM1 gene in mice, rats, and humans (17, 23–26). Extensive 3′- and 5′-splicing generates three classes of variants. One set involves full-length variants generated by 3′-splicing that differ only in the tip of the intracellular C terminus. Structurally, they are identical through the transmembrane regions because they all contain exons 1, 2, and 3, which encode all seven transmembrane domains (27, 28) that define the binding pocket (29). These C-terminal variants have distinct region- and cell-specific expressions, agonist-induced G-protein coupling, receptor phosphorylation, internalization, and postendocytic sorting, and involve morphine analgesia (23, 24, 30–34).

The second set involves truncated versions of MOR-1 that contain only six transmembrane domains (6-TM) due to the absence of exon 1, which encodes the first transmembrane domain (TM1). These were isolated following the discovery of exon 11 located at ~30 kb upstream of exon 1 in mice (23, 27), rats (36, 37), and humans (37). Expression of all the exon 11-associated variants is controlled by the exon 11 promoter (27, 28). The functional importance of these truncated 6-TM variants has been elucidated in an exon 11 KO mouse model (38, 39). Contrary to the exon 1 KO mice, morphine and methadone analgesia was not significantly affected in the exon 11 KO mice, whereas the analgesic responses to morphine-6β-glucuronide, fentanyl, and heroin were significantly attenuated. These observations suggested that different sets of variants may play a role in the analgesic actions of μ-opioids. Recent reports reveal that these truncated 6-TM variants also generate a target totally distinct from the traditional μ-receptors capable of producing analgesia without the side effects typically associated with μ-opioids (39, 40).

There is another set of splice variants that are generated through exon skipping or insertion. These variants comprise truncated receptor proteins containing only a single transmembrane domain encoded by exon 1 (i.e. TM1), the same TM lacking in the exon 11-associated 6-TM variants (41, 42). The current study identifies and characterizes this set of single TM splice variants and assesses their functional role as molecular chaperones that modulate expression of the full-length 7-TM receptors.

EXPERIMENTAL PROCEDURES

Materials—Male CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). [3H][d-Ala2,N-MePhe4,Gly(ol)2]Enkephalin (DAMGO; 53.4 Ci/mmol), [35S]GTPγS (1250 Ci/mmol), and EasyTag EXPRE35S35S protein labeling mix (>1,000 Ci/mmol) were purchased from PerkinElmer Life Sciences. Opiates and opioid peptides were the generous gift of the Research Technology Branch of NIDA, National Institutes of Health (Rockville, MD). Human post-mortem prefrontal cortex was from the Forensic Medicine Department at the Karolinska Institutet (Stockholm, Sweden), under guidelines approved by the ethics committee and the Swedish Board of Health and Social Welfare. Be(2)C, SH-SY5Y, and HEK293 cells were obtained from ATCC. Tet-Off Chinese hamster ovary (CHO) cells were obtained from Clontech (Mountain View, CA). All oligodeoxynucleotide synthesis and purification, anti-FLAG antibody, EZview Red anti-FLAG M2 and EZview Red anti-HA affinity gels, 3× FLAG peptide, HA peptide and brefeldin A were purchased from Sigma-Aldrich. Anti-HA antibody (Y-11), anti-protein-disulfide isomerase (PDI) antibody (C-2), and anti-EDM1 (ER degradation-enhancing α-mannosidase-like protein 1) (C-19) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-calnexin and anti-ubiquitin antibodies were from Novus Biologicals (Littleton, CO) and Lifesensors (Malvern, PA), respectively. Anti-μ-opioid receptor antibody (UMB3) was from Epitomics (Burlingame, CA). All other materials were obtained from the sources listed.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Cloning—Total RNAs were isolated from mouse and rat brain and human prefrontal cortex by the guanidinium thiocyanate phenol-chloroform extraction method. Total RNAs were isolated from Be(2)C, SH-SY5Y, and Tet-Off CHO cells, the mouse periaqueductal gray (PAG) and spinal cord by using the RNeasy kit (Qiagen). Total RNAs from the mouse and rat brain and the human prefrontal cortex were reverse transcribed with random primers and Superscript II reverse transcriptase (Invitrogen), amplified, subcloned, and sequenced, as described previously (24, 43). Primers used for cloning are listed in supplemental Table S1.

Expression of the Single TM Variant mRNAs by RT-SYBR Green qPCR—Total RNAs from human prefrontal cortex, Be(2)C, and Tet-Off cells and selected brain regions of mice and rats (from ZYAGEN, San Diego, CA) as well as the PAG and spinal cord were first treated with DNase I using Turbo DNA-free reagents (Invitrogen) and reverse transcribed with random primers and Superscript II reverse transcriptase. The first-strand cDNAs were used as template in SYBR qPCR using Hot Start SYBR Green Master Mix (Affymetrix, Santa Clara, CA). The succinate dehydrogenase subunit A or the TATA box-binding protein or 18 S ribosome was used as a reference gene for normalization. PCR primers and conditions are listed in supplemental Table S1. Expression levels of the single TM variants and exons 1 and 2 (E1–2) were calculated as $2^{-ΔC(t)}$, where the $ΔC(t)$ value was the difference of $C(t)$ values between the single TM variant or E1–2 and the reference gene. E1–2 qPCR determined the expression level of the total full-length MOR-1 mRNAs.

Cell Culture, Plasmid Constructs, and Stable or Transient Transfection—HEK293 cells were maintained in DME with non-essential amino acids–F12 plus high glucose medium supplemented with 10% fetal calf serum and Tet-Off CHO cells, maintained in MEM plus 2 mM glutamine medium supplemented with 10% Tet-free fetal calf serum (FCS) (Clontech) and 0.1 μg/ml G418. All of the cells were grown at 37 °C in a 5% CO2, 95% air humidified atmosphere. To express the single TM variants in Tet-Off CHO, the single TM variants cDNAs in the
pCRII-TOPO plasmids were subcloned into pTRE2hyg vector (Clontech) with appropriate restriction enzymes. N-terminal HA-tagged single TM variant constructs were made by using PCR with primers containing HA tag sequence and subsequently subcloning the PCR fragments into pTRE2hyg vector or pcDNA3.1 vector (Invitrogen). N-terminal and C-terminal FLAG-tagged mMOR-1 constructs were made by subcloning mMOR-1 cDNA into p3XFLAG-CMV-10 and p3XFLAG-CMV-14 vectors (Sigma-Aldrich), respectively. To generate stably transfected Tet-Off CHO cells, Tet-Off CHO cells were co-transfected with mMOR-1 and a single TM variant construct with or without the tags using Lipofectamine reagent (Invitrogen) and stable transfectants obtained 2 weeks after selection with G418 (0.9 μg/ml) and hygromycin (0.6 mg/ml). Transient transfections involved a co-transfection with the tagged mMOR-1 and single TM variant constructs in HEK293 cells, using Effectene reagent (Qiagen).

**Receptor Binding Assays**—Membranes were prepared from the stable transfected Tet-Off cells grown under the indicated concentrations of doxycycline, as described previously (24). [3H]DAMGO binding was performed at 25 °C for 60 min in 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM magnesium sulfate. Specific binding was defined as the difference between total binding and nonspecific binding, defined by levallophan (1 μM). Kd and Kt values were calculated by nonlinear regression analyses (Prism 5; GraphPAD Software, San Diego, CA). Protein concentration was determined by the Lowry method using BSA as the standard.

**Immunoprecipitation and Western Blot Analyses**—Whole cells from transient transfection or stable transfectants were solubilized in lysis buffer A (phosphate-buffered saline (PBS), pH 7.4, 8 mM CHAPS), and a protease inhibitor mixture containing 2 μg/ml each leupeptin, pepstatin, aprotinin, and bestatin and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) with shaking at 4 °C for 5 h. The mixture was centrifuged at 13,000 g for 15 min at 4 °C. The supernatant was incubated with EZview Red anti-FLAG M2 or EZview Red anti-HA affinity gels with shaking overnight at 4 °C. After washing with washing buffer (PBS, pH 7.4, 5 mM CHAPS), the affinity gels were eluted with 3× FLAG peptide or HA peptide. The elutions were mixed with SDS sample buffer containing 0.15 M dithiothreitol (DTT) and heated at 100 °C for 10 min. The samples were separated on a 4–20% gradient SDS-polyacrylamide gel and transferred onto PVDF membranes. The membranes used for the anti-ubiquitin antibody were first treated with 0.5% glutaraldehyde in PBS for 20 min at room temperature before immunoblotting. The membranes were blocked in a block solution containing TTBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), 4% nonfat dried milk, and 1% BSA at room temperature for 1 h and incubated with anti-HA antibody, anti-FLAG antibody, anti-calnexin antibody, anti-PDI antibody, the anti-EMDM1 antibody and anti-ubiquitin antibody (1:1000 dilution) in the block solution at 4 °C overnight. After washing with TTBS buffer, the membrane was incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibody (1:10,000 dilution, Jackson Immunoresearch) in TTBS buffer at room temperature for 1 h. After washing with TTBS buffer, the signals were determined by using ChemiGlow reagents (Proteinsimple, Santa Clara, CA), exposed on Eastman Kodak Co. BioMax film, imaged, and analyzed on an FC8000 Image System (Proteinsimple). For immunoprecipitation (IP) of the PAG and spinal cord from antisense-treated mice, membrane proteins were isolated as described previously (24) and solubilized in lysis buffer B (PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, the protease inhibitor mixture, and PMSF) at 4 °C for 1.5 h. The mixture was centrifuged at 13,000 × g for 10 min. The supernatant was incubated with an anti-μ antibody raised against a peptide containing 12 amino acids encoded by exon 4 (1:50 dilution; UMB3, Epitomics) (44) at 4 °C overnight, followed by incubation with Dynabeads protein A (Invitrogen) for an additional 3 h. After washing with washing buffer (PBS, pH 7.4, 0.3% Nonidet P-40), the beads were eluted with the washing buffer containing the exon 4 peptide (0.25 μg/ml). The elutions were used in Western blots as described above with UMB3 antibody (1:1,000 dilution).

Images were captured with the ChemiDoc system and analyzed with Image Lab version 4.1 (Bio-Rad).

**Metabolic Pulse-Chase Labeling with [35S]Methionine/**

**Cysteine**—The Tet-Off CHO cells stably transfected with C-terminal FLAG-tagged mMOR-1 and N-terminal HA-tagged single TM variant were grown in 100-mm culture plates in complete medium in the absence or presence of doxycycline (100 ng/ml) to 80–85% confluence. Both conditions were then examined in pulse-chase studies. Cells were incubated in Met/Cys-free MEMα containing 10% dialyzed Tet-free FCS at 37 °C for 90 min and then pulse-labeled in fresh MEMα containing 10% dialyzed Tet-free FCS and 150 μCi/ml [35S]Met/Cys (EasyTag EXPRESS35S35S protein labeling mix, PerkinElmer Life Sciences) at 37 °C for 60 min. After washing twice with MEMα containing 5 mM Met and 5 mM Cys, cells were then chased by incubating in chase medium (MEMα, 10% dialyzed Tet-free FCS, 5 mM Met, and 5 mM Cys) at 37 °C for the indicated times. Following each time point, cells were lifted with PBS, pH 7.4, plus 1 mM EDTA and solubilized with PTC lysis buffer (PBS, pH 7.4, 2.5% Triton X-100, 1.5 mM CHAPS, and protease inhibitor mixture) at 4 °C for 3 h. Following centrifugation at 4 °C at 13,000 × g for 30 min, supernatants were first precleaned by incubating with EZview Protein A Gel in PTC lysis buffer for 2 h and then incubated with EZview anti-FLAG M2 or EZview anti-HA affinity gel in PTC lysis buffer at 4 °C overnight. After washing three times with PTC lysis buffer, the precipitated tagged proteins were eluted with IgG elution buffer (0.1 M glycine/HCl, pH 2.8). After neutralization with 1 M Tris/HCl, pH 8.5, elutions were incubated with new batches of EZview anti-FLAG M2 or EZview anti-HA affinity gel in PTC lysis buffer at 4 °C for 4 h for a second round of purification. The precipitated proteins were finally eluted with SDS sample buffer containing 5% meceptoethanol and 0.15 mM DTT, and separated on 4–20% SDS-polyacrylamide gels. The gels were treated with Amplify fluorographic reagent (GE Healthcare), dried, and exposed on Kodak BioMax MS film. The intensities of the radiolabeled protein bands were imaged and quantified with an FC8000 image system.

**Brefeldin A (BFA) Treatment**—The Tet-Off cells stably transfected with C-terminal FLAG-tagged mMOR-1 and N-terminal HA-tagged single TM variants were grown in the complete...
MEMα containing 5 μg/ml BFA with or without doxycycline (100 ng/ml) for 40 h. After harvesting, cell membranes isolated were used for immunoprecipitation and Western blot analysis as described above, except that 8–16% SDS-polyacrylamide gels were used.

In Vivo Antisense Oligodeoxynucleotide Studies—A 23-mer antisense oligodeoxynucleotide (E1–4/AS; 5’–CAG ATT TTC TAG CTT ACA ATC AC–3’) was designed against the mMOR-1S cDNA sequence, where the first 12-mer was derived from exon 4 and the last 11-mer were derived from exon 1. Two 23-mer mismatched control antisense oligodeoxynucleotides, E4-MIS/AS (5’–CTT GAA TCT AGT CTT ACA ATC–3’) and E1-MIS/AS (5’–CAG ATT TTC TAG TCA ATC TAA CC–3’), were designed such that the 12-mer sequence targeted to exon 4 in E4-MIS AS and the 11-mer sequence targeted to exon 1 in E1-MIS AS were scrambled, where the other halves of the sequences were kept the same as the E1–4/AS. Groups of mice received the antisense (E1/4 AS; 10 μg) or mismatched oligodeoxynucleotide or saline (0.9% NaCl) intracerebroventricularly (i.c.v.) or intratermally (i.t.) under light halothane anesthesia on days 1, 3, and 5 as described previously (20). Analgesia was assessed on day 6 in the radiant heat tail flick assay 15 min after the injection of morphine (0.6 μg, i.c.v. and i.t.). A base-line latency was typically between 2 and 3 s. A maximal latency of 10 s was used to minimize any tissue damage. Tail flick latencies were converted to percentage of maximum possible effect scores [(postdrug latency – base-line latency/cut-off latency – base-line latency) × 100]. Significance among groups was analyzed using one-way ANOVA. All procedures were approved by our institutional animal care and use committee.

RESULTS

Cloning of Single TM Variants—The first two single TM variants were isolated from two human neuroblastoma cell lines, SHSY-5Y and Be(2)C. Amplification of the full-length hMOR-1 using a sense primer from exon 1 and an antisense primer from exon 4 yielded two bands of 0.65 and 1 kb in size, clearly distinguished from the 1.4 kb band of the full-length hMOR-1. Sequence analyses of the fragments indicated that both fragments are exon-skipping variants of hMOR-1, with the 0.65-kb fragment lacking exons 2 and 3 (hMOR-1S) and the 1-kb fragment lacking exon 2 (hMOR-1Z) (Figs. 1 and 2). Similar approaches yielded both exon skipping variants in mouse (mMOR-1S and mMOR-1Z) and rat brains (rMOR-1S and rMOR-1Z) (Figs. 1 and 2), demonstrating conservation of the exon-skipping variants from rodents to humans. These differ from the two single TM variants generated by exon insertions (42).

In mMOR-1S, exon 1 was directly spliced to exon 4, skipping exons 2 and 3. This yields the same amino acid sequences as exon 1, but a reading frame shift in exon 4 leads to only one serine residue due to the early termination of translation due to a reading frame shift. Thus, MOR-1S encodes a protein containing the identical N-terminal sequence and the first transmembrane domain as the full-length MOR-1. In mMOR-1Z, the exon composition contains exons 1, 3, and 4, skipping exon 2. Again, the amino acid sequences of MOR-1Z were identical to the N terminus and the first transmembrane domain of the full-length MOR-1. However, translating it through exon 3 extends the protein for 90 residues in humans (hMOR-1Z) and 128 residues in the mouse and rat homologs (mMOR-1Z and rMOR-1Z). These extended amino acid sequences encoded by exon 3 differ from those in the full-length MOR-1 due to reading frame shifts. The deduced amino acid sequences from exon 3 have high homologies among three species, with 84.4% between mMOR-1Z and rMOR-1Z, and 53.1 and 50.8% between hMOR-1Z and mMOR-1Z and rMOR-1Z, respectively, and contain several potential casein kinase II and protein kinase C phosphorylation sites and N-myristoylation sites (Fig. 2).

Mice express three additional single TM variants. mMOR-1Q and mMOR-1R were isolated using RT-PCR with sense primers from exon 1 and antisense primers from exons 7b and 9, respectively. Both are exon 2-skipping variants with nucleotide sequences identical to those of mMOR-1O and mMOR-1D, respectively, except for the absence of exon 2. However, at the amino acid level, these two variants predict the same protein as mMOR-1Z because the coding regions (exons 1 and 3) in these two variants are identical.

mMOR-1T was isolated using sense primers from exon 11 and antisense primers from exon 4. Included in the cDNA clone was an insertion of 103 bp of a new exon (exon 16) between exons 1a and 2, mMOR-1T contains two potential translational initiation codons, one in exon 11 and the other in exon 1a. Translation starting with the exon 1a AUG produces a protein with the exact same N terminus and first transmembrane domain as the other single TM variants with a unique sequence of 21 amino acids generated from exon 16 downstream of the transmembrane region. Initiating translation with the exon 11 AUG predicts a protein with only 84 amino acids and no transmembrane domains, which is identical to that using the exon 11 translation start site in mMOR-1H. Of the 84 amino acids, the first 27 amino acids are encoded by exon 11, and the remaining 57 amino acids are encoded by exon 1a. However, it is not clear if the protein is actually expressed in the mouse brain.

Differential Expression Levels of Single TM Variant mRNAs—We examined expression of the human single TM variant mRNAs in Be(2)C cells and human prefrontal cortex using SYBR Green qPCR. The expression level of hMOR-1S mRNA was quite high in both Be(2)C cells and the prefrontal cortex at 66 and 46%, respectively, of the total full-length hMOR-1 level measured by qPCR with primers from exons 1 and 2, when converted from $2^{-ΔΔCt}$ values using the $2^{-ΔΔCt}$ format (Fig. 3A). In contrast, a much lower level of hMOR-1Z mRNA relative to hMOR-1 was seen in Be(2)C cells (4%) and the prefrontal cortex (3%) when converted from $2^{-ΔΔCt}$ values using the $2^{-ΔΔCt}$ format. The reasons for these different expression levels are unclear, but with a stop codon in hMOR-1Z located in the middle of exon 3, it may result from a nonsense-mediated degradation mechanism that targets mRNAs containing a stop codon located more than 50 nucleotides upstream of the last exon-exon junction (45, 46).

The regional distribution of the single TM variants also differed among several brain regions in mice and rats (Fig. 3, B and
C). Both mMOR-1S and rMOR-1S were highly expressed in the thalamus, with lower levels in the brainstem. In contrast, mMOR-1Q was far lower in the thalamus, with relatively high levels in other regions. These results suggested region-specific alternative splicing. The overall expression levels of all of the exon 2-skipping variants (mMOR-1Q, mMOR-1R, mMOR-1Z, and rMOR-1Z) were lower than the exon 2/3-skipping variant mMOR-1S and rMOR-1S. It was interesting that the expression levels of mMOR-1T in the striatum and whole brain were higher than that of mMOR-1S.

Co-expression of 7-TM MOR-1 with Single TM Variants in the CHO Tet-Off System—When expressed in CHO and HEK293 cells, the single TM variants alone did not bind any of a series of radiolabeled opioid agonists or antagonists (data not shown), a result that was expected because they lack the other six transmembrane domains that are important for opioid
binding (29). We next established a stable Tet-Off CHO cell line in which the full-length variant mMOR-1 was constitutively expressed, whereas the expression of the single TM variant was regulated by doxycycline. Expression of the single TM variant modulated [3H]DAMGO binding in a dose-dependent manner (Fig. 4A). The highest binding levels were seen with cells fully expressing the single TM variant while lowering expression of either mMOR-1S or mMOR-1R with doxycycline-decreased receptor binding in a dose-dependent manner (Fig. 4A).

Saturation and competition [3H]DAMGO binding studies revealed that the increased binding resulting from the co-expression of the single TM and full-length variants reflected an increase in receptor number (B_max), with no change in affinity (K_D) or its binding selectivity (Table 1). The increased binding sites were functionally active in a [35S]GTPγS binding assay. In addition to increasing the B_max of binding, co-expressing the truncated and the full-length variants increased the maximal stimulation of [35S]GTPγS binding in a similar manner with no change in potency (EC_{50} value) (Table 2).

Varying the concentration of doxycycline altered the level of expression of the single TM variant mRNA in a dose-dependent manner as anticipated (Fig. 4C). Although the expression levels of the single TM variants correlated well with the changes in receptor binding, the single TM variants did not alter the mRNA levels of the full-length mMOR-1 mRNA as determined by qPCR (Fig. 4B). Thus, the enhanced [3H]DAMGO binding seen with co-expression was not due to changes in mMOR-1 mRNA levels.

Enhanced Stability of MOR-1 Protein by Co-expression of Single TM Variants—Because the single TM variants did not influence mRNA expression levels of the full-length variant MOR-1, we then examined whether they might increase binding by enhancing the stability of the mMOR-1 protein. To explore this possibility, we established stable CHO Tet-Off cell lines with a C-terminal FLAG-tagged mMOR-1 and N-terminal HA-tagged single TM variant to allow monitoring protein expression through IP and Western blot analysis. Similar to the Tet-Off cells with non-tagged constructs, these Tet-Off cell lines with non-tagged constructs, these Tet-Off cell lines...
lines constitutively expressed FLAG-tagged mMOR-1, whereas the HA-tagged single TM variants were under the control of doxycycline. Full expression of the HA-tagged single TM variant was seen without the doxycycline. As the concentration of doxycycline increased, the expression of the HA-tagged single TM variant decreased with constant expression levels of mMOR-1 mRNA. There was an excellent correlation between increasing single TM variant expression and increasing [3H]DAMGO binding (data not shown). Western blot analyses further confirmed the increased expression of the tagged mMOR-1 along with increased levels of the single TM variant (Fig. 4, D and E).

Physiological Association of Single TM with 7-TM MOR-1—We next assessed the physiological association of the single TM and the full-length mMOR-1 using co-IP of transiently co-transfected HEK293 cells using FLAG-tagged mMOR-1 and HA-tagged single TM variants. Co-IP of the co-transfected HEK293 cells confirmed that the single TM variants co-purify with the 7-TM variants revealed physical association between two proteins (Fig. 6A, lanes 1 and 5). The specificity of the physiological association was confirmed by using membranes from a transfection with only a single tagged construct (lanes 2, 3, 6, and 7) and a mixture of membranes from cells transfected only with the FLAG-tagged mMOR-1 or the HA-tagged single TM variants (lanes 4 and 8), respectively. Co-IP studies using the Tet-Off cells stably expressing the tagged constructs confirmed that the single TM variants co-purify with the 7-TM.
mMOR-1, implying a physical interaction (Fig. 6B). The dimerization between the single TM variants and the 7-TM mMOR-1 dissociated in the presence of SDS, as shown on the SDS-PAGE.

Brefeldin A (BFA) blocks transport of proteins from the ER to the Golgi, leading to an accumulation of proteins in the ER. Thus, BFA would be expected to prevent dimerization in compartments downstream from the ER. Brefeldin A did not prevent the dimerization of the HA-tagged mMOR-1S and FLAG-tagged mMOR-1 (Fig. 7, A (second panel) and C), implying that dimerization can occur within the ER. Brefeldin A also failed to prevent the increased expression of the FLAG-tagged mMOR-1 protein by the HA-tagged mMOR-1S (Fig. 7, A (first panel) and B), suggesting that the dimerization within the ER contributed to the increased expression of the 7-TM mMOR-1 protein.

**FIGURE 4.** Effect of co-expression of mMOR-1 and mMOR-1R or mMOR-1S on mMOR-1 expression in Tet-Off CHO cells. A, [3H]DAMGO binding. [3H]DAMGO binding was performed using membranes isolated from the Tet-Off cell lines stably co-expressing mMOR-1 and mMOR-1R or mMOR-1S in the presence or absence of the indicated concentration of doxycycline (Doxycycline), as described under “Experimental Procedures.” Because the expression of the single TM variants was under control of a Tet-off system, the highest expression levels of the single TM variants are seen in the absence of doxycycline. Increasing concentrations of doxycycline yield progressively lower levels of the single TM variant expression. Triangles show the expression levels of mMOR-1S or mMOR-1R. B and C, expression of mMOR-1 (B) and mMOR-1R or mMOR-1S mRNAs (C). Total RNAs isolated from the same Tet-Off cells were used for RT-qPCR as described under “Experimental Procedures.” TBP was used as RNA loading control for normalization. Expression levels were calculated through 2^(-ΔΔCt) format using ΔCt from Tet-Off cells with doxycycline (100 ng) as control. D and E, expression of mMOR-1, and mMOR-1R and mMOR-1S protein. Western blot analysis (D) was performed with FLAG antibody, HA antibody, or actin antibody using membrane proteins isolated from the Tet-Off cells stably transfected with C-terminal FLAG-tagged mMOR-1 and N-terminal HA-tagged single TM variants, and grown in the medium containing the indicated concentration of doxycycline. Quantification of Western blots (E) was performed by using the FC8000 image system (Alpha Innotech) to measure the band intensities. The actin bands were used for normalization. Bars, mean ± S.E. (error bars) of at least three independent experiments. Significant difference was calculated by one-way ANOVA with Tukey’s post hoc analysis. Red asterisks, compared with 0 ng/ml; blue asterisks, compared with 0.01 or 0.05 ng/ml; green asterisks, compared with 0.1 ng/ml. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Western blots revealed a smaller size for mMOR-1 after brefeldin A treatment, suggesting that brefeldin A inhibited further maturation of the FLAG-tagged mMOR-1, presumably in the Golgi. This maturation in the Golgi was important to generate a functional receptor because blockade of the maturation by brefeldin A eliminated [3H]DAMGO binding despite the continued elevated level of expression of the mMOR-1 protein on Western blot by the single TM variant (Fig. 7D).

Association of the 7-TM mMOR-1 with ER Chaperone Proteins and Ubiquitination of the 7-TM mMOR-1—N-Linked glycoproteins are tightly regulated in the ER through an ER quality control (ERQC) system that allows the sorting of properly folded proteins to the Golgi for further maturation and routing.

### TABLE 1
Saturation and competition studies with [3H] DAMGO

| Saturation | mMOR-1/mMOR-1R | mMOR-1/mMOR-1S | mMOR-1/mMOR-1T |
|------------|----------------|----------------|----------------|
| K_D (nM)   | 0.46 ± 0.04    | 0.53 ± 0.09    | 0.50 ± 0.07    |
| B_max (fmol/mg protein) | 43.2 ± 1.8** | 136 ± 6.8** | 103 ± 11.7** |
| Increase   | 186%           | 176%           | 390%           |

### Table 2
Stimulation of [35S]GTPγS binding by opioids

| Ligand       | EC_50 | % max | EC_50 | % max | EC_50 | % max | EC_50 | % max |
|--------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Morphine     | 0.5 ± 0.1 | 45 ± 7’ | 0.2 ± 0.1 | 12 ± 5 | 0.2 ± 0.1 | 12 ± 5 | 0.2 ± 0.1 | 12 ± 5 |
| Increase     | 375%  |       | 228%  |       | 228%  |       | 228%  |       |
| DAMGO        | 0.1 ± 0.0 | 44 ± 3’ | 0.2 ± 0.1 | 18 ± 6 | 0.2 ± 0.1 | 18 ± 6 | 0.2 ± 0.1 | 18 ± 6 |
| Increase     | 244%  |       | 223%  |       | 223%  |       | 223%  |       |
| M6G          | 0.2 ± 0.0 | 35 ± 5’ | 0.4 ± 0.2 | 20 ± 3 | 0.3 ± 0.1 | 102 ± 9’ | 0.2 ± 0.1 | 110 ± 5’ |
| Increase     | 173%  |       | 242%  |       | 242%  |       | 242%  |       |
| β-Endorphin  | 0.3 ± 0.0 | 58 ± 4’ | 0.2 ± 0.3 | 27 ± 3 | 0.3 ± 0.1 | 136 ± 8’ | 0.7 ± 0.3 | 81 ± 2 |
| Increase     | 207%  |       | 168%  |       | 168%  |       | 168%  |       |
| Dynorphin A  | 0.3 ± 0.1 | 55 ± 5’ | 0.8 ± 0.3 | 24 ± 2 | 0.4 ± 0.1 | 134 ± 5’ | 0.6 ± 0.3 | 90 ± 5 |
| Increase     | 229%  |       | 149%  |       | 149%  |       | 149%  |       |

Saturation and competition studies with [3H] DAMGO

[3H]DAMGO binding was performed in membranes isolated from the stable Tet-Off cells containing the indicated cDNA clones under conditions with or without doxycycline (− Doxy or + Doxy), as described under “Experimental Procedures.” The binding parameters were established by nonlinear regression analysis. Results are the mean ± S.E. of at least three independent determinations. Student’s t test was used to calculate the differences between − Doxy and + Doxy groups. *, p < 0.05; **, p < 0.01.

### TABLE 2
Stimulation of [35S]GTPγS binding by opioids

EC_50 and maximal stimulation values were calculated by nonlinear regression analysis (Prism 5.0). Results are the means ± S.E. of at least three independent determinations. Significant differences between − Doxy and + Doxy groups were analyzed by Student’s t test. *, p < 0.05; **, p < 0.01.
of misfolded proteins to the ubiquitin-proteasome for degrada-
tion through an ER-associated degradation (ERAD) pathway.
ERQC functions through a number of chaperone proteins, such
as calnexin, calreticulin, ERp57, PDI, and ER degradation-en-
hancing/9251-mannosidase-like (EDEM) proteins (47, 48). Mis-
folded or ER retention mutant proteins increase or prolong the
association with these chaperone proteins, promoting their
degradation by ERAD (49, 50).

&9262;Opioid receptors contain conserved
N-glycosylation sites
and are subject to regulation by ERQC. Because the physical
association of the single TM variants with the 7-TM mMOR-1
protein occurs in the ER, we investigated whether or not
dimerization of the single TM and full-length mMOR-1 protein
modulates their association with the ER chaperone proteins,
thus contributing to the increased expression of the 7-TM
mMOR-1 protein. Western blot analyses for several ER chap-
erone proteins, including calnexin, PDI, and EDEM1, using
immunoprecipitated mMOR-1 fractions from the Tet-Off cells
co-expressing the tagged mMOR-1 and mMOR-1S, revealed
that all of the chaperone proteins tested co-immunoprecipita-
ted with mMOR-1 protein when the HA-tagged mMOR-1S
was suppressed (Fig. 8). However, as the level of expression
of the tagged mMOR-1S increased by lowering levels of doxycy-
cline (Fig. 8, A–D), there was a corresponding decrease in the
association of the FLAG-tagged mMOR-1 protein with all of
the chaperone proteins tested, as indicated by a ratio of co-
immunoprecipitated chaperone proteins with FLAG-tagged
mMOR-1 (Fig. 8, A–D). We observed similar results with
colocation of the tagged mMOR-1S and mMOR-1R (Fig. 8, B–D).
Together, these results demonstrate that expression of the single TM variants decreases chaperone
association with 7-TM mMOR-1, implying that the single TM
mMOR-1S variant facilitates the proper folding of mMOR-1
protein and reduces degradation through ERAD.

UBiquitination of 7-TM mMOR-1 also was influenced by
expression of the single TM variant. The levels of ubiquitinated
FLAG-tagged mMOR-1 decreased as the levels of expression of mMOR-1S and mMOR-1R increased (Fig. 8, A and E). These results further support the hypothesis that co-expression of single TM variants promotes proper folding of mMOR-1 protein and thereby reduces its degradation by ERAD.

Attenuation of Morphine Analgesia by an Antisense Oligodeoxynucleotide Probe against mMOR-1S—We next used an in vivo antisense oligodeoxynucleotide approach to explore the functional significance of the single TM variant, mMOR-1S. Traditional antisense mapping models in which specific exons are targeted (51) are unable to selectively down-regulate...
Actions of Single Transmembrane MOR-1 Variants

**A**

| mMOR-1_Flag/mMOR-1S_HA |
|-------------------------|
| Doxy (ng/ml) |
| 0 | 0.5 | 100 |

- Flag
- Lysate
- Lysate
- Lysate
- elution from Flag ab-IP
- Calnexine
- Lysate
- elution from Flag ab-IP
- PDI
- Lysate
- elution from Flag ab-IP
- EDEM1
- Lysate
- elution from Flag ab-IP
- Ubiquitin
- Lysate

**B**

| Ratio of calnexin/mMOR-1_Flag |
|-------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of PDI/mMOR-1_Flag |
|--------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of EDEM/mMOR-1_Flag |
|---------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

**C**

| Ratio of calnexin/mMOR-1_Flag |
|-------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of PDI/mMOR-1_Flag |
|--------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of EDEM/mMOR-1_Flag |
|---------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

**D**

| Ratio of calnexin/mMOR-1_Flag |
|-------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of PDI/mMOR-1_Flag |
|--------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of EDEM/mMOR-1_Flag |
|---------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

**E**

| Ratio of ubiquitinated mMOR-1_Flag |
|-----------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of ubiquitinated mMOR-1_Flag |
|-----------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |

| Ratio of ubiquitinated mMOR-1_Flag |
|-----------------------------------|
| Doxy (ng/ml) |
| 0 | 0.05 | 100 |
mMOR-1S without also lowering full-length variants because the sequences in the single TM variant mRNA are also present in the full-length variant mRNA. However, in the past, we successfully utilized an approach in which an oligodeoxynucleotide spanning a splice site selectively down-regulated an exon-skipping variant of neuronal nitric-oxide synthase (52). Using an antisense oligodeoxynucleotide that spanned the junction between exon 1 and exon 4, we observed a selective down-regulation of mMOR-1S mRNA using qRT-PCR when the antisense was administered either spinally or supraspinally, whereas the mismatch controls were without significant effect (Fig. 9, C and D). Furthermore, neither the antisense nor the mismatch affected the expression of the 7-TM mMOR-1 mRNA (Fig. 9, E and F). This down-regulation was functionally relevant. Reduction of mMOR-1S mRNA by antisense treatment significantly reduced morphine analgesia both supraspinally and spinally, whereas two mismatch controls were inactive (Fig. 9, A and B).

We next examined the effect of the antisense oligodeoxynucleotides on expression of the 7-TM mMOR-1 proteins using Western blots with a rabbit monoclonal antibody raised against exon 4 epitope that mainly targets mMOR-1 proteins. We detected a specific band of 70–76 kDa, similar to those reported and verified using a MOR-1 KO mouse model (44). We observed that in contrast to no changes in their mRNA levels, administration of the mMOR-1S antisense oligodeoxynucleotide i.c.v. and i.t. selectively reduced mMOR-1 protein expression in both PAG (Fig. 9, G and I) and spinal cord (Fig. 9, H and J), respectively, whereas the mismatch controls were ineffective. The reduction of mMOR-1 proteins was consistent with attenuation of morphine analgesia by the antisense oligodeoxynucleotide, suggesting that sensitivity to morphine is proportional to the levels of the μ-receptor.

**DISCUSSION**

Three general patterns of μ-opioid receptor splicing have been identified (17, 24, 26, 30, 31, 34, 38). The first to be reported were the full-length variants with 3'-splicing leading to changes at the intracellular C terminus. The second encompasses the truncated 6-TM variants, which lack the first transmembrane domain and represent a novel target for opioid drugs lacking side effects (39, 40). The present studies address a series of single TM variants containing the first transmembrane domain of the full-length MOR-1 variants, some of which were initially observed years ago (41, 42, 58). Our current studies indicate that these truncated proteins also are pharmacologically relevant.

The single TM variants are generated through exon skipping and insertions. Of these, MOR-1S and MOR-1Z are the most highly conserved from rodent to human. Like the full-length 7-TM C-terminal variants and truncated 6-TM variants, these single TM variants display region-specific expression of their RNAs, further suggesting region-specific alternative splicing of the OPRM1 gene. MOR-1S mRNA is quite abundant in human prefrontal cortex and Be(2)C cells as well as in the thalamus of mouse and rat, whereas the expression levels of the exon 2-skipping variants, such as hMOR-1Z, mMOR-1Z, rMOR-1Z, mMOR-1Q, and mMOR-1R, are low. The reasons for these differences are not clear but may involve nonsense-mediated degradation. However, the high levels of mMOR-1T mRNA in striatum and whole brain raise questions regarding region-specific nonsense-mediated degradation or other RNA processing mechanisms.

The single TM variants physically associate with full-length MOR-1 variants, as illustrated in the co-IP studies. Using a Tet-Off CHO cell system, we demonstrate that the single TM variants increase the expression of the functional 7-TM full-length mMOR-1 at the protein level using both receptor binding assays with the μ agonist [3H]DAMGO, Western blot analysis and a [35S]GTPγS binding assay. The association of the single TM variant does not alter the binding affinity of the full-length variant. Rather, it increases the number of receptors, as indicated by the increase in $B_{max}$.

The increased expression of the full-length MOR-1 protein induced by the single transmembrane variants was not associated with changes in mRNA levels. Instead, the pulse-chase studies reveal that the single transmembrane variants increase mMOR-1 expression by slowing the turnover rate of the receptor. At least a portion of this effect is mediated within the ER, as shown by the effect of the BFA treatment and the reduced association of mMOR-1 with several ER chaperone proteins in the ER, including calnexin, PDI, and EDEM1. Calnexin belongs to the lectin family and plays an important role in ERQC by retaining unfolded or misfolded N-linked glycoproteins in the ER (47). PDI is a chaperone that corrects misfolded proteins through its ability to engage in disulfide exchange, whereas EDEM1 is a mannosidase that can create substrates for ERAD by demannosylation of misfolded glycoproteins (47, 48). The single TM variants reduce the association of mMOR-1 with these chaperone proteins, implying that they facilitate the proper conformation or folding of mMOR-1 in the ER, allowing its escape from the ERAD pathway and thereby increasing its overall expression. However, we do not know if the single TM variants remain associated with the full-length MOR-1 on the cell surface and whether or not this association further extends the half-life of mMOR-1 protein. Studies with a MORT1-TAT fusion peptide analogous to the single TM variants suggest that they may associate with full-length variants at the cell surface (59).

**FIGURE 8.** Effect of co-expression of mMOR-1S and mMOR-1R with mMOR-1 on the association of mMOR-1 with ER chaperone proteins. A, co-IP of chaperone proteins with FLAG antibody in the Tet-Off cells stably co-expressing mMOR-1_FLAG and mMOR-1S_HA grown with the indicated concentration of doxycycline, as described under “Experimental Procedures.” The sample source used for immunoblots is indicated on the right of the graph, whereas antibodies used are indicated on the left of the graph. B–E, quantification of ratios of indicated chaperone and mMOR-1_FLAG proteins. Intensities of the bands were determined by densitometric analyses using the FC8000 image system (Alpha Innotech) from A and images from co-IP of co-expression of mMOR-1_FLAG and mMOR-1R_HA in the Tet-Off cells (images not shown) and were normalized by the actin bands. Values are the mean ± S.E. of three independent experiments. Significant difference was calculated by one-way ANOVA with Tukey’s post hoc analysis. Red asterisks, compared with 0 ng/ml; green asterisks, compared with 0.05 ng/ml. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 

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Actions of Single Transmembrane MOR-1 Variants

Stabilization of opioid binding sites through chaperone mechanisms is not limited to the single TM proteins. Opioid ligands themselves can have chaperone-like actions. Both agonists and antagonists can up-regulate opioid receptors at the protein level through promoting correct conformation or folding of the receptor proteins by escaping the ERAD pathway (60–64). Similar observations were made as early as 1973, when it was reported that administration of opiates in vivo increased opioid receptor binding in the brain by as much as 70% with a concomitant enhanced sensitivity toward opioids (65–69).

Presumably, the single TM variants act through a physical association with the full-length variant, supported by our evidence that they can physically associate. The crystal structure of the µ-opioid receptor (29) reveals that TM1 is important in the formation of MOR-1 oligomers. It is interesting to speculate on whether or not a similar association occurs between the single TM and full-length variants and, if so, whether they can modulate oligomer or even heterodimer formation, as suggested by He et al. (59).

The other full-length MOR-1 splice variants are identical to MOR-1, with the exception of the very tip of the C terminus. Thus, it seems likely that the 1-TM variants will regulate the other full-length variants in a way similar to how they did on MOR-1 itself. Another question is whether the single TM variants can partner other receptors. A 1-TM TAT construct (MORTM1-TAT) structurally similar to the cloned single TM MOR-1 itself. Another question is whether the single TM variants can partner other receptors. A 1-TM TAT construct (MORTM1-TAT) structurally similar to the cloned single TM variants interrupts the dimerization of MOR-1 and DOR-1 receptors and thereby modulates trafficking, degradation, and tolerance (59). Heterodimers have been reported among the opioid receptor families (70–73). It will be interesting to see if the single TM MOR-1 variants can also serve as molecular chaperones for DOR-1, KOR-1, or other classes of GPCRs and if they physiologically modulate dimerization.

The single TM variants are pharmacologically relevant in vivo. An antisense spanning the exon 1–4 junction lowers the expression of mMOR-1S mRNA without altering the mRNA levels of the full-length 7-TM variant MOR-1. This selective down-regulation of the single TM variant lowers morphine analgesia both spinally and suparapinally and reduces the protein levels of the full-length 7-TM variant, closely mimicking our in vitro studies using the Tet-Off CHO cells. It is interesting that the MOR7TM-TAT protein, which is highly analogous to the single TM variants, also enhanced morphine analgesia (59).

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Although the authors suggested that the fusion protein disrupted heterodimerization between DOR-1 and MOR-1, it might also have had a chaperone-like function similar to that of the single TM variants that contribute to the increased morphine analgesia.

Single TM variants are not limited to μ-opioid receptors. In the mouse, a DOR-1 variant with a 243-bp insertion (exon 1') between exons 1 and 2 has been reported that generates a truncated DOR-1 receptor only containing the first TM encoded by exon 1 (35), a situation similar to that with MOR-1. Three single TM ORL₁, (also known as KOR-3) splice variants (mKOR-3a, mKOR-3b, and mKOR-3c) produced by insertion of additional exons between exons 1 and 2 have also been isolated from the mouse OPRK₁ gene (12). Wakamatsu and colleagues identified a single TM KOR-1 splice variant (GenBank™ number AK310407) from the human κ-opioid receptor (OPRK₁) gene through their human cDNA sequencing project. The variant has an insertion of a 174-bp exon between exons 1 and 2. Translation from the AUG in exon 1 predicts a truncated KOR-1 protein with only the first TM due to early translation termination within the inserted exon. All of the single TM domain variants from the various opioid receptors encode their respective TM₁, raising the question of whether or not these DOR-1, KOR-1, and ORL₁ (KOR-3) single TM variants function similarly as the single TM MOR-1 variants.

Single TM variants are not restricted to the opioid receptor family. Approximately 7% of the human GPCR genes (excluding olfactory receptor genes) have one or more potential single TM domain splice variants predicted by searching the genomic databases.⁴ Although the expressions of these single TM variants need to be verified experimentally, our studies raise the possibility that truncated single TM variants might also serve similar functional roles in other GPCR families.

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