JCI The Journal of Clinical Investigation

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J Clin Invest. 2015;125(7):2626-2630. https://doi.org/10.1172/JCI81070.

Brief Report **Genetics** **Neuroscience** **Therapeutics**

The generation of potent opioid analgesics that lack the side effects of traditional opioids may be possible by targeting truncated splice variants of the μ-opioid receptor. μ-Opioids act through GPCRs that are generated from the *Oprm1* gene, which undergoes extensive alternative splicing. The most abundant set of *Oprm1* variants encode classical full-length 7 transmembrane domain (7TM) μ-opioid receptors that mediate the actions of the traditional μ-opioid drugs morphine and methadone. In contrast, 3-iodobenzoyl-6β-naltrexamide (IBNtxA) is a potent analgesic against thermal, inflammatory, and neuropathic pain that acts independently of 7TM μ-opioid receptors but has no activity in mice lacking a set of 6TM truncated μ-opioid receptor splice variants. Unlike traditional opioids, IBNtxA does not depress respiration or result in physical dependence or reward behavior, suggesting it acts through an alternative μ-opioid receptor target. Here we demonstrated that a truncated 6TM splice variant, mMOR-1G, can rescue IBNtxA analgesia in a μ-opioid receptor–deficient mouse that lacks all *Oprm1* splice variants, ablating μ-opioid activity in these animals. Intrathecal administration of lentivirus containing the 6TM variant mMOR-1G restored IBNtxA, but not morphine, analgesia in *Oprm1*-deficient animals. Together, these results confirm that a truncated 6TM GPCR is both necessary and sufficient for IBNtxA analgesia.

Mediation of opioid analgesia by a truncated 6-transmembrane GPCR

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Introduction

Traditional opioids are effective against many types of severe pain, but side effects are problematic and opioid efficacy against neuropathic pain is limited. Using biased agonism to develop drugs lacking side effects is one approach to address these limitations (1, 2). An alternative is to pursue novel targets. Recently, a new class of opioid analgesics was described with an unusual pharmacological profile (3–6). IBNtxA (3-iodobenzoyl-6β-naltrexamide), a member of this group, is a potent analgesic against thermal, inflammatory and neuropathic pain, but does not depress respiration or produce physical dependence and shows no reward behavior.

These drugs, exemplified by IBNtxA (4), act through mechanisms distinct from traditional μ drugs. The single-copy μ-opioid receptor *Oprm1* creates an array of splice variants through alternative pre-mRNA splicing with patterns conserved from rodents to humans (refs. 7, 8, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI81070DS1). The major set of variants are full-length 7 transmembrane domain (7TM) variants associated with exon 1. A second set of variants is characterized by the replacement of the 94 amino acids encoded by exon 1 with 27 amino acids encoded by exon 11 (Supplemental Figure 1). Unlike exon 1, exon 11 lacks a transmembrane domain, resulting in a truncated form of the receptor with only 6TMs. Disruption of exon 1 of the μ-opioid receptor MOR-1 (exon 1 KO) eliminates all full-length 7TM variants and all morphine activity (9). The 6TM variants are still expressed in this mouse, and IBNtxA retains full analgesic activity (4). Conversely, IBNtxA is inactive in an exon 11 KO mouse lacking the truncated 6TM splice variants (4) while morphine retains full analgesic activity (10). Furthermore, IBNtxA labels a binding site in the brain that is lost in exon 11 KO mice and is pharmacologically distinct from the classical opioid receptors.

The 6TM variants were an unanticipated drug target, since they do not conform to the traditional structure of GPCRs. Truncated forms have been reported for over 20 GPCRs, with most acting as dominant-negative mutations (11). In addition to MOR-1, α_{1A} adrenergic, calcitonin, histamine H_3, and prostaglandin F_{2A} receptors generate 6TM variants. Unlike the others, the 6TM MOR-1 variants are functionally active. They provide a target for drug development, with the possibility of potent analgesics lacking many of the problems of current medications. We now demonstrate that restoration of a truncated 6TM splice variant through a lentivirus infection in a KO mouse lacking all MOR-1 splice variants can rescue IBNtxA analgesia, confirming that truncated GPCRs are both necessary and sufficient for IBNtxA analgesia.

▶ Related Commentary: p. 2558

Results and Discussion

The μ-opioid receptor gene *Oprm1* contains 2 independent promoters associated with either exon 1 or exon 11, which is located approximately 30 kb upstream of exon 1 (Supplemental Figure 1 and refs. 7, 8). A number of μ-receptor KO mice eliminate morphine actions by targeting different regions of the gene (9, 12–15), but individual mouse models may not completely eliminate all *Oprm1* transcripts. Examples exist in which disruption of exon 1 does not impair the expression of the exon 11 variants (9) and in which elimination of exon 11 does not impact the expres-

Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: January 23, 2015; **Accepted:** April 9, 2015.
Reference information: *J Clin Invest*. 2015;125(7):2626–2630. doi:10.1172/JCI81070.

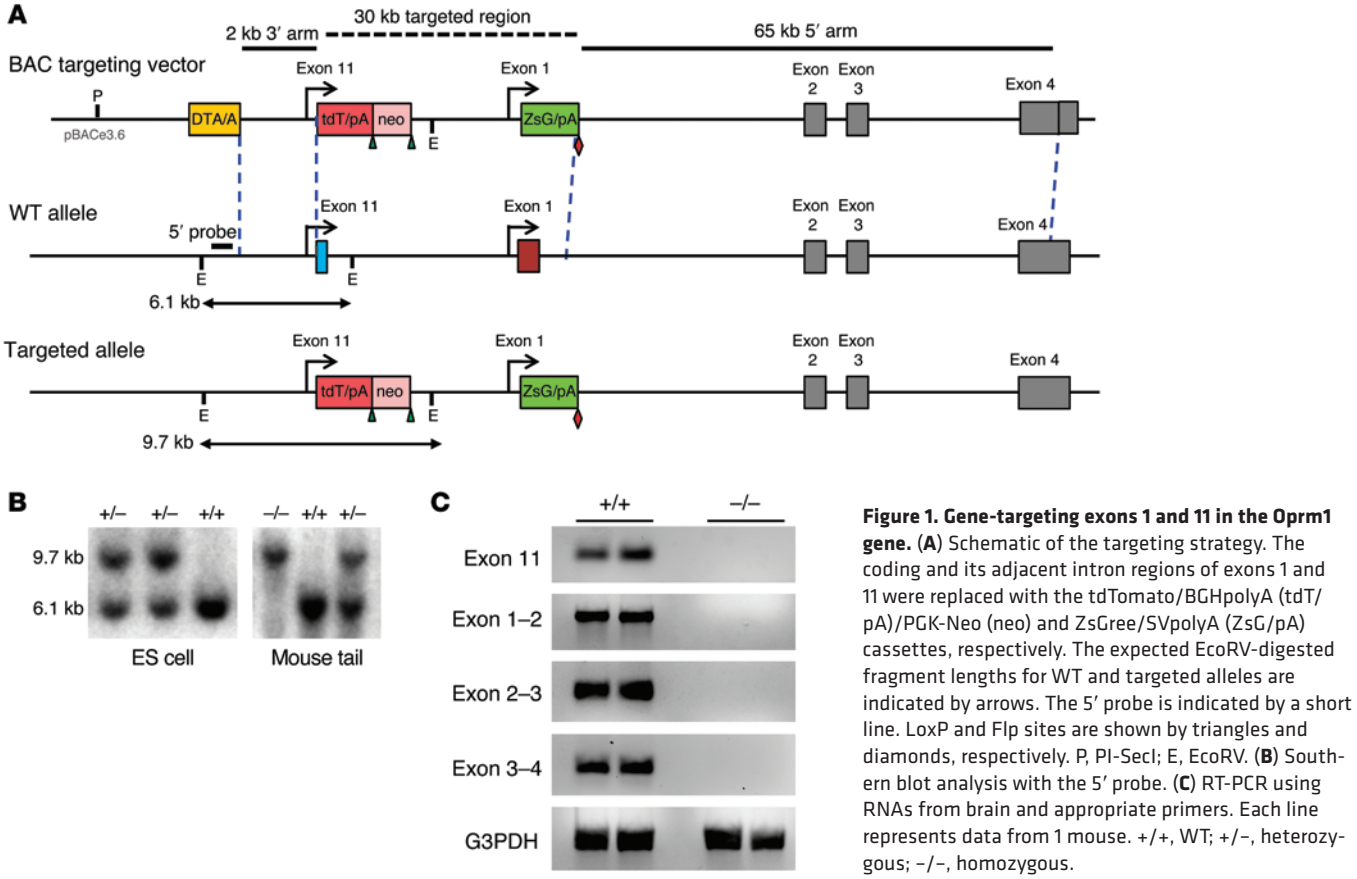

Figure 1. Gene-targeting exons 1 and 11 in the Oprm1 gene. (**A**) Schematic of the targeting strategy. The coding and its adjacent intron regions of exons 1 and 11 were replaced with the tdTomato/BGHpolyA (tdT/pA)/PGK-Neo (neo) and ZsGree/SVpolyA (ZsG/pA) cassettes, respectively. The expected EcoRV-digested fragment lengths for WT and targeted alleles are indicated by arrows. The 5′ probe is indicated by a short line. LoxP and Flp sites are shown by triangles and diamonds, respectively. P, PI-SecI; E, EcoRV. (**B**) Southern blot analysis with the 5′ probe. (**C**) RT-PCR using RNAs from brain and appropriate primers. Each line represents data from 1 mouse. +/+, WT; +/–, heterozygous; –/–, homozygous.

sion of exon 1 variants (4, 10). We therefore generated a complete MOR-1 KO mouse (exon 1/exon 11 KO) lacking all *Oprm1* splice variants by targeting both exon 1 and exon 11 coding and adjacent intron regions (Figure 1, A and B), providing a valuable model to assess the functional role of individual splice variants. Homozygous mice were produced through heterozygous mating, yielding 27.6% WT, 49.4% heterozygous, and 23.0% homozygous KO mice (Figure 1B, n = 87), consistent with the lack of embryonic or postnatal lethality. Homozygous mice were healthy and fertile, with no obvious morphological abnormalities. Reverse transcriptase PCR (RT-PCR) confirmed the absence of both exon 1– and exon 11–containing transcripts in the homozygous mice (Figure 1C). No μ opioids tested, including IBNtxA, were active in the exon 1/exon 11 KO mice, even at high doses ($P < 0.001$; Table 1). None of the mice showed other typical opioid behaviors, such as hyperlocomotion or Straub tail.

Loss of IBNtxA actions in exon 11 KO mice implicated 6TM variants in its actions. To determine if a truncated 6TM exon 11 MOR-1 variant could restore IBNtxA analgesia in the exon 1/exon 11 KO mice, we generated a lentivirus expressing the 6TM variant mMOR-1G, the predominant 6TM variant in the spinal cord (16), along with a coexpressed EGFP marker (Supplemental Figure 2). A lentivirus expressing the EGFP marker but lacking the mMOR-1G variant served as a control. Since IBNtxA analgesia is mediated predominantly at the spinal cord, the virus was administered intrathecally on days 1, 3, and 5 (Supplemental Figure 3A).

Viral expression was seen as early as 1 week; it gradually increased over 4 weeks and then remained stable for at least 14

Table 1. Opioid analgesia in WT and exon 1/exon 11 KO mice

	Drug dose		Analgesia (% MPE)	
	mg/kg, s.c.	Relative to ED_{50}	WT (n)	Exon 1/exon 11 KO (n)
Morphine	100	>50-fold	100 ± 0 (6)[A]	–3.3 ± 1.6[A] (6)
M6G	50	>50-fold	100 ± 0 (7)	1.6 ± 2.2[A] (7)
Fentanyl	0.08	>4-fold	95 ± 5 (7)	0.5 ± 1.9[A] (13)
	0.2	>8-fold	100 ± 0 (6)	–3.5 ± 2.1[A] (8)
Heroin	50	>50-fold	100 ± 0 (6)	1.2 ± 2.1[A] (6)
Buprenorphine	1	>30-fold	89 ± 8 (9)	1.5 ± 1.9[A] (8)
Ketocyclazocine	2	>10-fold	67 ± 16 (5)	11 ± 3[A] (13)
Methadone	50	>50-fold	93 ± 7 (6)	4.2 ± 2.2[A] (5)
IBNtxA	10	>20-fold	100 ± 0 (8)	1.6 ± 2.9[A] (10)

[A]$P < 0.0001$. Groups of WT and exon 1/exon 11 KO mice received the indicated dose of opioid, and analgesia was assessed at peak effect. To obtain the dose relative to the ED_{50}, the dose given was compared the ED_{50} in WT mice (4, 10). All responses in the exon 1/exon 11 KO mice were significantly different from their respective WT controls ($P < 0.0001$; ANOVA and Tukey). None of the opioids displayed significant analgesia in the KO mice, compared with their individual baseline latencies.

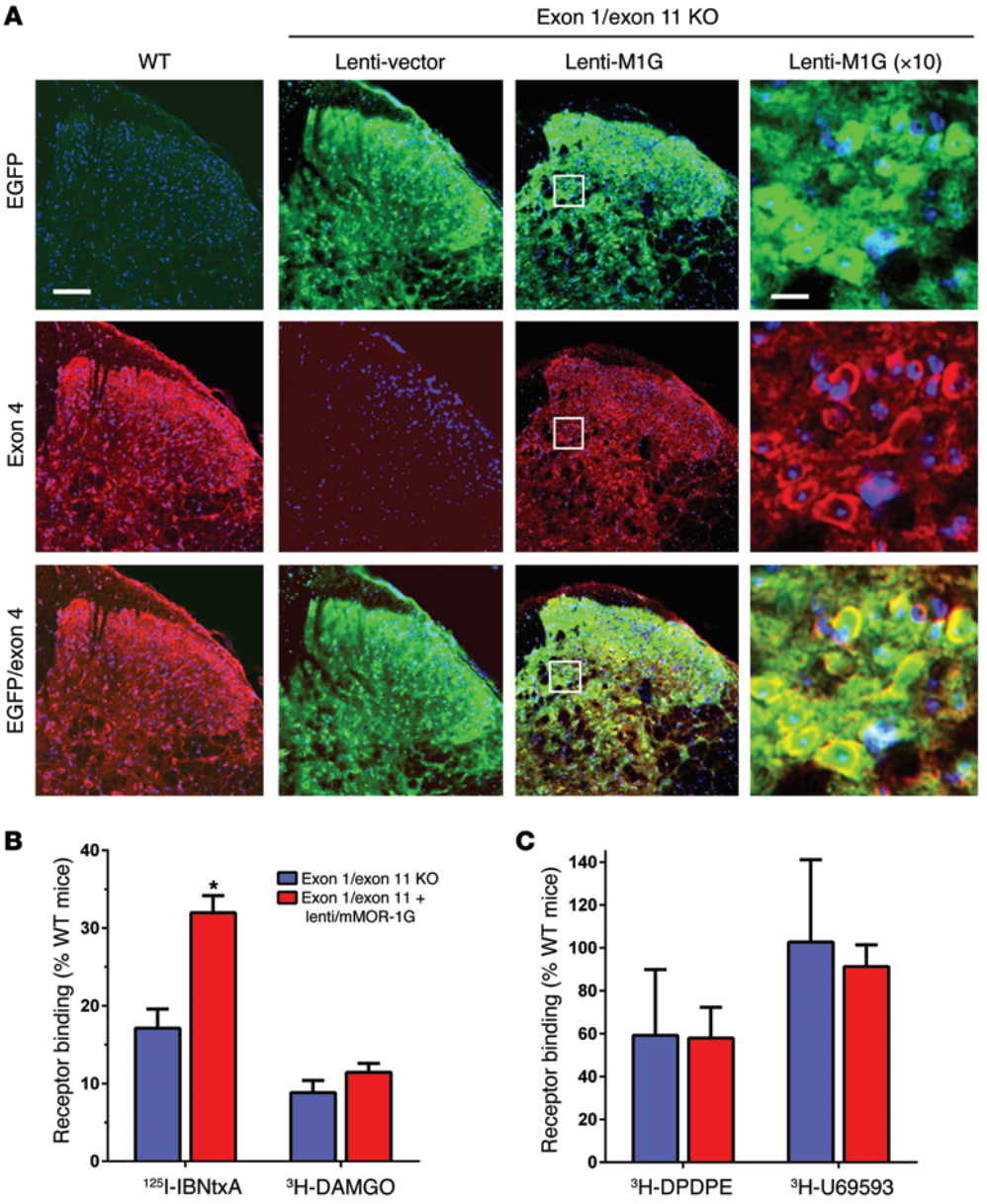

Figure 2. Lentivirus expression in the spinal cord following intrathecal administration. (**A**) Distribution of lentiviral-expressed mMOR-1G (lenti–mMOR-1G) and EGFP. An exon 4 antibody was used for staining mMOR-1G. Nuclei were stained with DAPI. Scale bars: 100 μm in first three columns and 10 μm in last column. (**B** and **C**) Receptor binding in the spinal cord of exon 1/exon 11 KO mice without and following lenti–mMOR-1G (percent of WT; mean ± SEM from 3–4 pooled samples. Each pool contains the spinal cord from 2–3 mice). Significant differences between WT, exon 1/exon 11 KO, and lenti–mMOR-1G groups were seen with ANOVA for ^{125}I-IBNtxA and ^{3}H-DAMGO (*$P < 0.001$) (**A**) but not ^{3}H-DPDPE and ^{3}H-U69,593 (**B**). For ^{125}I-IBNtxA, the exon 1/exon 11 KO and the lenti–mMOR-1G groups differed from WT ($P < 0.001$) and from each other ($P < 0.05$; Tukey). For ^{3}H-DAMGO, the exon 1/exon 11 KO and the lenti–mMOR-1G groups differed from WT ($P < 0.001$) but not from each other ($P > 0.05$).

weeks (Supplemental Figure 3B). EGFP labeling was highest in the lumbar/sacral levels, near the site of injection; was colocalized with the neuronal nuclear marker NeuN; and was prominent in the dorsal horns, a region important in pain modulation (Supplemental Figure 3C). WT mice that did not receive virus showed no EGFP staining but showed exon 4 antibody labeling in the superficial laminae of the dorsal horn, reflecting the endogenous expression of a number of exon 4–containing variants (Figure 2A, first column, and Supplemental Figure 1) Administration of the control lentivirus expressing only EGFP (i.e., no mMOR-1G) to exon 1/exon 11 KO mice revealed robust EGFP staining, but no exon 4 staining (Figure 2A, second column), confirming the absence of those variants. Both antibodies revealed staining following the lentivirus expressing both EGFP and mMOR-1G, which indicated the coexpression of both proteins in lentiviral-transduced cells (Figure 2A, third column). Higher power showed EGFP labeling throughout the cell, whereas exon 4 staining was localized to the surface (Figure 2A, fourth column), suggesting cell surface expression of the mMOR-1G.

Lentivirus infection restored ^{125}I-IBNtxA binding to exon 11 targets. In WT mice, ^{125}I-IBNtxA labels traditional μ binding sites, as well as the exon 11 target. This contrasts with ^{3}H-DAMGO ([D-Ala^{2},MePhe^{4},Gly(ol)^{5}]enkephalin), which only labels traditional μ sites. In the spinal cord of the exon 1/exon 11 KO mice, ^{125}I-IBNtxA binding was decreased by more than 80% and ^{3}H-DAMGO by more than 90%, compared with WT controls (Figure 2B). These decreases reflect the loss of traditional full-length 7TM μ receptors as well as exon 11–associated targets; the residual ^{125}I-IBNtxA binding likely reflects cross-labeling of δ and/or κ sites. Administration of the lentivirus with the mMOR-1G insert significantly increased ^{125}I-IBNtxA binding but not ^{3}H-DAMGO binding (Figure 2B). Similarly, neither the δ ligand ^{3}H-DPDPE nor the κ_1 ligand ^{3}H-U69,593 showed an increase in binding following virus administration (Figure 2C). The absence of any increase in traditional μ, δ, or κ_1 binding (Figure 2B)

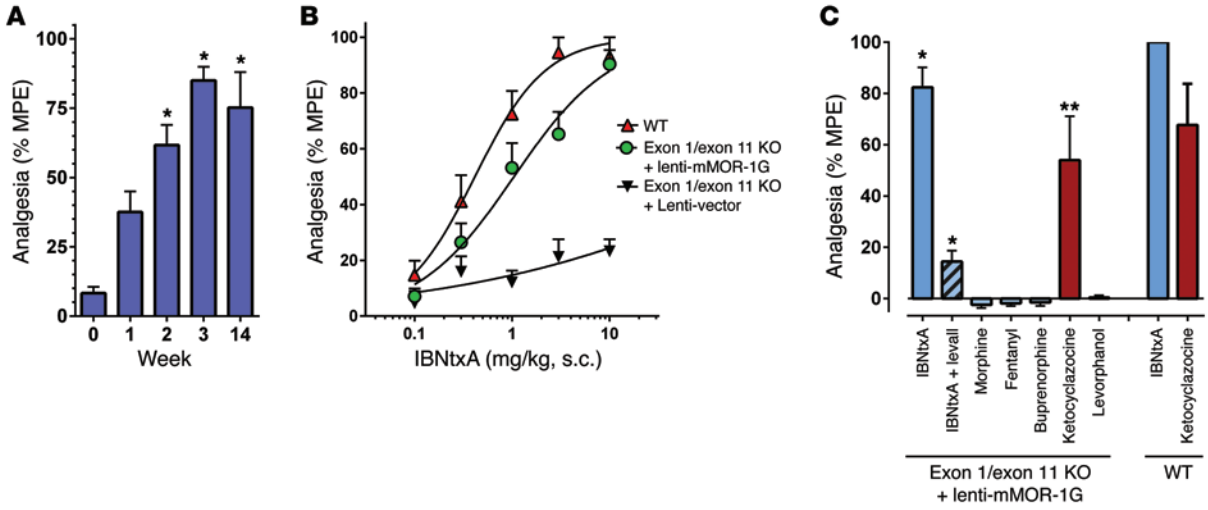

Figure 3. Lentivirus rescue of IBNtxA analgesia. (**A**) Opioid analgesia. Analgesia was determined in groups of mice (n = 6–13) at the stated time. (*P < 0.0001 compared with week 0; ANOVA followed by Tukey). (**B**) IBNtxA cumulative dose–response curves were carried out in exon 1/exon 11 KO mice with lentivirus vector alone (n = 4), lenti–mMOR-1G (ED_{50} 1.1 mg/kg [95% CI, 0.72–1.53], n = 18) and WT mice (ED_{50} 0.42 mg/kg [95% CI, 0.29–0.58], n = 16). (**C**) Single doses of IBNtxA (2.5 mg/kg, s.c. n = 5), morphine (10 mg/kg, s.c. n = 7), fentanyl (0.08 mg/kg, s.c. n = 7), buprenorphine (1 mg/kg, s.c. n = 7), ketocyclazocine (2 mg/kg, s.c. n = 7), or levorphanol (0.8 mg/kg, s.c. n = 7) were administered to groups of either WT or exon 1/exon 11 KO mice infected with lenti–mMOR-1G. The mice were assessed for analgesia. Another group of mice received IBNtxA with levallorphan (2.5 mg/kg, s.c. n = 7). ANOVA shows that the IBNtxA group and the ketocyclazocine group were significantly different (*P < 0.001 and **P < 0.006 by ANOVA, respectively). The IBNtxA WT and lenti–mMOR-1G animals were not significantly different from each other but were different from both the exon 1/exon 11 KO alone and the lenti–mMOR-1G/levallorphan groups (Tukey). Ketocyclazocine in WT and lenti–mMOR-1G were not significantly different from each other but were different from exon 1/exon 11 alone (Tukey).

indicated that expression of mMOR-1G restored ^{125}I-IBNtxA binding to an exon 11–associated target.

The expressed mMOR-1G was functionally active. IBNtxA analgesia returned as early as 1 week following lentivirus infection, gradually increasing until it reached a peak response after 3 weeks (Figure 3A) and remained constant for at least 14 weeks, a time course similar to that of EGFP expression (Supplemental Figure 3B). Mice infected with a control lentivirus that did not contain mMOR-1G failed to display an analgesic response (Figure 3B). IBNtxA was fully efficacious in the mice infected with the lentivirus containing mMOR-1G with an ED_{50} (1.1 mg/kg, s.c.) similar to that of WT mice (0.42 mg/kg, s.c.; Figure 3B).

IBNtxA analgesia was readily reversed by levallorphan, confirming an opioid mechanism (Figure 3C). Ketocyclazocine is a nonselective opioid that produces an exon 11–dependent analgesia (4) that was lost in the exon 1/exon 11 KO (Table 1). As with IBNtxA, ketocyclazocine analgesia returned in the lentivirus-infected mice, with a response similar to that seen in WT mice (Figure 3C). Although expression of mMOR-1G rescued IBNtxA and ketocyclazocine analgesia, it failed to rescue high doses of the other opiates. Morphine analgesia is independent of exon 11 variants (4), so this result was anticipated. Although the diminished activity of the other drugs in exon 11 KO mice in earlier studies implicated exon 11 variants in their actions, the inability to rescue their response in the exon 1/exon 11 KO indicated that a 6TM variant was necessary but not sufficient. Other factors may be involved.

The current studies describe a new exon 1/exon 11 KO mouse with disruptions of both exon 1 and exon 11, yielding a full μ receptor–null mouse lacking all *Oprm1* splice variants (Supplemental Figure 1) that is insensitive to μ opioids. The absence of all the variants provides an excellent model to assess the functional significance of individual variants. The 6TM variant mMOR-1G restored both IBNtxA and ketocyclazocine analgesia in the exon 1/exon 11 KO mouse, indicating that a 6TM variant is both necessary and sufficient for their actions. The failure of mMOR-1G to restore morphine analgesia is consistent with a mechanism independent of exon 11 variants. The lack of rescue of the others has several potential explanations. Since the mouse generates 5 different 6TM variants, one of the other 6TM variants may be needed. The spinal injection site may be important, since the other drugs may act supraspinally. Finally, some drugs may require expression of both 6TM and 7TM MOR-1 variants for activity. For example, buprenorphine analgesia is lost in the exon 11 KO mouse (4) and is also markedly reduced in an exon 1 KO mouse lacking only 7TM variants, implying a need for both sets of variants.

The 6TM MOR-1 splice variant mMOR-1G is both necessary and sufficient for IBNtxA analgesia. With its broad analgesic activity in thermal, inflammatory, and neuropathic pain models (5), as well as its lack of respiratory depression, physical dependence, and reward, IBNtxA may represent a new class of analgesic distinct from those associated with traditional opiates and 6TM variants may prove valuable targets for future analgesic drug development. These results also raise the broader question of the significance of truncated GPCRs (8, 11, 17).

Methods

Detailed information is described in the Supplemental Methods.

Exon 1/exon 11 KO mice. KO mice with disruptions of exons 1 and 11 were generated using a targeting vector in W4 ES cells (a gift from

Alexandra Joyner, Memorial Sloan-Kettering Cancer Center, New York, NY, USA; Figure 1 and Supplemental Methods). Heterozygotes were crossbred to obtain WT and homozygous mice.

Lentivirus. Lentivirus (a gift from Didler Trono, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) was generated using the constructs with and without the mMOR-1G sequence in HEK293T cells (Supplemental Figure 3). The 2 μl of concentrated lentiviral particles expressing mMOR-1G or vector alone without insertion (1.5×10^9 transducing units/ml) were administrated intrathecally on days 1, 3, and 5, as previously described (18, 19).

Statistics. Analysis of the EGFP qPCR and analgesia utilized one-way ANOVA with post hoc Tukey's multiple comparisons test. Opioid binding was assessed using 2-tailed Student's *t* test. Data are represented as the mean ± SEM.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Memorial Sloan-Kettering Cancer Center.

Acknowledgments

This work was supported by the NIH with grants from NIDA (DA013997 and DA02944) to Y.-X. Pan and (DA06241 and DA07242) to G.W. Pasternak, a Harrington Discovery Institute Scholar-Innovator Grant to G.W. Pasternak and a core grant from the NCI to MSKCC (CA08748). We thank Didler Trono for providing the lentiviral vectors, Chingwen Yang of Rockefeller University for help with ES cells, and Ke Xu, Margaret Leversha, and Willie Mark from Memorial Sloan-Kettering Cancer Center for help with imaging, ES clone karyotyping, and generating the targeted mice, respectively.

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