Functional characterization of human variants of the mu-opioid receptor gene

Ajay Ravindranathan, Geoff Joslyn, Margaret Robertson, Marc A. Schuckit, Jennifer L. Whistler, and Raymond L. White

Contribution by Raymond L. White, April 30, 2009 (sent for review January 26, 2009)

Opioids and their receptors have an important role in analgesia and alcohol and substance use disorders (ASUD). We have identified several naturally occurring amino acid changing variants of the human mu-opioid receptor (MOR), and assessed the functional consequences of these previously undescribed variants in stably expressing cell lines. Several of these variants had altered trafficking and signaling properties. We found that an L85I variant showed significant internalization in response to morphine, in contrast to the WT MOR, which did not internalize in response to morphine. Also, when L85I and WT receptor were coexpressed, WT MOR internalized with the L85I MOR, suggesting that, in the heterozygous condition, the L85I phenotype would be dominant. This finding is potentially important, because receptor internalization has been associated with development of tolerance to opiate analgesics. In contrast, an R181C variant abolished both signaling and internalization in response to saturating doses of the hydrolysis-resistant enkephalin [D-Ala2,N-MePhe4,Gly5-ol]enkephalin (DAMGO). Coexpression of the R181C and WT receptor led to independent trafficking of the 2 receptors. S42T and C192F variants showed a rightward shift in potency of both morphine and DAMGO, whereas the S147C variant displayed a subtle leftward shift in morphine potency. These data suggest that these and other such variants may have clinical relevance to opioid responsiveness to both endogenous ligands and exogenous drugs, and could influence a broad range of phenotypes, including ASUD, pain responses, and the development of tolerance to morphine.

Identification of Variants. DNA sequencing of each of the coding regions of OPRM1 in the San Diego Sibling Pair study cohort of 550 subjects revealed significant variability in genetic sequence. Sixty-eight variants were identified in the coding region, flanking sequences, and conserved noncoding regions. Eight predicted a change in amino acid sequence, and are shown in Fig. 1. Sequencing of OPRM1 has identified a number of nonsynonymous SNPs which can significantly alter receptor function (10, 22). Sixty-eight variants were identified in the coding region, flanking sequences, and conserved noncoding regions. Eight predicted a change in amino acid sequence, and are shown in Fig. 1.

Author contributions: A.R., J.L.W., and R.L.W. designed research; A.R., G.J., and M.R. performed research; and A.R., M.A.S., J.L.W., and R.L.W. wrote the paper.

The authors declare no conflict of interest.

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T205M) to be deleterious to the normal functioning of the receptor. L85I was predicted to be benign.

**Functional Analysis of Variants.** HEK293 cells stably expressing FLAG-tagged, WT MOR1, or the variants L85I, R181C, or T205M were initially investigated for alterations in receptor trafficking. Live cells were incubated with anti-FLAG M1 antibody to selectively label surface receptors and then stimulated with either 10 μM [D-Ala²,N-MePhe⁴,Gly⁵-ol]enkephalin (DAMGO) or morphine, fixed, permeabilized, and incubated with a fluorescent secondary antibody. As shown in Fig. 2, the WT MOR1 remained primarily on the surface after activation with morphine or with no drug, whereas DAMGO induced substantial endocytosis (Fig. 2A). T205M showed a trafficking pattern similar to that of the WT MOR (Fig. 2B). In contrast, the L85I variant was internalized in response to not only DAMGO, but also morphine (Fig. 2C), whereas the R181C variant showed little to no internalization in response to DAMGO (Fig. 2D). These results were replicated in multiple clones, confirming that the observed phenotype was not due to clonal variation (Fig. S1). To confirm these trafficking profiles, we performed biotin-protection assays. Cells stably expressing FLAG-tagged, WT MOR, or the variants L85I, R181C, or T205M were labeled with a thio-cleavable biotin label and treated with agonist. After agonist stimulation with morphine, receptors remaining at the surface were “stripped” of biotin with a membrane-impermeant reducing agent; internalized “protected” receptors were visualized with streptavidin overlay. Lanes from left to right are: 100% (total), strip, internalized protected biotinylated receptors visualized by SDS/PAGE followed by streptavidin overlay. Images shown are representative from 4 different experiments. (A–D) Live antibody feeding experiment to assay agonist-mediated internalization in HEK cells expressing FLAG-tagged variant MOR receptors. Stable cell lines expressing the MOR1 or the variants T205M, L85I, and R181C were left untreated (Left), treated with 10 μM morphine (Center), or treated with 10 μM DAMGO (Right). Images shown are representative from 4 different experiments. (E–G) Biotin protection assay confirming aberrant internalization profiles of L85I and R181C when compared with MOR1. Drug treatment for 30 min was followed by strip of remaining surface receptors, with the remaining internalized protected biotinylated receptors visualized by SDS/PAGE followed by streptavidin overlay. Lanes from left to right are: 100% (total), strip, no treatment (NT), morphine-treated (MS), or DAMGO-treated (DG). Results are from at least 3 independent experiments.

**Fig. 1.** Nonsynonymous variants identified by sequencing the coding region of the OPRM1 gene are superposed on a structural model of the MOR. Vertical bars represent transmembrane domains. The 2 tails signify variants caused by C-terminal splicing. Previously undescribed variants are shown in black. Variants in gray have been previously described. Functional studies were not performed on N40D, as its function has been extensively characterized. In each case, allelic frequencies of the variants are provided as well. Predictive programs of functional consequence of the novel variants (Polyphen) indicate the variants may be benign (italics), possibly damaging (underline), or probably damaging (bold + underline).

**Activity Assays.** We next examined whether altered trafficking of the variants was accompanied by changes in signaling, testing G protein signaling in our MOR cell lines with a calcium-based activity assay (27). As seen in Table 1, the EC₅₀ of both DAMGO and morphine were similar and in the nM range for MOR1, L85I, and T205M (2-way ANOVA). Thus, the internalization of the L85I variant receptor by morphine cannot be attributed to enhanced potency at the L85I receptor. Also, statistical analysis indicated that the maximal effect (E₅₀) values for MOR1, T205M, and L85I did not differ significantly from each other. R181C showed no agonist response to either DAMGO or morphine in either of the independent stable clones tested, suggesting that lack of internalization could reflect the inability of the receptor to signal through G protein, although G protein coupling is not an absolute requirement for MOR internalization (28), and there is even evidence of some antagonists promoting G protein-coupled receptor (GPCR) internalization (29). The dose-response curves are shown in Fig. S2.

**Fig. 2.** Agonist-mediated internalization of variant mu opioid receptors. (A–D) Live antibody feeding experiment to assay agonist-mediated internalization in HEK cells expressing FLAG-tagged variant MOR receptors. Stable cell lines expressing the MOR1 or the variants T205M, L85I, and R181C were left untreated (Left), treated with 10 μM morphine (Center), or treated with 10 μM DAMGO (Right). Images shown are representative from 4 different experiments. (E–G) Biotin protection assay confirming aberrant internalization profiles of L85I and R181C when compared with MOR1. Drug treatment for 30 min was followed by strip of remaining surface receptors, with the remaining internalized protected biotinylated receptors visualized by SDS/PAGE followed by streptavidin overlay. Lanes from left to right are: 100% (total), strip, no treatment (NT), morphine-treated (MS), or DAMGO-treated (DG). Results are from at least 3 independent experiments.

**Binding Assays.** Failure to bind agonist could explain the absence of signaling observed in R181C. To examine this possibility, we assessed DAMGO binding in the R181C and other variants. DAMGO bound to all variants, including R181C, in the nM affinity range (Table 2), and no significant differences in Kᵦ were observed (1-way ANOVA). Differences in B₅₀ were observed, with clones for T205M showing lower levels of expression (P <
MOR in response to 10 protection assay demonstrates that internalization of the WT assays confirmed these findings (Fig. 3). Also, mice expressing these mutant receptors show reduced morphine tolerance and dependence (33). Here, we have identified a naturally occurring variant that undergoes internalization with morphine without showing changes in morphine potency. We predicted that cells expressing the L85I MOR variant would, therefore, show reduced tolerance and dependence as assessed by cAMP responses. To examine this possibility, we ascertained acute drug responses before and after chronic morphine treatment, as well as cAMP superactivation in cells expressing MOR or L85I. Chronic morphine treatment of cells expressing WT superactivation, are thought to underlie, at least in part, tolerance and dependence to morphine (32). It has been shown previously that MOR mutants engineered to undergo morphine-induced internalization demonstrate reduced propensity to cause these adaptive responses in heterologous cells (9). Also, mice expressing these mutant receptors show reduced morphine tolerance and dependence (33). Hence, it is possible that only half of the MORs in each heterozygous individual expressing a rare variant would drive aberrant trafficking or signaling (assuming identical transcription and translation), it is also possible that either the WT or the variant could show dominance, resulting in either no trafficking of either or trafficking of both. To examine whether trafficking of WT receptors was altered in the presence of an allelic variant or vice versa, we simulated a heterozygote by generating a heterozygous individual expressing a rare variant would drive aberrant trafficking or signaling (assuming identical transcription and translation), it is also possible that either the WT or the variant could show dominance, resulting in either no trafficking of either or trafficking of both. To examine whether trafficking of WT receptors was altered in the presence of an allelic variant or vice versa, we simulated a heterozygote by generating HEK293 cells stably expressing both a FLAG-tagged mutant receptor and an HA-tagged WT receptor. In cells expressing both WT and R181C receptors, WT, but not the R181C variant, internalizes in response to DAMGO (Fig. 3A). Biotin protection assay confirmed these findings (Fig. 3B). Also, the biotin protection assay demonstrates that internalization of the WT MOR in response to 10 μM DAMGO is not adversely affected by the presence of the noninternalizing R181C variant. These data suggest either that WT MOR and R181C do not form dimers with each other, or that any such dimerization is labile and the receptors separate at the surface and traffic independently. In contrast, cells expressing the L85I variant together with the WT MOR internalize both receptors in response to morphine as shown by both immunofluorescence (Fig. 4A) and biotin protection assay (Fig. 4B). Thus, L85I appears to be a dominant mutation with respect to the trafficking phenotype.

The cAMP Superactivation and Tolerance in the L85I Variant. Cells in culture expressing MORs show compensatory changes in signal transduction, including up-regulation of cAMP, after chronic treatment with morphine (31). These changes, including cAMP

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<th>Table 1. Table with EC50s and Emax (mean ± SEM) for morphine and DAMGO in WT and variant MORs</th>
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Stably expressing MOR variant clones were transiently transfected with a mutant GPCR construct such that activation of the receptor would result in an efflux of intracellular calcium that could be measured with a fluorescent dye. Each data point is an average of at least 3 independent experiments in triplicate. NA, not applicable.

Table 2. Membranes from stably expressing MOR variant clones were tested for binding to 3H-DAMGO

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<th>Kd, nM</th>
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<td>MOR1</td>
<td>3</td>
<td>1.02 ± 0.20</td>
<td>5.56 ± 0.59</td>
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<tr>
<td>T205M</td>
<td>3</td>
<td>0.59 ± 0.20</td>
<td>2.23 ± 0.22</td>
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<td>R181C</td>
<td>3</td>
<td>1.66 ± 0.37</td>
<td>3.54 ± 0.46</td>
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<td>L85I</td>
<td>3</td>
<td>1.45 ± 0.42</td>
<td>5.22 ± 0.53</td>
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Non-specific binding was ascertained in the presence of 10 μM naltrexone. Values for Kd and Bmax are an average of 5 replicates from 3 independent experiments.

0.001; 1-way ANOVA, Tukey post hoc test). Binding curves are shown in Fig. S3.

Functional Consequence of Having a Single Mutant Allele. With rare mutations, minor allele homozygotes will likely not be seen outside of highly inbred populations. Indeed, both L85I and R181C have been found only in the heterozygous condition in our population. In several instances, mutant GPCRs with altered trafficking properties have been shown to affect the trafficking of the WT receptor, presumably due to receptor oligomerization (10), such as the CCR5 mutant that protects against HIV (30). Thus, it is possible that only half of the MORs in each heterozygous individual expressing a rare variant would drive aberrant trafficking or signaling (assuming identical transcription and translation), it is also possible that either the WT or the variant could show dominance, resulting in either no trafficking of either or trafficking of both. To examine whether trafficking of WT receptors was altered in the presence of an allelic variant or vice versa, we simulated a heterozygote by generating HEK293 cells stably expressing both a FLAG-tagged mutant receptor and an HA-tagged WT receptor. In cells expressing both WT and R181C receptors, WT, but not the R181C variant, internalizes in response to DAMGO (Fig. 3A). Biotin protection assay confirmed these findings (Fig. 3B). Also, the biotin protection assay demonstrates that internalization of the WT MOR in response to 10 μM DAMGO is not adversely affected by the presence of the noninternalizing R181C variant. These data suggest either that WT MOR and R181C do not form dimers with each other, or that any such dimerization is labile and the receptors separate at the surface and traffic independently. In contrast, cells expressing the L85I variant together with the WT MOR internalize both receptors in response to morphine as shown by both immunofluorescence (Fig. 4A) and biotin protection assay (Fig. 4B). Thus, L85I appears to be a dominant mutation with respect to the trafficking phenotype.

The cAMP Superactivation and Tolerance in the L85I Variant. Cells in culture expressing MORs show compensatory changes in signal transduction, including up-regulation of cAMP, after chronic treatment with morphine (31). These changes, including cAMP...
MOR1 led to increased cAMP levels or “superactivation” (see diamonds in Fig. 5A). Significant superactivation was observed after pretreatment with 10 and 100 nM morphine. In contrast, DAMGO at these doses did not promote superactivation, consistent with the hypothesis that internalization prevents this effect (see triangles in Fig. 5A). Importantly, superactivation was significantly reduced (P < 0.001; 2-way ANOVA, Bonferroni post hoc test) in response to morphine at the 10 and 100 nM doses in cells expressing the L85I variant. In each case, the cAMP levels in naive cells are normalized to agonist-naive controls and represent 3 independent experiments. To assess whether tolerance to morphine was likewise reduced in cells expressing L85I, we used a morphine rechallenge experiment. Cells were treated chronically with morphine (100 nM, with chronic 100 nM morphine for 14 h followed by a 4-hr acute 10 nM morphine rechallenge (bars 4 and 8). The treatment received by each group is designated above each bar. Gray bars (bars 1–4) represent WT MOR1 expressing cells, whereas the white bars (bars 5–8) represent cAMP levels in cells expressing the L85I variant. In each case, the cAMP levels in naive cells are designated as 1, and cAMP levels for the other 3 conditions are expressed relative to naive levels. Data represents 3 independent experiments.

The other 4 variants we identified (A6V, S42T, S147C, and C192F) showed no changes in morphine or DAMGO-induced internalization by immunofluorescence (Fig. S4), although each of these variants was examined only in the context of the MOR1A splice variant backbone that lacks 12 aa coded by exon 4 (34, 35). To determine whether the MOR1A background altered the activity of the MOR protein, we compared FLAG-tagged MOR1 receptors to MOR1A receptors. We also included 4 additional variants on the MOR1A backbone. DAMGO and morphine had potency in the nanomolar range on MOR1, MOR1A, A6V-MOR1A, and S147C-MOR1A, but only >150 nM potency on S42C-MOR1A and C192F-MOR1A, indicated by a rightward shift in the dose-response curves for both DAMGO and morphine in the S42T and C192F variants (Table 3; Fig. S5A). Also, morphine was more potent on the S147CMOR1A variant than on the WT receptor (P < 0.01; 2-way ANOVA, Bonferroni post hoc test). No significant differences in efficacies (Emax) of DAMGO or morphine were observed when MOR1A, A6V-MOR1A, S42T-MOR1A, and S147C-MOR1A were compared with MOR1. However, Emax of both morphine and DAMGO were significantly different between S147C and MOR1A (P < 0.01 and P < 0.05, respectively; 2-way ANOVA, Bonferroni post hoc test). Emax of DAMGO also differed significantly between the A6V variant and MOR1A...
internalize in response to 10% of the subjects sequenced carrying at least 1 receptor. Thus, the MOR appears to be quite polymorphic, with identified 68 variants, 8 of which alter the protein sequence of the sequenced the coding regions of the human OPRM1 gene and been put forth (37). Extending such studies, we have rese-

ASUD, a concerted effort to resequence the OPRM1 gene has in amino acid sequence in the MOR gene (36). Given the Individual variability to therapeutically administered opiates. Some of this variability may be due to variations in the R181C variant in our cell system. Also, R181C did expression of MOR1 did not lead to DAMGO-mediated internal-

ization of these variants should help provide valuable insight into the functioning of the MOR. Functional differences have been reported between C-terminal splice variants of the OPRM1 gene in mice (45) and rats (17). When we compared MOR1 receptors to MOR1A receptors lacking the 12 aa coded by exon 4, we found that the 2 receptors had similar binding, activation, and trafficking profiles. However, we cannot exclude the possibility that closer examination of MOR1 and MOR1A could reveal important functional differences between the 2 isoforms. Interestingly, the S42T-MOR1A and C192F MOR1A variants demonstrated reduced potency for both DAMGO and morphine, whereas morphine had increased potency at the S147C-MOR1A receptor. Thus, 5 of the 8 nonsynonymous variants tested appear to have consequences on receptor function.

In conclusion, we and others have identified a number of polymorphisms in OPRM1 that may explain the variability seen in the clinical response to opiates, both from an analgesic and ASUD stand-point. Further functional and clinical characterization of these variants should help provide valuable insight into the functioning of the MOR.

Materials and Methods
Identification of Variants. The subjects were used from the San Diego Sibling Pair study (46, 47), and consisted of college age students from the San Diego

| Table 3. EC50 and Emax (mean ± SEM) for morphine and DAMGO in MOR1, MOR1A, A6V-MOR1A, S42T-MOR1A, S147C-MOR1A, or C192F-MOR1A receptors |
|---|---|---|
| n | DAMGO | Morphine | DAMGO | Morphine |
| MOR1 | 3 | 32.3 ± 10.4 | 39.27 ± 5.21 | 1140 ± 170 | 941 ± 96 |
| MOR1A | 3 | 35.40 ± 4.11 | 39.43 ± 9.08 | 877 ± 166 | 776 ± 38 |
| A6V | 3 | 13.28 ± 0.79 | 51.62 ± 9.02 | 1345 ± 30 | 1081 ± 84 |
| S42T | 4 | >150 | >150 | NA | NA |
| S147C | 3 | 13.13 ± 6.46 | 4.83 ± 0.09 | 1341 ± 46 | 1297 ± 89 |
| C192F | 4 | >150 | >150 | NA | NA |

Stably expressing MOR variant clones were transiently transfected with a mutant Gi construct such that activation of the receptor would result in an efflux of intracellular calcium that could be measured with a fluorescent dye. Each data point is an average of at least 3 independent experiments in triplicate. NA, not applicable.

Discussion
Individuals vary in their sensitivity to therapeutically administered opiates. Some of this variability may be due to variations in amino acid sequence in the MOR gene (36). Given the importance of the MOR in clinical pain treatment, as well as ASUD, a concerted effort to resequence the OPRM1 gene has been put forth (37). Extending such studies, we have resequenced the coding regions of the human OPRM1 gene and identified 68 variants, 8 of which alter the protein sequence of the receptor. Thus, the MOR appears to be quite polymorphic, with almost one-third of the subjects sequenced carrying at least 1 nonsynonymous SNP in the OPRM1 sequence. The R181C variant is a signaling dead variant that does not internalize in response to 10 μM DAMGO. However, this variant receptor does show a similar agonist binding profile to WT, indicating that the amino acid substitution does not alter ligand binding. This result is perhaps not surprising given the intracellular location of the variant. However, once bound, the agonist does not result in G protein mediated signaling and subsequent events in the cascade that culminate in internalization. Also, the proband carrying this variant is a heterozygote and, thus, likely has only half the functional receptors present in a normal individual. Previous experiments have demonstrated a 60% decrease in MOR binding (38), as well as reduced sensitivity to morphine in heterozygous MOR deficient mice (39, 40). Thus, an individual carrying this variant may demonstrate an altered response to therapeutically administered opiates or endogenous beta endorphin.

Although morphine treatment of HEK cells cotransfected with WT MOR1 and a recycling chimeric MOR1 (D-MOR) results in internalization of the WT MOR protein (10), coexpression of MOR1 did not lead to DAMGO-mediated internalization of the R181C variant in our cell system. Also, R181C did not prevent the WT MOR1 from DAMGO-mediated internalization. Together, these data suggest that R181C does not form stable oligomers with WT MOR1, possibly due to the variant cysteine residue forming inappropriate disulfide bonds and altering the topology and/or function of R181C. Morphine-stimulated MORs have been shown, with few exceptions (41, 42), to elude internalization (43). It has been suggested that this aberrant trafficking of MORs results in prolonged activation of the MORs, leading to compensatory changes that result in tolerance to morphine. The L851 variant is interesting in that, although this variant receptor internalized normally in response to DAMGO in a HEK cell system, internalization was also seen in response to morphine. This finding was surprising, given that the replacement of a leucine by an isoleucine was not predicted to have a major consequence for the structure or function of the variant protein. However, there are other reports of seemingly benign amino acid substitutions dramatically altering protein function of GPCRs. Substitution of a leucine for an isoleucine at position 542 in the luteinizing hormone receptor, for example, leads to elevated cAMP production (44). Coexpression of WT with the L851 variant does not mitigate the internalization to morphine. Also, both cAMP up-regulation and tolerance to morphine are reduced in cells expressing the L851 variant. These in vitro findings can now be extended to animal systems by creating L851 knock-in mice. Also, we are in the unique position of being able to ascertain morphine tolerance in human carriers of the L851 allele, thus, exploring the relationship between receptor endocytosis and tolerance to opiates in humans.

Functional differences have been reported between C-terminal splice variants of the OPRM1 gene in mice (45) and rats (17). When we compared MOR1 receptors to MOR1A receptors lacking the 12 aa coded by exon 4, we found that the 2 receptors had similar binding, activation, and trafficking profiles. However, we cannot exclude the possibility that closer examination of MOR1 and MOR1A could reveal important functional differences between the 2 isoforms. Interestingly, the S42T-MOR1A and C192F MOR1A variants demonstrated reduced potency for both DAMGO and morphine, whereas morphine had increased potency at the S147C-MOR1A receptor. Thus, 5 of the 8 nonsynonymous variants tested appear to have consequences on receptor function.

Non-synonymous binding was ascertained in the presence of 10 μM naloxone. Values for KG and Bmax are an average of 5 replicates from at least 2 independent experiments.
metropolitan area. This collection was designed to identify families enriched for alcoholism susceptibility genes. Genomic DNA was extracted from anticoagulated venous blood samples using the PureGene DNA extraction kit (Genta) according to manufacturer protocols. PCR primers for the coding regions of the MOR were generated using Exon Primer (http://ihg.gsf.de/ihg/Exon Primer.html) and synthesized commercially. PCRs were performed in 10-μL volumes using AmpliTaq Gold (ABI), and the PCR products then sequenced on an ABI 3730XL sequencer using standard reaction mixtures. The sequence data obtained was imported into Mutation Surveyor and polymorphisms identified. Each polymorphism was manually checked for confirmation.

Generation of Variants. WT MOR1 or MOR1A was cloned into pCDNA3.1 Zeo or Hygro (Invitrogen), downstream of an in-frame signal sequence and FLAG epitope as described previously (48). Quicheck (Stratagene) was used to generate FLAG-tagged constructs of the point mutations identified. The constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) and individual clones selected using appropriate antibiotics. Double-stable cell lines were generated by cotransfecting cDNAs for FLAG-tagged mutant receptors concomitantly with HA-tagged MOR1 into HEK293 cells and selecting for clones with 2 antibiotics.

Characterization of Variants. Experimental details for immunohistochemistry, biotin protection assays, binding assays, activity assays, and cAMP and tolerance assays are provided as SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Asma Asayed, Karen Chang, and Mohammad Naeemuddin for technical assistance; Selena Bartlett, Li He, Dawn Thompson, and Lene Martini for helpful discussions; and Claudia Yu for helping in the preparation of this manuscript. R.L.W. was supported by funds provided by the State of California for Medical Research on Alcohol and Substance Abuse through the University of California, San Francisco.

Supporting Information

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SI Materials and Methods

Immunocytochemistry. For immunocytochemistry, cells were grown on polylysine coated chamber-slides and incubated with 3.5 μg/mL M1 anti-FLAG (Sigma) or anti-HA (Covance) antibody for 30 min. Cells were then treated with 10 μM agonist as specified, fixed in 4% formaldehyde in PBS, permeabilized in 0.1% Triton X-100, and incubated with appropriate fluorescently labeled secondary antibodies (Invitrogen). Images were acquired using a Zeiss confocal microscope with a 63× oil objective.

Biotin Protection Assay. Cells stably expressing FLAG-tagged mu-opioid receptors (MORs), HA-tagged MORs, or both, were grown to 80% confluence in 10-cm plates, washed with PBS, and then treated with 3 μg/mL dithiole-cleavable biotin (Pierce) in PBS at 4 °C for 30 min. They were then washed in PBS and placed in prewarmed media for 15 min, before treatment with specified ligands for the time indicated. Concurrent with ligand treatment, plates were washed 5 times and strip plates remained at 4 °C. After ligand treatment, plates were washed in PBS, and remaining cell surface biotinylated receptors stripped in 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, and 1% BSA at 4 °C for 30 min for all ligand-treated plates, as well as control plates. Cells were then quenched with iodoacetamide for 20 min. Cells were lysed in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris-HCl, pH 7.4, and cellular debris removed by centrifugation at 10,000 × g for 10 min at 4 °C. Lysates were immunoprecipitated overnight with anti-FLAG M2 (Sigma) or anti-HA (Covance), rabbit anti-mouse linker antibody (Jackson Immunoresearch), and protein A Sepharose (Amersham Pharmacia). Immunoprecipitates were washed 5× with PBS containing 1% Triton X-100, and deglycosylated with PNGase F (NEB) for 2 h. Samples were then denatured in SDS sample buffer with no reducing agent, resolved by SDS/PAGE, transferred to nitrocellulose, and biotinylated proteins detected with Vectastain ABC immunoperoxidase reagent (Vector Laboratories), developed with ECL reagents (Amersham), visualized on a Storm 860 (Molecular Dynamics), and quantified with ImageQuant software.

Binding Assays. Membranes were prepared as follows: transfected cells (4 140-mm dishes at 100% confluence) were washed twice in PBS, scraped off the plates in PBS, pelleted by spinning at 1,500 × g for 10 min at 4 °C, frozen at −80 °C for 30 min, and thawed in 30 mL of cold 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, with 0.1 mM phenylmethylsulfonyl fluoride. The cell lysate was kept on ice for 15 min, Dounce-homogenized, and spun at 2,500 × g for 10 min at 4 °C. The pellet was resuspended in 15 mL of buffer, Dounce-homogenized, and spun again at 2,500 × g for 10 min at 4 °C. Both supernatants were pooled and centrifuged at 40,000 × g for 30 min at 4 °C. The supernatant was removed, and the pellet was re-suspended in 25 mL of 50 mM Tris-HCl, pH 7.4, Dounce-homogenized, and spun again at 40,000 × g for 30 min at 4 °C. The pellet was then resuspended in 4 mL of 50 mM Tris-HCl, pH 7.4, 0.32 M sucrose, and the protein concentration was measured using the Bradford assay (Pierce). Opioid binding experiments were performed on membrane preparations as follows: 10 μg of membrane proteins were diluted in 50 mM Tris-HCl, pH 7.4, in a final volume of 0.25–0.5 mL and incubated with variable concentrations of [3H]-DAMGO (0.05–6 nM) for 1 h at 25 °C. Nonspecific binding was determined in the presence of 10 μM naltrexone and represented less than 15% of total binding. Samples were incubated for 90 min at 25 °C, and incubation was terminated by ice-cold phosphate buffer followed by filtration over GF/B glass fiber filters. Scintillation mixture was added before counting. Data were graphed using Prism 4 software (Graphpad Software) and Kd and Bmax values calculated using the same. One-way ANOVAs followed by Tukey posthoc tests were used to test for significance.

Activity Assays. Activation was measured in HEK-293 cells stably expressing the respective receptors transiently-transfected with a chimeric G protein Δ6-Gq44-myr (200 ng for every 40,000 cells). One day after transfection, cells were loaded for 60 min with a Ca2+-fluorophore (Molecular Devices), and stimulated with increasing amounts of ligand. Intracellular Ca2+ release due to activation of the receptor was measured immediately after agonist application in a Flex apparatus (Molecular Devices) for 2 min. Graphing of the data and calculations of EC50 were performed with the Prism 4 software package (Graphpad Software). Two-way ANOVAs followed by Bonferroni posthoc tests were used to test for significance.

cAMP Up-Regulation and Tolerance Assays. Cells stably expressing WT or variant L851 MORs were transfected with a CRE-luciferase reporter (Promega) along with a renilla luciferase construct (transfection control). Experiments were carried out 24 h after transfection. For the cAMP up-regulation assay, cells were treated with morphine (1 to 100 nM) or left untreated for 14 h and rinsed 3 times in drug-free media to simulate a withdrawal phase. Both treated and untreated cells were subsequently treated with 2 μM forskolin and luciferase activity measured; 14 h was chosen as a time point, because it had been previously demonstrated that superactivation at this time point was highly reproducible (1). To assay for tolerance, cells containing WT or variant receptors were treated with 2 μM Forskolin to ascertain baseline naive response. Cells were treated with 10 nM acute morphine for 4 h, 100 nM chronic morphine for 14 h or 100 nM chronic morphine for 14 h followed by an acute 10 nM rechallenge for 4 h. In all treatment conditions, cells were rinsed once in PBS immediately before luciferase measurement; 100 μL Cell Culture Lysis Reagent (Promega) was added to each well; a 20 μL cell lysate aliquot was transferred to an opaque 96-well plate; 100 μL substrates (LAR-2 and Stop and Glo) were added per well using an LD400 luminometer (Beckman-Coulter); and light measurements were collected. Data were exported to GraphPad Prism 4.0 for graphical display, nonlinear regression curve-fitting, and subsequent statistical analyses.

Fig. S1. Live antibody feeding experiment to assay agonist-mediated internalization in HEK cell clones stably expressing FLAG-tagged R181C or L85I receptors. (Upper) Three different clones of R181C show no internalization to a 10 μM DAMGO challenge. (Lower) Independent clones of L85I show robust internalization in response to 10 μM morphine treatment.
Fig. S2. Activation profiles of WT and variant MORs. Stably expressing MOR variant clones were transiently transfected with a mutant Gi construct such that activation of the receptor would result in an efflux of intracellular calcium that could be measured with a fluorescent dye. Curves with unbroken lines denote responses to DAMGO, whereas those with dashed lines denote response to morphine. Each data point is an average of at least 3 independent experiments in triplicate.
Fig. S3. Binding profiles of WT and variant MORs. Membranes from stably expressing MOR variant clones were tested for binding to 3H-DAMGO. Nonspecific binding was ascertained in the presence of 10 μM naloxone. Each data point is an average of 5 replicates from 3 independent experiments.
Fig. S4. Live antibody feeding experiment to assay agonist-mediated internalization in HEK cells expressing FLAG-tagged MOR1, MOR1A, A6V-MOR1A, S42T-MOR1A, S147C-MOR1A, or C192F-MOR1A receptors. Stable cell lines expressing each of the constructs were left untreated (Left), treated with 10 μM morphine (Center), or treated with 10 μM DAMGO (Right). Images shown are representative from 3 independent experiments.
Fig. S5. Activity and binding of MOR1, MOR1A, A6V-MOR1A, S42T-MOR1A, S147C-MOR1A, and C192F-MOR1A. (A) Activation profiles of MOR1, MOR1A, A6V-MOR1A, S42T-MOR1A, S147C-MOR1A, and C192F-MOR1A. Stably expressing MOR variant clones were transiently transfected with a mutant G\textsubscript{i} construct such that activation of the receptor would result in an efflux of intracellular calcium that could be measured with a fluorescent dye. Curves with unbroken lines denote responses to DAMGO, whereas curves with dashed lines denote response to morphine. Each data point is an average of three independent experiments in triplicate. EC\textsubscript{50} data are shown in Table 3. (B) [3H]-DAMGO binding to membranes isolated from HEK cells stably expressing the same. K\textsubscript{d} and B\textsubscript{max} values are shown in Table 4. Data represents at least 2 independent experiments in quintuplicate.