Alternatively spliced mu opioid receptor C termini impact the diverse actions of morphine

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Extensive 3′ alternative splicing of the mu opioid receptor gene OPRM1 creates multiple C-terminal splice variants. However, their behavioral relevance remains unknown. The present study generated 3 mutant mouse models with truncated C termini in 2 different mouse strains, C57BL/6J (B6) and 129/SvEv (129). One mouse truncated all C termini downstream of Oprm1 exon 3 (mE3M mice), while the other two selectively truncated C-terminal tails encoded by either exon 4 (mE4M mice) or exon 7 (mE7M mice). Studies of these mice revealed divergent roles for the C termini in morphine-induced behaviors, highlighting the importance of C-terminal variants in complex morphine actions. In mE7M-B6 mice, the exon 7–associated truncation diminished morphine tolerance and reward without altering physical dependence, whereas the exon 4–associated truncation in mE4M-B6 mice facilitated morphine tolerance and reduced morphine dependence without affecting morphine reward. mE7M-B6 mutant mice lost morphine-induced receptor desensitization in the brain stem and hypothalamus, consistent with exon 7 involvement in morphine tolerance. In cell-based studies, exon 7–associated variants shifted the bias of several mu opioids toward β-arrestin 2 over G protein activation compared with the exon 4–associated variant, suggesting an interaction of exon 7–associated C-terminal tails with β-arrestin 2 in morphine-induced desensitization and tolerance. Together, the differential effects of C-terminal truncation illustrate the pharmacological importance of OPRM1 3′ alternative splicing.

Introduction

Most clinically used opiates, including morphine, act through the mu opioid receptor (1), producing both potent analgesia and a number of side effects, such as tolerance, physical dependence, constipation, respiratory depression, and addiction. The different responses to various mu drugs seen clinically (2, 3) and in animal models (4–6) suggested the existence of multiple mu opioid receptors, a concept initially proposed from classic pharmacological studies (7) and reinforced with the identification of an array of mu opioid receptor variants produced by alternative pre-mRNA splicing of the single-copy mu opioid receptor gene (OPRM1) (see review, refs. 8, 9).

Alternative splicing is commonly seen among G protein–coupled receptors (GPCRs) (10–12). However, the extensive alternative splicing of OPRM1 is quite unusual, creating 3 structurally distinct classes of splice variants that are conserved from rodent to human: (a) full-length 7-transmembrane (7TM) C-terminal variants; (b) truncated 6TM variants that lack exon 1 and the first TM; and (c) truncated single TM variants containing the first TM (ref. 9 and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI88760DS1). The relevance of the truncated variants has been extensively explored and validated (13–17), but few studies have examined the pharmacological consequences of alternative splicing of the C terminus in full-length 7TM variants.

Full-length 7TM C-terminal variants all contain the same 386-aa sequences in mouse and rat or 388-aa in humans that are encoded by OPRM1 exons 1/2/3, but alternative splicing from exon 3 to various downstream exons produces unique alternative aa sequences at the tip of the intracellular C terminus. For example, the murine mu opioid receptor mMOR-1 has a C-terminal tail with 12 aa encoded by Oprm1 exon 4, whereas the C-terminal tails in mMOR-1C and mMOR-1O contain 52 aa and 31 aa encoded by exons 7/8/9 and exons 7a/7b, respectively (8). Increasing evidence implies that the full-length 7TM C-terminal splice variants are pharmacologically important. At the mRNA level, they show region-specific expressions in rodents and humans (18–20), and long-term morphine treatment selectively increases C-terminal variant mRNA expression in various brain regions by as much as 300-fold (21). At the protein level, different C-terminal epitopes also differ in their cellular (i.e., pre- vs. postsynaptic) and regional distributions (22, 23). Additionally, heterodimerization of 7TM C-terminal variant mMOR-1D with gastrin-releasing peptide receptor has been implicated in morphine-induced itch (24).

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At the cellular level, all the 7TM C-terminal variants expressed in CHO cells bind \[^{3}H\]D-Ala\(^2\),MePhe\(^4\),Gly(ol)\(^5\)]\(^\text{(DAMGO)}\), a mu agonist, with high affinity and with only subtle differences in selectivity for the endogenous opioid peptides dynorphin A and β endorphin (18, 25, 26). However, in \[^{35}S\]GTP\(\gamma\)S-binding assays, these C-terminal splice variants displayed marked differences in mu opioid-induced G protein coupling in both potency (EC\(_{50}\)) and efficacy (% of maximal stimulation) (26, 27), suggesting that the distal carboxyl terminal sequences influence mu agonist-induced receptor–G protein coupling and signal transduction. Multiple protein kinase phosphorylation sites are predicted from various alternative C-terminal sequences, and in vitro studies revealed differences in mu agonist–induced phosphorylation, internalization, and postendocytic sorting among several C-terminal splice variants (28, 29). However, in vivo functions of these C-terminal splice variants remain largely unknown.

The current study explores the functional significance of alternative C termini by generating 3 mutant mouse models with truncations of the distal end of the C terminus in 2 different inbred strains. By truncating either all C-terminal tails or selectively truncating C-terminal tails encoded by Oprml exon 4 or exon 7, we now show the importance of these C-terminal tails in morphine tolerance, physical dependence, locomotor activity, and rewarding behavior, and suggest a role for exon 7–associated C terminus with β-arrestin 2 and morphine-induced desensitization and tolerance.

Results

**Targeting intracellular carboxyl termini of the mu opioid receptors in mice.** To target intracellular C-terminal tails that are generated through alternative splicing downstream from Oprml exon 3 to the different downstream exons, we introduced a stop codon at the end or beginning of the targeted exon to produce 3 targetted mouse models without deletion of any individual exons (Supplemental Figure 1 and Supplemental Figure 2, A and B). The first mouse model (mE3M) was generated by inserting a stop codon at the 3′ end of Oprml exon 3 that prevents translation of all exons downstream of exon 3, even though their mRNAs are expressed. Thus, the mE3M homozygous mice express only truncated mu opioid receptors lacking any distal C-terminal tails. In the other 2 mouse models (mE4M and mE7M), we introduced a stop codon at the 5′ end of Oprml exon 4 or exon 7 to selectively terminate translation at the end of exon 3 only in exon 4–or exon 7–associated variants in mE4M or mE7M mice, respectively (Supplemental Figure 1 and Supplemental Figure 2, A and B).

All the stop codon mutations were introduced into W4 embryonic stem (ES) cells derived from 129/SvEv (129) or C57.2 E5 cells from C57BL/6J (B6) mice through homologous recombination using targeting vectors. Positive ES cells determined by Southern blot analysis were used to generate germ line–transmitting chimeras (Supplemental Figure 2C; see complete unedited blots in the supplemental material). WT and homozygous mice were produced through heterozygous breeding, identified by PCR restriction enzyme digestion (Supplemental Figure 2D) and confirmed by sequencing (Supplemental Figure 2E). The selection cassette (pgk-neo) was then removed by breeding with a CAG-Cre transgenic line to eliminate the potential influence of the cassette on transcription and splicing. As B6 and 129 mice have markedly different responses to mu opioids (30–32), we generated 2 sets of congenic mice for each mutant model on a B6 strain background (mE3M-B6, mE4M-B6, and mE7M-B6 WT and homozygous mice) and on a 129 strain background (mE3M-129, mE4M-129, and mE7M-129 WT and homozygous mice) by using traditional breeding combined with speed congenic breeding. Sequencing the exon-exon junctions of reverse-transcription PCR (RT-PCR) products from brain total RNAs confirmed that all the stop codon mutations were present, as expected (Supplemental Figure 2F).

The WT control mice for each set of congenic lines and their corresponding homozygous mice were generated through heterozygous breeding and used for in vivo behavioral and ex vivo studies.

**Expression of the mu opioid receptors in the targeted mouse models.** To examine the effect of the stop codon mutations on mRNA expression, we quantified the mRNA levels of a number of splice variants in brains of the mutant mouse models using quantitative PCR (qPCR). The overall mRNA expression levels of the combined repertoire of full-length 7TM splice variants, determined by the primer set from exon 1 to exon 2 (mE1-2), failed to demonstrate significant differences in any of the mouse models regardless of the strain backgrounds (Supplemental Tables 1 and 2). Similarly, the mRNA levels of individual 7TM variants mMOR-1A, mMOR-1D, mMOR-1I, and mMOR-1O, 6TM variants mMOR-1G and mMOR-1K, and the single TM variants mMOR-1S and mMOR-1R were not appreciably altered in homozygous mice of all the models (Supplemental Tables 1 and 2). These results suggest that the stop codon mutations in mE3M, mE4M, and mE7M models did not have a generalized impact on their transcription and alternative splicing. However, the mRNA levels of mMOR-1C and mMOR-1M were greatly reduced in both the mE3M and mE7M mouse models. These reductions were probably due to nonsense-mediated mRNA decay, a process that degrades mRNA with a stop codon located more than 50 nucleotides upstream of the last exon-exon junction, because the stop codon insertions in the mE3M and mE7M models produce such a premature stop codon.

Protein expression of the full complement of 7TM variants was accessed by opioid receptor binding, recognizing that it measured the total pool of 7TM variants. Receptor expression levels are best assessed with antagonist binding, since the ligand labels both agonist and antagonist conformations to yield the full \(B_{\text{max}}\) (total density [concentration] in a sample of tissue) value. The maximal number of \[^{3}H\]naltrexone–binding sites (\(B_{\text{max}}\)), an indicator of receptor protein expression, was significantly reduced in mE3M and mE4M homozygous mice on both 129 and B6 backgrounds (Table 1), but not in the mE7M mice. We observed no changes in binding affinity in either \[^{3}H\]naltrexone saturation (Table 1) or competition studies with the mu agonists morphine, morphine-6-glucuronide (M6G), dynorphin A, and β-endorphin, and the antagonist naloxone against \[^{3}H\]DAMGO binding (Supplemental Table 3).

**Effect of the C-terminal truncation on mu agonist–induced \[^{35}S\]GTP\(\gamma\)S binding.** The C terminus is critical for receptor–G protein interaction and signal transduction. To determine whether the C-terminal truncation alters mu agonist–induced G protein coupling, we examined DAMGO- and morphine-stimulated \[^{35}S\]GTP\(\gamma\)S binding in whole brain membranes from the mutant mouse models. The truncations had little effect on ED\(_{50}\) values, but significantly reduced DAMGO- and morphine-induced maxi-
Table 1. Saturation studies using [3H]naloxone

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Genotype</th>
<th>$K_v$ (nM)</th>
<th>$B_{	ext{max}}$ (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mE3M-129</td>
<td>WT</td>
<td>0.63 ± 0.02</td>
<td>86 ± 6</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.73 ± 0.09</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>mE3M-B6</td>
<td>WT</td>
<td>0.62 ± 0.06</td>
<td>58 ± 5</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.66 ± 0.09</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>mE4M-129</td>
<td>WT</td>
<td>0.72 ± 0.02</td>
<td>86 ± 5</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>1.48 ± 0.36</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>mE4M-B6</td>
<td>WT</td>
<td>1.02 ± 0.36</td>
<td>64 ± 6</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.99 ± 0.29</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>mE7M-129</td>
<td>WT</td>
<td>1.41 ± 0.33</td>
<td>81 ± 16</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>1.21 ± 0.22</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>mE7M-B6</td>
<td>WT</td>
<td>0.89 ± 0.05</td>
<td>64 ± 3</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.99 ± 0.12</td>
<td>59 ± 9</td>
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</table>

[3H]naloxone saturation studies were performed in membranes prepared from whole brains of indicated mouse models as described in Supplemental Methods. The $K_v$ and $B_{	ext{max}}$ values were determined by nonlinear regression analysis (Prism). Results are shown as mean ± SEM of at least 3 independent determinations. *P < 0.05; **P < 0.01, compared with WT. 1-way ANOVA with Bonferroni’s post hoc test. Mut, homozygous mice.

Table 2. [35S]GTPγS binding

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Genotype</th>
<th>EC$_{50}$ (μM)$^a$</th>
<th>% Maximum stimulation$^a$</th>
<th>EC$_{10}$ (μM)</th>
<th>% Maximum stimulation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mE3M-129</td>
<td>WT</td>
<td>0.41 ± 0.1$^b$</td>
<td>78 ± 3</td>
<td>0.22 ± 0.1</td>
<td>57 ± 3</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.20 ± 0.1</td>
<td>49 ± 2</td>
<td>0.28 ± 0.1</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>mE3M-B6</td>
<td>WT</td>
<td>0.37 ± 0.2</td>
<td>73 ± 3</td>
<td>0.22 ± 0.1</td>
<td>56 ± 5</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.64 ± 0.3</td>
<td>39 ± 10</td>
<td>0.18 ± 0.1</td>
<td>29 ± 9$^d$</td>
</tr>
<tr>
<td>mE4M-129</td>
<td>WT</td>
<td>0.33 ± 0.1</td>
<td>74 ± 12</td>
<td>0.26 ± 0.1</td>
<td>51 ± 2</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.36 ± 0.3</td>
<td>37 ± 3</td>
<td>0.22 ± 0.1</td>
<td>38 ± 3$^d$</td>
</tr>
<tr>
<td>mE4M-B6</td>
<td>WT</td>
<td>0.21 ± 0.1</td>
<td>66 ± 4</td>
<td>0.53 ± 0.1</td>
<td>49 ± 5</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.43 ± 0.0</td>
<td>33 ± 1$^f$</td>
<td>0.23 ± 0.1</td>
<td>26 ± 1$^f$</td>
</tr>
<tr>
<td>mE7M-129</td>
<td>WT</td>
<td>0.11 ± 0.0</td>
<td>75 ± 1</td>
<td>0.45 ± 0.1</td>
<td>57 ± 7</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.20 ± 0.1</td>
<td>77 ± 6</td>
<td>0.14 ± 0.1</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>mE7M-B6</td>
<td>WT</td>
<td>0.33 ± 0.2</td>
<td>71 ± 8</td>
<td>0.28 ± 0.2</td>
<td>52 ± 14</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>0.41 ± 0.2</td>
<td>65 ± 7</td>
<td>0.14 ± 0.1</td>
<td>44 ± 2</td>
</tr>
</tbody>
</table>

[35S]GTPγS binding was performed in membranes from whole brains of indicated mouse models prepared from whole brains of indicated mouse models as described in Supplemental Methods. $^a$EC$_{50}$ and percentage of maximal stimulation values were determined by nonlinear regression analysis (Prism). $^b$Results are shown as mean ± SEM of at least 3 independent determinations. $^*P < 0.05; ^**P < 0.01, compared with WT. 1-way ANOVA with Bonferroni’s post hoc test. Mut, homozygous mice.
mE7M-B6 homozygous mice exhibited significantly lower preference for the drug-paired compartment than the mE7M WT control mice (Figure 2B), suggesting that exon 7–associated variants are involved in morphine reward behavior in B6 mice. Morphine CPP in WT and homozygous 129 mice at the same dose was not robust and was not significant (Supplemental Figure 4), consistent with previous findings (36).

Effect of the C-terminal truncation on morphine locomotor activity. In WT B6 and 129 mice, morphine significantly increased locomotion, with a greater effect in B6 compared with 129 mice (Figure 3A and Supplemental Figure 5), consistent with the literature (37). Both mE4M-B6 and mE7M-B6 homozygous mice showed a modest, but significant, decrease in morphine-induced locomotion measured as total distance traveled compared with their respective WT B6 control mice, while the locomotor activity in mE3M-B6 homozygous mice was not significantly changed (Figure 3A and Supplemental Figure 5). A similar pattern was observed in all the mouse models on the 129 background, but the differences were not significant (Figure 3A). When analyzed by distance per minute, locomotor activity was significantly lower in mE4M-129 homozygous mice than in WT control mice (Supplemental Figure 5). These results indicate a modest effect of the C-terminal truncations in morphine-induced locomotor activity.

Effect of the C-terminal truncation on morphine inhibition of gastrointestinal transit. Morphine modulates gastrointestinal (GI) transit through activation of receptors on the myenteric plexus and centrally (38). Morphine (5 mg/kg) decreased charcoal transit in both B6 and 129 WT mice (Supplemental Figure 6). All the C-terminal truncations had little effect, except for a small, but significant, decrease in morphine’s inhibition of GI transit in mE4M-B6 homozygous mice compared with the WT controls (Supplemental Figure 6), consistent with our early observation that an antisense oligo targeting exon 4 reduced morphine inhibition of GI transit (39).

Effect of the C-terminal truncation on morphine-induced catalepsy. High doses of morphine induce catalepsy (40, 41), a condition of diminished responsiveness characterized by a trance-like state and immobility. In B6 mice, morphine produced dose–dependent and time-dependent cataleptic responses (Supplemental Figure 7). We therefore examined morphine catalepsy 30 minutes after mor-
Effect of the truncation of exon 7–associated C-terminal tails on \([^{[35]}\text{S}]\text{GTP}\gamma\text{S}\) binding in brain regions of mice chronically treated with morphine.

Receptor desensitization has been proposed as one cellular mechanism contributing to morphine tolerance in both in vitro cell lines and in vivo rodent models (42–45). The reduced morphine tolerance with exon 7 truncation in mE7M-B6 mice raised the question of whether or not the truncation altered receptor desensitization in these mice. We evaluated receptor desensitization by measuring morphine-stimulated \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding with membranes from 6 brain regions in the mE7M-B6 models after the 5 days of morphine treatment. There were no significant differences in the EC50 values among brain regions or groups of mE7M-B6 mice, and most regions failed to show morphine-induced changes in maximal stimulation in either WT or homozygous mice (Table 5 and Supplemental Figure 8), although the maximal stimulation in the periaqueductal gray (PAG) of saline-treated mE7M-B6 homozygous mice was significantly higher than in WT controls (Table 5 and Supplemental Figure 8). However, morphine treatment reduced the stimulation in the hypothalamus and brain stem in mE7M-B6 WT mice, but not in

**Figure 1. Effect of the C-terminal truncation on morphine tolerance.** (A) Morphine tolerance in the mutant mice. Tolerance was induced and assessed as described in Methods. The number of mice used were as follows: mE3M-B6, 15 WT and 14 homozygous (Mut) in 2 independent experiments; mE4M-B6, 18 WT and 17 Mut in 2 independent experiments; mE7M-B6, 20 WT and 19 Mut in 3 independent experiments; mE3M-129, 15 WT and 11 Mut in 2 independent experiments; mE4M-129, 13 WT and 13 Mut in 2 independent experiments; mE7M-129, 7 WT and 7 Mut in 1 experiment. *P < 0.05; **P < 0.001; ***P < 0.0001, 2-way ANOVA with Bonferroni’s post hoc test. Mut, homozygous mice. (B) Morphine dose-response curve in the mutant mice. Cumulative dose–response studies were performed before (day 1) and after (day 5) morphine treatment (ED50 values and number of mice in Table 4). (C) ANT-vMO (ANT-vMO targeting intron/exon 7) study in CD-1 mice. Top: mRNA expression of \(\text{Oprm1}\) transcripts containing exons 1–2 (mE1-2), exons 3–4 (mE3-4), and exons 3–7 (mE3-7). RNAs from the PAG dissected on day 6 (see bottom panel) were used in RT-qPCRs. All 2Δ\(\text{Ct}\) values are normalized with PBS group. Results are shown as the mean ± SEM of at least 3 individual samples. *P < 0.05; **P < 0.01, 1-way ANOVA with Bonferroni’s post hoc test. Bottom: morphine tolerance. Group of mice were i.c.v. injected with 10 \(\mu\)g of ANT-vMO (\(n = 18\)) or MIS-vMO (\(n = 16\)), or PBS (\(n = 19\)), for 4 days (days 1–4). Tolerance was induced by twice-daily morphine injection (10 mg/kg, s.c.) for 5 days (days 2–6). Morphine analgesia was tested on days 1, 4, and 6. Results are from 2 independent experiments. *P < 0.0001, compared with PBS; †P < 0.0001, compared with MIS-vMO; *P < 0.05, compared with MIS-vMO, 2-way ANOVA with Bonferroni’s post hoc test.
mE7M-B6 homozygous mice (Figure 4 and Table 5). Binding studies from the same hypothalamus and brain stem tissues revealed no change in [\(^{3}H\)]DAMGO binding after morphine treatment or between genotypes (Supplemental Table 4). Thus, these results suggest that morphine treatment desensitized mu opioid receptor coupling with G proteins in the hypothalamus and brain stem in WT mE7M-B6 mice and that this desensitization was lost in truncated mutant mE7M-B6 mice.

mE7M-129 WT mice did not develop tolerance to morphine, so it was not surprising that morphine treatment failed to significantly alter the maximal stimulation in any brain regions (Table 5). However, we observed elevated stimulation levels in saline-treated mE7M-129 homozygous mice compared with WT controls in both the hypothalamus and brain stem (Table 5). Similar elevation was seen in the PAG of mE7M-B6 homozygous mice (Table 5).

We did not examine the mE3M and mE4M mouse models because the decreased receptor expression and [\(^{35}S\)]GTP\(\gamma\)S binding in the naive truncate mutant mice (Tables 1 and 2) might have made interpretation difficult.

**Effect of the truncation of exon 7–associated C-terminal tails on ERK1/2 activation in brain regions of mice chronically treated with morphine.** ERK1/2 activation has been linked to receptor desensitization through arrestin-dependent and/or arrestin-independent mechanisms, which may contribute to morphine tolerance (42, 46). We investigated ERK activation with Western blots from the same 6 brain regions in mE7M-B6 mice after 5 days of morphine or saline treatment. While chronic morphine failed to alter ERK phosphorylation levels in most regions, including the striatum, PAG, hypothalamus, and brain stem in either WT control or homozygous mice (Supplemental Figure 9), it increased ERK1/2 phosphorylation in the thalamus of WT B6 control mice, raising questions regarding the role of ERK activation in the thalamus in morphine tolerance and its relationship with receptor desensitization in the hypothalamus and brain stem. Similarly to WT B6 controls, mE7M-B6 homozygous mice showed increased ERK activation in the thalamus after morphine treatment, with no changes in other regions (Supplemental Figure 9), suggesting it is unlikely that exon 7–associated C-terminal sequences are involved in the ERK1/2 activation.

**In vitro characterization of exon 7–associated 7-TM variants.** In view of the potential role of exon 7–containing variants in morphine action, we compared the exon 7–associated 7-TM variants mMOR-1C and mMOR-1O with the exon 4–associated mMOR-1 in stably transfected CHO cells. First, we examined signaling bias. Using appropriately engineered stable CHO cell lines, we examined mu agonist–induced \(\beta\)-arrestin recruitment using a PathHunter \(\beta\)-arrestin 2 assay (DiscoverX) and G protein activation by [\(^{35}S\)]GTP\(\gamma\)S binding. Overall, the mu agonists morphine, endomorphin 1, and fentanyl stimulated [\(^{35}S\)]GTP\(\gamma\)S binding with full efficacy relative to DAMGO, with the exception of mMOR-1O, in which the maximal stimulation by morphine was significantly reduced (Figure 5A and Supplemental Table 5). We observed marked potency differences among the variants. For example, DAMGO was most potent against mMOR-1, with an EC\(_{50}\) of 17 nM, compared with 195 and 157 nM against mMOR-1C and mMOR-1O, respectively. Endomorphin 1 and fentanyl also were far less potent against the exon 7–associated variants. The compounds also stimulated \(\beta\)-arrestin 2 recruitment with varying efficacies relative to DAMGO. Although morphine was the least effective against all 3 variants, it stimulated \(\beta\)-arrestin 2 binding more efficaciously in mMOR-1O than in mMOR-1 and mMOR-1C, with no significant changes in potencies. Endomorphin 1 also had higher \(E_{\text{max}}\) (maximum response achievable) values against mMOR-1O than mMOR-1, with no difference in their EC\(_{50}\) values. Both DAMGO and endomorphin 1 had higher EC\(_{50}\) values in mMOR-1C than in mMOR-1 and mMOR-1O.

Using those 2 assays, we calculated the bias factors of the drugs among the variants using the Black and Leff operational model (refs. 47, 48, and Figure 5B). Compared with the bias of DAMGO against mMOR-1, which was normalized to a value of 1, we observed marked variability among the drugs and among the variants, with the greatest difference being a bias factor of ~44.3 for fentanyl against mMOR-1O (Figure 5B). When each drug was normalized to mMOR-1 (Figure 5B), they all displayed a great-
The Journal of Clinical Investigation

Figure 3. Effect of the C-terminal truncation on morphine locomotor activity and catalepsy in the mutant mice. (A) Morphine locomotor activity. Morphine locomotor activity of the mutant mice on B6 and 129 backgrounds was measured in an open-field chamber (Med Associates) as described in Supplemental Methods. Following morphine injection (10 mg/kg, i.p.), the total distance traveled (cm) within 60 minutes was measured. Results are shown as mean ± SEM. The number of mice used were as follows: mE3M-B6, 6 WT and 8 Mut; mE4M-B6, 6 WT and 12 Mut; mE7M-B6, 6 WT and 8 Mut; mE3M-129, 6 WT and 8 Mut; mE4M-129, 7 WT and 10 Mut; mE7M-129, 9 WT and 8 Mut. *P < 0.001, **P < 0.0001, compared with WT treated with morphine, 2-way ANOVA with Bonferroni’s post hoc test. Mut, homozygous mice. (B) Morphine catalepsy. Morphine catalepsy in the mutant mice was assessed by using a horizontal bar test as described in Supplemental Methods. Mice were tested 30 minutes after morphine injection (60 mg/kg, s.c.). The time (s) spent in cataleptic position was recorded. Results are shown as mean ± SEM. The number of mice used were as follows: in mE3M-B6, 6 WT-Saline, 8 WT-morphine, 6 Mut-Saline, 8 Mut-morphine; in mE4M-B6, 6 WT-Saline, 12 WT-morphine, 6 Mut-Saline, 6 Mut-morphine; in mE7M-B6, 8 WT-morphine, 10 Mut-Saline, 10 Mut-morphine; in mE3M-129, 6 WT-Saline, 8 WT-morphine, 6 Mut-Saline, 8 Mut-morphine; in mE4M-129, 5 WT-Saline, 10 WT-morphine, 5 Mut-Saline, 9 Mut-morphine; in mE7M-129, 5 WT-Saline, 7 WT-morphine, 6 Mut-Saline, 11 Mut-morphine. *P < 0.05, **P < 0.001, compared with WT treated with Saline; ***P < 0.001, compared with WT treated with morphine. 2-way ANOVA with Bonferroni’s post hoc test.

Finally, we compared the sodium sensitivity of [3H]DAMGO binding in the 3 CHO cell lines. Sodium acts allosterically to stabilize receptors in an antagonist conformation, which has lower affinity for agonist binding (50–52). Sodium ions lowered [3H]DAMGO binding in a dose-dependent manner for all 3 receptors (Supplemental Figure 10B). However, mMOR-1O stood apart from the other 2, displaying a decreased sensitivity to the ion. This was quite interesting in that the sodium site is located within the transmembrane region of the receptor (51), which is common to all 3 variants. These studies revealed a general pattern in vitro of differences between exon 7-associated and exon 4-associated 7-TM C-terminal variants.

Discussion

The current study used 3 mutant mouse models, mE3M, mE4M, and mE7M, on 2 inbred strain backgrounds to investigate the importance of Oprm1 3’-splicing and the distal C-terminal tails of mu opioid receptors in mediating complex morphine actions (see summary in Table 6). These truncation models did not substantially affect morphine analgesia, but differentially altered morphine-induced tolerance, physical dependence, reward behavior, and locomotor activity profiles. The loss of morphine-induced receptor desensitization in the hypothalamus and brain stem of mE7M-B6 mutant mice further implicated the involvement of exon 7-associated variants in morphine-induced receptor desensitization and tolerance. The similarity in several morphine-induced behaviors and receptor desensitization between mE7M-B6 homozygous and β-arrestin 2 KO mice suggests a physical and functional association of exon 7-associated C-terminal tails with β-arrestin 2, a hypothesis further supported by our in vitro data showing that several mu agonists displayed greater β-arrestin bias against exon 7-associated variants than against the exon 4-associated mOR-1.

Loss of the exon 4 epitope reduced both [3H]naloxone receptor binding and maximal mu agonist–stimulated [35S]GTPγS binding levels despite the lack of significant changes in mRNA levels. The 12 amino acids encoded by exons 8 and 9 beyond the 30 aa encoded by exon 7. This extended sequence is likely responsible for the diminished bias relative to exon 7 coding sequences alone. When the drugs were normalized to DAMGO within each variant, they all showed similar β-arrestin 2 bias patterns (Figure 5B).

We then examined DAMGO- and morphine-induced receptor desensitization in a [35S]GTPγS-binding assay in CHO cells stably transfected with either mOR-1, mMOR-1C, or mMOR-1O, as previously described (25, 49). Although both DAMGO and morphine induced rapid desensitization in all variants, the desensitization was slightly more rapid in the mOR-1O cells (Supplemental Figure 10A).

er β-arrestin 2 bias with the exon 7-associated variants. Of the 2 exon 7-associated variants, mMOR-1O showed greater β-arrestin 2 bias than mMOR-1C. For example, relative to mMOR-1, the bias factor for mMOR-1C was approximately 4-fold lower (–2.2) than for mMOR-1O (–10.0) in response to fentanyl. Structurally, both variants have exon 7, but mMOR-1C is extended for an additional 12 aa encoded by exons 8 and 9 beyond the 30 aa encoded by exon 7. This extended sequence is likely responsible for the diminished bias relative to exon 7 coding sequences alone. When the drugs were normalized to DAMGO within each variant, they all showed similar β-arrestin 2 bias patterns (Figure 5B).

We then examined DAMGO- and morphine-induced receptor desensitization in a [35S]GTPγS-binding assay in CHO cells stably transfected with either mOR-1, mMOR-1C, or mMOR-1O, as previously described (25, 49). Although both DAMGO and morphine induced rapid desensitization in all variants, the desensitization was slightly more rapid in the mOR-1O cells (Supplemental Figure 10A).

suggests a physical and functional association of exon 7-associated C-terminal tails with β-arrestin 2, a hypothesis further supported by our in vitro data showing that several mu agonists displayed greater β-arrestin bias against exon 7-associated variants than against the exon 4-associated mOR-1.

Loss of the exon 4 epitope reduced both [3H]naloxone receptor binding and maximal mu agonist–stimulated [35S]GTPγS binding levels despite the lack of significant changes in mRNA levels. The 12 amino acids encoded by exon 4 contain a MRS–derived recycling sequence (MRS) that facilitates recycling of internalized MOR-1 to the plasma membrane following DAMGO treatment in HEK293 cells (29). Loss of the MRS enhanced agonist-induced receptor desensitization in the hypothalamus and brain stem of mE7M-B6 mutant mice further implicated the involvement of exon 7-associated variants in morphine-induced receptor desensitization and tolerance. The similarity in several morphine-induced behaviors and receptor desensitization between mE7M-B6 homozygous and β-arrestin 2 KO mice suggests a physical and functional association of exon 7-associated C-terminal tails with β-arrestin 2, a hypothesis further supported by our in vitro data showing that several mu agonists displayed greater β-arrestin bias against exon 7-associated variants than against the exon 4-associated mOR-1.
Morphine (M) tolerance was induced in mE7M-B6 and mE7M-129 mouse models by the same paradigms used in Figure 1A. A control group injected with saline (S) was also included for both WT and homozygous mice. [35S]Morphine (M) tolerance was induced in mE7M-B6 and mE7M-129 mouse models by the same paradigms used in our mE3M mice (53). As with our mice, the DMOR animals developed tolerance to morphine more rapidly than WT controls.

The role of the exon 7–associated C-terminal truncation in morphine tolerance was opposite that of the exon 4–encoded C-terminal truncation. Loss of the exon 7 sequence attenuated morphine tolerance in the mutated animals. Furthermore, this association of the exon 7–associated variants with morphine tolerance was further supported by the reduction of morphine tolerance by the downregulation of exon 7–associated variants by an ANT-vMO. Thus, the exon 7–associated and exon 4–encoded C-terminal tails appear to have opposing functions, with the exon 7–associated tails facilitating tolerance and the exon 4–encoded tails diminishing it.

Receptor desensitization in specific brain regions, including brain stem nuclei, has been previously observed autoradiographically using DAMGO-stimulated [35S]GTPγS in chronic morphine tolerance mouse and rat models (54, 55), with similar results in membrane-binding studies from morphine-tolerant mouse brain stem (56). In our study, morphine-tolerant control WT mE7M-B6 mice exhibited reduced morphine-stimulated G protein coupling in the brain stem following chronic morphine treatment, consistent with previous observations. Our study also revealed receptor desensitization in the hypothalamus. Mu opioid receptor agonist–induced desensitization involves many proteins in various cellular processes, such as receptor phosphorylation, β-arrestin 2 binding, and internalization (42). These studies raised questions regarding regional expression and function of these proteins as well as regional processing of these cellular events. The prevention of receptor desensitization in both the brain stem and hypothalamus by the exon 7–associated C-terminal truncation in B6 mice suggests a contribution of the exon 7–associated C-terminal variants to morphine-induced receptor desensitization and tolerance. Also, the WT control mE7M-129 mice failed to show the effects to this enhanced sensitivity to the production of tolerance. This possibility is further supported by a mutant DMOR mouse model in which a portion of exon 3–encoded sequences in mMOR-1 was replaced by a portion of the C terminus of the δ opioid receptor mDOR-1 with the elimination of all the C-terminal downstream from exon 3, similarly to our mE3M mice (53).

Table 5. Morphine-stimulated [35S]GTPγS binding in membranes from brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Mouse</th>
<th>mE7M-B6 % Maximum stimulation</th>
<th>EC50 (μM)</th>
<th>mE7M-129 % Maximum stimulation</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>WT-Saline</td>
<td>38 ± 2</td>
<td>0.3 ± 0</td>
<td>41 ± 3</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td></td>
<td>WT-morphine</td>
<td>30 ± 5</td>
<td>0.4 ± 0.1</td>
<td>39 ± 3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mut-Saline</td>
<td>49 ± 4^1</td>
<td>0.3 ± 0.1</td>
<td>41 ± 2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Mut-morphine</td>
<td>37 ± 4</td>
<td>0.3 ± 0</td>
<td>43 ± 2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>WT-Saline</td>
<td>77 ± 2</td>
<td>0.5 ± 0.0</td>
<td>95 ± 5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>WT-morphine</td>
<td>77 ± 5</td>
<td>0.7 ± 0.2</td>
<td>95 ± 11</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Mut-Saline</td>
<td>73 ± 7</td>
<td>0.9 ± 0.3</td>
<td>95 ± 16</td>
<td>0.3 ± 0.1</td>
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<tr>
<td></td>
<td>Mut-morphine</td>
<td>83 ± 10</td>
<td>0.5 ± 0.2</td>
<td>86 ± 6</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Thalamus</td>
<td>WT-Saline</td>
<td>61 ± 5</td>
<td>0.2 ± 0.1</td>
<td>66 ± 8</td>
<td>0.2 ± 0.0</td>
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<tr>
<td></td>
<td>WT-morphine</td>
<td>49 ± 2</td>
<td>0.3 ± 0.1</td>
<td>76 ± 5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mut-Saline</td>
<td>53 ± 3</td>
<td>0.2 ± 0.1</td>
<td>76 ± 9</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mut-morphine</td>
<td>57 ± 7</td>
<td>0.2 ± 0.1</td>
<td>79 ± 3</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>WT-Saline</td>
<td>61 ± 2^1</td>
<td>0.3 ± 0.0</td>
<td>86 ± 8</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>WT-morphine</td>
<td>43 ± 5</td>
<td>0.2 ± 0.1</td>
<td>104 ± 13</td>
<td>0.2 ± 0.0</td>
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<td></td>
<td>Mut-Saline</td>
<td>62 ± 3^1</td>
<td>0.2 ± 0.0</td>
<td>106 ± 13^1</td>
<td>0.2 ± 0.1</td>
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<td>Mut-morphine</td>
<td>72 ± 1^1</td>
<td>0.2 ± 0.0</td>
<td>96 ± 8</td>
<td>0.2 ± 0.1</td>
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<td>PAG</td>
<td>WT-Saline</td>
<td>42 ± 3</td>
<td>0.5 ± 0.2</td>
<td>31 ± 4</td>
<td>0.3 ± 0.0</td>
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<tr>
<td></td>
<td>WT-morphine</td>
<td>49 ± 3</td>
<td>0.5 ± 0.1</td>
<td>33 ± 8</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Mut-Saline</td>
<td>59 ± 4^1</td>
<td>0.3 ± 0.1</td>
<td>33 ± 7</td>
<td>0.2 ± 0.1</td>
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<tr>
<td></td>
<td>Mut-morphine</td>
<td>54 ± 1</td>
<td>0.4 ± 0.2</td>
<td>24 ± 4</td>
<td>0.6 ± 0.2^2</td>
</tr>
<tr>
<td>Brainstem</td>
<td>WT-Saline</td>
<td>50 ± 3^1</td>
<td>0.4 ± 0.2</td>
<td>45 ± 2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>WT-morphine</td>
<td>31 ± 7</td>
<td>0.3 ± 0.1</td>
<td>62 ± 2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Mut-Saline</td>
<td>50 ± 6^1</td>
<td>0.3 ± 0.1</td>
<td>71 ± 7^1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mut-morphine</td>
<td>48 ± 1^1</td>
<td>0.3 ± 0.1</td>
<td>71 ± 7^1</td>
<td>0.2 ± 0.0</td>
</tr>
</tbody>
</table>

Distal C-terminal sequences markedly affected morphine tolerance, with opposite effects seen between exon 4– and exon 7–associated C-terminal truncation models. This was particularly interesting in view of the lack of change in morphine analgesia among the truncation models, despite receptor binding decreases of 30% to 50% in mE4M and mE3M models. Maintaining analgesic activity with reduced receptor expression has been reported previously in mu receptor KO models in which loss of half the receptor in the heterozygotes failed to change morphine’s analgesic ED50 values (33, 34). Removal of the exon 4–encoded 12 aa in mMOR-1, the predominant 7TM variant, facilitated morphine tolerance in mE4M homozygous mice on both B6 and 129 backgrounds, implying that expression of the exon 4–encoded sequence impedes the development of morphine tolerance. It is interesting to consider whether the loss of the MRS sequence and its ability to recycle mMOR-1 contrib-
receptor desensitization in any of the brain regions, including the brain stem and hypothalamus, consistent with the lack of tolerance in these animals in this tolerance paradigm.

Intriguingly, several morphine-induced responses in a β-arrestin 2 KO mouse were similar to those in a mE7M-B6 homozygous mouse (Table 6), including reduced morphine tolerance (56, 57), loss of the receptor desensitization in the brain stem (56), no alteration of physical dependence (56, 58) and GI transit inhibition in the small intestine (59), and reduced locomotor activity (60). However, β-arrestin 2 KO mice also showed some differences from mE7M-B6 homozygous mice, such as enhanced morphine analgesia (61) and CPP (ref. 60 and Table 6). Nevertheless, these similar responses in both models are consistent with a physical and functional interaction of the exon 7–associated C-terminal tails with β-arrestin in producing morphine-induced receptor desensitization in specific regions and morphine tolerance. Our in vitro observations of greater β-arrestin 2 bias with exon 7–associated variants, particularly mMOR-1O, further support this possibility.

Mu agonist-induced receptor phosphorylation and subsequent β-arrestin binding have been postulated as one mechanism of receptor desensitization and mu opioid tolerance (42–44). For example, serine 375 (S375) of MOR-1 in exon 3–encoded C-terminal sequences shared by all C-terminal variants can be phosphorylated mainly by GRK5 (62), contributing to β-arrestin recruitment (63). A S375A mutant mouse displayed reduced tolerance to fentanyl, but not to morphine (64). Phosphorylation of threonine 394 (T394) in the exon 4–encoded C-terminal sequence was involved in mu agonist–induced receptor internalization and desensitization (65, 66). A T394A mutant mouse showed attenuated acute morphine tolerance and increased heroin-taking behavior (67).

β-arrestin 2 phosphorylation is also involved in the post-receptor signaling of β-arrestin 2, such as GPCR kinases (GRKs), are predicted in exon 7–associated C-terminal tails (Supplemental Figure 11 and ref. 68), raising the possibility of enhanced phosphorylation and increased β-arrestin recruitment in these variants. The exon 7–encoded C-terminal sequences also contain a consensus β-arrestin 2 binding motif, PXpXXE or PXXpXXE (Supplemental Figure 11), that interacts with positively charged residues at the N terminus of β-arrestin 2, based on homology modeling with the recent crystal structure of the rhodopsin-arrestin complex as a template (ref. 69 and H. Eric Xu, personal communication). Although only a prediction, it will be interesting to see how this motif influences β-arrestin 2 binding and receptor signaling as well as morphine actions in vivo.

The exon 7 truncation also selectively and markedly reduced morphine CPP in the B6 mice, while the other 2 truncation models did not. Conversely, the exon 7–associated C-terminal tails did not affect naloxone-precipitated jumping in B6 mice, while the other 2 B6 truncation models did. However, some behaviors were similarly altered by the truncations, such as the lowered locomotor activity in B6 mice. Together, the divergent effects seen in these mutant mice among various morphine actions strongly support the functional significance of 3′ alternative splicing of the Oprml gene and illustrate selective roles for specific C-terminal tails in various actions.

While the models dissociate the actions of different groups of C-terminal splice variants, assigning a specific variant is not possible due to concurrent 5′ alternative splicing, which yields multiple variants with identical C-terminal sequences (Supplemental Figure 1 and Supplemental Figure 2B). For example, the exon 4 epitope is contained within a number of 7TM variants (mMOR-1, mMOR-1H, mMOR-1i, and mMOR-1J) as well as a 6TM variant (mMOR-1G). The exon 7–encoded sequence is present in 7TM variants (mMOR-1G, mMOR-1O, and mMOR-1U) as well as the 6TM variant mMOR-1M. While the far greater affinity of morphine for 7TM receptors in binding assay and the retention of morphine analgesia in an E11-KO mouse lacking 6TM variants (34, 70–73) implies that morphine is more likely to interact with 7TM receptors, our recent studies revealed a decrease in mor-
C-terminal splice variants are not limited to the OPRM1 gene. Genomic database analysis suggests that as many as 12% of human GPCR genes (excluding olfactory receptor genes) generate alternatively spliced intracellular C-terminal tail variants (our unpublished observation). However, the expression and function of the majority of these C-terminal variants remain unknown. Our studies suggest that the different C termini generated from 3′ alternative splicing of the mu opioid receptor gene are pharmacologically relevant, raising the more general question of whether 3′ alternative splicing may expand the pharmacological repertoire of GPCRs in general.

Methods

Detailed experimental procedures for generation of gene-targeted mutant mice, RT-qPCR, opioid receptor binding, [35S]GTPγS binding, receptor desensitization study, Western blot, locomotor activity, GI motility assay, and catalepsy are described in the Supplemental Methods.
Table 6. Comparison of behavioral and biochemical studies in targeted mice

| Strain/morphine         | mE3M Truncation of all C termini | mE4M Truncation of exon 4–encoded C termini | mE7M Truncation of exon 7–encoded C termini | β-arrestin 2 KO
|-------------------------|----------------------------------|-------------------------------------------|-------------------------------------------|----------------
| Analgesia               | 129                              | 129                                       | 129                                       | Enhanced (61)
| Locomotor activity      | No change                        | No change                                | No change                                | Reduced (60)
| Tolerance               | No change                        | Reduced                                  | No change                                | Reduced (60)
| Physical dependence     | Reduced                          | Reduced                                  | Reduced                                  | Reduced (60)
| Reward (CPP)            | No change                        | No change                                | No change                                | Reduced (60)
| Inhibition of GI transit| No change                        | Reduced                                  | No change                                | Reduced (60)
| Catalysis               | No change                        | Reduced                                  | No change                                | Reduced (60)
| Receptor desensitization in hypothalamus | No change | Lost                                    | No change                                | Reduced (60)
| Receptor desensitization in brain stem | No change | Lost                                    | No change                                | Reduced (60)

A all changes are compared with respective WT controls. B Data from β-arrestin 2 KO mouse model were from indicated literature. C Similar results seen in mE7M-B6 homozygous mice. D Divergent results from mE7M-B6 homozygous mice. E The physical dependence data in ref. 56 were determined using morphine pellets. There was no change in the physical dependence in β-arrestin 2 KO mice. In ref. 58, when administered using osmotic pump, 2 of 3 doses (12 and 48 mg/kg/d) of morphine did not induce significant reduction of naloxone-precipitated withdrawal (jumps and global score), but 1 dose (24 mg/kg/d) did, in β-arrestin 2 KO mice. F Morphine-induced receptor desensitization was assessed by morphine-stimulated (mE7M-B6) or DAMGO-stimulated (β-arrestin 2 KO) [35S]GTPγS binding (56).
PathHunter detection reagents for 60 minutes. Chemiluminescence was measured with an Infinite M1000 Pro plate Reader (Tecan).

Statistics. All mice were randomized and assigned to groups. Some, but not all, experiments were performed under blinded conditions. All statistical analysis was carried out using GraphPad Prism 6. A 1-way ANOVA or 2-way ANOVA was performed with post hoc Bonferroni’s multiple comparisons test as described in the figure legends. Data represented the mean ± SEM of at least 3 independent experiments. Statistical significance was set at P < 0.05.

Study approval. All animal studies were approved by the IACUC of Memorial Sloan Kettering Cancer Center.

Author contributions. LC, AM Rajadhyaksha, GWP, and YXP designed experiments. JX, ZL, VPLR, AN, AH, MX, TGB, WFH, GCR, RCR, A Martínez-Rivera, DLB, and YXP performed experiments. LC, AM Rajadhyaksha, DLY, and YXP contributed new reagents/analytic tools. LX, ZL, VPLR, AN, AH, TGB, WFH, GCR, RCR, A Martínez-Rivera, AM Rajadhyaksha, and YXP analyzed data. JX, ZL, AN, GWP, and YXP wrote the manuscript.

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The Journal of Clinical Investigation

for common genetic mechanisms in acute and chronic morphine physical dependence. Neuro-
science. 2002;115(2):463–469.


33. Hall FS, et al. Congenic C57BL/6 μ opioid receptor (MOR) knockout mice: baseline and opio-

34. Schuller AG, et al. Retention of heroin and mor-


37. Murphy NP, Lam HA, Maidment NT. A comparison of morphine-induced locomotor activity and meso-

38. Ceese I, Snyder SH. Receptor binding and pharma-
cological activity of opiates in the guinea-pig intes-

39. Rossi GC, Pan YX, Brown GP, Pasternak GW. Antisense mapping the MOR-1 opioid receptor: evidence for alternative splicing and a novel morph-

40. VanderWende C, Spoelner LT. Morphine-
induced catalepsy in mice. Modification by drugs acting on neurotransmitter systems. Neurophar-

41. Zarrindast MR, Samadi P, Haeri-Rohani A, Moa-


43. Raehal KM, Walker JK, Bohn LM. Differential mechanisms of morphine antinociceptive toler-

44. Bohn LM, Gainetdinov RR, Lin FT, LeKowitz RJ, Caron MG. Mu-opioid receptor desensiti-

45. Sim LJ, Suter PY, Bartlett S, Ferwerda M, Whis-
le JR. A novel knock-in mouse reveals mecha-
nistic distinction of mu-opioid receptor-mediated G protein activation in mouse CNS as a func-


52. Bengtsson J, et al. Role for the C-terminus of agonist-induced mu opioid receptor phospho-

53. Gokalp U, et al. Replacement of threonine 394 by alanine facilitates internalization and resensitiza-


55. Baptista S, et al. Morpholino oligomers: a comprehensive tool for phospho-


57. Bohn LM, et al. Differential mechanisms of morphine antinociceptive tolerance and physical dependence induced by different opioid pain ther-


60. Bohn LM, LeKowitz RJ, Gainetdinov RR, Pep-

61. Mann A, Illing S, Meiss E, Schulz S. Differential mechanisms of homologous and heterologous µ opioid receptor phosphorylation. Br J Pharma-

62. Lau EK, et al. Quantitative encoding of the effect of a partial agonist on individual opioid recep-


64. Bengtsson J, et al. Role for the C-terminus of agonist-induced mu opioid receptor phos-

65. Wolf R, et al. Replacement of threonine 394 by alanine facilitates internalization and resensitiza-