

## μ-Opioid receptor 6-transmembrane isoform: A potential therapeutic target for new effective opioids



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### ABSTRACT

The μ-opioid receptor (MOR) is the primary target for opioid analgesics. MOR induces analgesia through the inhibition of second messenger pathways and the modulation of ion channels activity. Nevertheless, cellular excitation has also been demonstrated, and proposed to mediate reduction of therapeutic efficacy and opioid-induced hyperalgesia upon prolonged exposure to opioids. In this mini-perspective, we review the recently identified, functional MOR isoform subclass, which consists of six transmembrane helices (6TM) and may play an important role in MOR signaling. There is evidence that 6TM MOR signals through very different cellular pathways and may mediate excitatory cellular effects rather than the classic inhibitory effects produced by the stimulation of the major (7TM) isoform. Therefore, the development of 6TM and 7TM MOR selective compounds represents a new and exciting opportunity to better understand the mechanisms of action and the pharmacodynamic properties of a new class of opioids.

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### 1. Introduction: biphasic cellular inhibitory and excitatory effects of MOR agonists

Opioids are the most frequently used and effective analgesics for the treatment of moderate to severe pain. Unfortunately, both acute and chronic use is frequently associated with a number of adverse side

effects such as respiratory depression, nausea, constipation, itching, xerostomia, drowsiness, as well as physical and psychological addiction. In addition, the prolonged use of opioids may lead to reduced efficacy (tolerance) and a clinically significant problem of post-dosing opioid-induced hyperalgesia (OIH) (Ballantyne and Shin, 2008; Chu et al., 2008; Lee et al., 2011). At a cellular level, MOR is a primary target for clinically used opioids, and MOR-induced analgesia is due to the initiation of presynaptic and/or postsynaptic inhibitory processes that decrease the electrical excitability, neurotransmitter release, and/or pro-nociceptive processes.

The canonical seven transmembrane (7TM) MOR is a member of the G-protein-coupled receptor (GPCR) family. MOR signaling involves activation of a pertussis toxin (PTX)-sensitive G-proteins ( $G_{\alpha_{i/o}}$ ). Receptor activation leads to the dissociation of the heterotrimeric G-protein complex: the release of the  $\alpha$  subunit inhibits adenylyl cyclase, while the  $\beta\gamma$  subunit activates  $K^+$  channels or inhibits voltage-gated  $Ca^{2+}$  channels (VGCC) (Crain and Shen, 2000; North, 1986; Williams et al., 2001). MOR-mediated inhibition of VGCC on the central presynaptic terminals of primary afferent nociceptors is thought to be one of the primary mechanisms mediating the spinal analgesic effects of opiates.  $K^+$  channel activation is a primary mechanism underlying the inhibitory actions of MOR on central nervous system neurons (Herz and Millan, 1990; Millan, 2002; Stanfa and Dickenson, 1995).

Similarly to the analgesic effects of opioids, tolerance and OIH are also manifested in both animals and human following acute or chronic

**Abbreviations:** MOR, μ-opioid receptor; 6TM MOR, six transmembrane helices μ-opioid receptor; 7TM MOR, seven transmembrane helices μ-opioid receptor; OIH, opioid induced hyperalgesia; VGCC, voltage-gated  $Ca^{2+}$  channels; cAMP, cyclic adenosine monophosphate; AC, adenylyl cyclase; *OPRM1*, μ-opioid receptor locus; DAMGO, Ala-2-MePhe-4-Glyol-5-Enkephalin; COS-1, CV-1 carrying the SV40 genetic material fibroblast-like cells; NO, nitric oxide; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism; MOR-1K, μ-opioid receptor carrying the SNP rs563649; TMH1, transmembrane helix 1; GPCR, G protein-coupled receptor; ORL1, orphanin FQ receptor; IBNTxA, iodobenzoylnaltrexamide; IBNaA, iodobenzoylnaloxamide; M6G, morphine 6-glucuronide; HEK-293, human embryonic kidney 293 cells; MYC-tag, N-EQKLISEEDL-C; Anti-MYC-FITC, fluorescein isothiocyanate-conjugated anti-MYC antibody; CHO, Chinese hamster ovary; DPDPE, D-Penicillamine-2-D-Penicillamine-5-Enkephalin; U69,593, N-methyl-2-phenyl-N-[(5R,7S,8S)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl]-acetamide; CTAP, D-Phe-Cys-D-Trp-Arg-Thr-Penicillamine-Thr-NH2; norBNI, norbinaltorphimine; 5'-GNTI, 5'-Guanidinonaltrindole; U50,488U, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-yl-cyclohexyl]-acetamide

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dosing (Crain and Shen, 2001; Kayser et al., 1987; Levine et al., 1979; Li et al., 2001). The neural mechanisms that underlie hyperalgesic effects are poorly understood, but are dependent on the concentration of the drug and the duration of exposure (Crain and Shen, 2000; Rubovitch et al., 2003). A biphasic effect of opioids on cAMP formation and substance P release has also been demonstrated (Crain and Shen, 2000; Rubovitch et al., 2003; Suarez-Roca and Maixner, 1992; Suarez-Roca and Maixner, 1995; Suarez-Roca et al., 1992; Wang et al., 2005). There is evidence that the excitatory actions of MOR reflect a switch in the G protein coupling profile of the MOR from  $G_i$  to both  $G_s$  (Crain and Shen, 2000; Esmaeili-Mahani et al., 2008; Mostany et al., 2008; Wang and Burns, 2006) and  $G_q$  (Rubovitch et al., 2003), as well as adenylyl cyclase (AC) activation by  $G_{\beta\gamma}$  (Wang and Burns, 2006; Wang et al., 2005). There is additional evidence for a critical role of  $\beta$ -arrestin in MOR desensitization and tolerance. It has been shown that in mice lacking  $\beta$ -arrestin-2, MOR desensitization does not occur after chronic morphine treatment and these animals fail to develop tolerance, although they still develop physical dependence (Bohn et al., 2000).

## 2. Genomic organization of *OPRM1* gene

The major isoform of MOR, also called MOR-1, is coded by exons 1, 2, 3 and 4 of MOR gene locus (*OPRM1*), where exon 1 codes for the first transmembrane domain and exons 2 and 3 code for all the other transmembrane domains. Gene structure is highly conserved between human, mouse and rat. Today, at least 30 splice variants of MOR in mice, 16 in rats and 19 in humans have been identified (Pasternak, 2014). There are two common splicing patterns of *OPRM1* that involve the C-terminus and N-terminus. C-terminus variants contain exons 1, 2 and 3 and code for all seven transmembrane domains, but differ structurally and functionally at the intracellular domains. The N-terminus also has a number of variants, some of which encode for truncated receptors with only six transmembrane domains (6TM, coded by exons 2 and 3). While other truncated receptors have been reported (Boise et al., 1993; Karpa et al., 2000; Nag et al., 2007; Zhu and Wess, 1998), analysis of the functional significance of truncated MOR receptors is still ongoing. Some truncated receptors can modulate the activity of the full version of receptor (Karpa et al., 2000; Nag et al., 2007; Zhu and Wess, 1998) or change the biological activity of the protein, sometimes in the opposite direction (Boise et al., 1993).

The 6TM MOR variants were first cloned from mouse (Pan et al., 2001). Five 6TM alternatively spliced isoforms were first reported, of which three (MOR-1G, MOR-1M, and MOR-1N) have an initial methionine positioned at exon 11 that codes for the N-terminus of the protein, and two isoforms (MOR-1K and MOR-1L) have the initial methionine positioned at the beginning of exon 2. The latter MOR mRNA splice variants also code for a short upstream peptide. The potential for these mRNA splice variants to code for 6TM MOR isoform has not been explored. Furthermore, none of the five variants have been shown to bind opioid ligands by [ $^3$ H]-DAMGO displacement experiments, and thus, the functionality of these receptor variants has been questioned (Pan et al., 2001).

The first described human 6TM *OPRM1* isoform (Cadet et al., 2003) was referred to as MOR-3. It has no reported analog in mouse or rat, and its first methionine is positioned at the beginning of exon 2. On a functional level, COS-1 cells transiently overexpressing MOR-3 receptor exhibit a dose-dependent release of nitric oxide (NO) following treatment with morphine. MOR-3 possesses a unique intracellular C-terminal amino acid sequence that has been hypothesized to serve as a coupling or docking domain required for constitutively expressed NO synthase activation (Kream et al., 2007). The expression of MOR-3 has been detected in human vascular tissue, mononuclear cells, polymorphonuclear cells, and human neuroblastoma cells by northern blot and reverse transcription polymerase chain reaction (RT-PCR) (Cadet et al., 2003). The physiological and cellular pathways whereby this isoform contributes to opioid responses have not been yet identified.

Using comparative genomic approaches we reported that the human *OPRM1* gene is much more complex than previously shown and has orthologs to all described mouse exons (Shabalina et al., 2009). Our association study analysis identified a new potentially functional single nucleotide polymorphism (SNP), namely rs563649, which showed strong contribution to variability in pain sensitivity and morphine analgesia. This finding allowed us to clone a new human isoform (MOR-1K) that carries SNP rs563649. The human MOR-1K is orthologous to mouse MOR-1K, containing an ortholog of mouse exon 13. Although similar to the human MOR-3 variant reported by Cadet et al. (2003) and initiated from methionine in exon 2, MOR-1K codes for a truncated 6TM receptor isoform with an intracellular domain identical to the major MOR-1 isoform, which is different from MOR-3. In contrast to MOR-3, MOR-1K is also expressed in neuronal cell lines, neuronal tissues, and brain regions that mediate the pharmacodynamic effects of MOR agonists. The minor T allele situated in the 5'-untranslated region (5'-UTR) of MOR-1K mRNA is associated with higher pain sensitivity as well as a poorer analgesic response to morphine. It codes for higher translation efficiency of the 6TM receptor isoform (Shabalina et al., 2009), suggesting that the expression of the 6TM variant produces a pronociceptive effect.

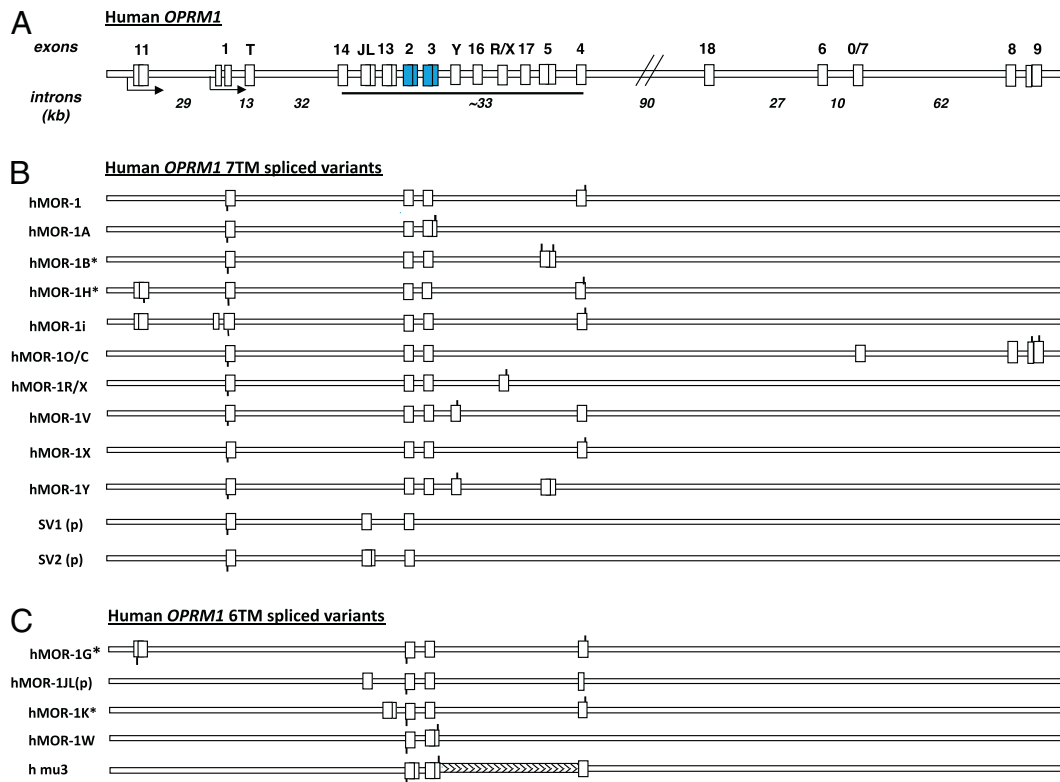
In line with these observations, cellular studies with MOR-1K suggest that, after agonist activation, this isoform drives excitatory rather than the classic inhibitory cellular responses following the stimulation of the 7TM isoform (Gris et al., 2010). Indeed, morphine-induced activation of MOR-1K increases the production of excitatory mediators (e.g.,  $Ca^{2+}$  and NO). Furthermore, immunoprecipitation experiments reveal that MOR-1K couples to  $G_{\alpha_s}$ , rather than  $G_{\alpha_{i/o}}$  (which classically couples with the major MOR1 isoform) (Gris et al., 2010). These data suggest that MOR-1K can function as a counterbalance to the actions mediated by the 7TM isoform and may mediate the molecular processes that underlie OIH and pharmacological tolerance.

The human ortholog of mouse exon 11 also has been cloned by Pan and coworkers (Xu et al., 2009). Four new alternatively spliced forms (MOR-1G1, MOR-1G2, MOR-1I and MOR-1H) have been identified that contain exon 11 spliced either directly to exon 2 or to exon 1 through a variable 3' acceptor splice site (Xu et al., 2009). These RNAs code for either the extended 7TM receptor isoforms (MOR-1I and MOR-1H) or for 6TM receptor isoforms, namely MOR-1G1 and MOR-1G2. While MOR-1G1 codes for the same amino acid sequence of the MOR-1K variant, MOR-1G2 is characterized by the presence of additional 10 residues at the N-terminal domain of the protein. Importantly, the human exon 11, which was cloned by Pan and coworkers, corresponds to the predicted exon 11 by Shabalina et al. (2009) revealed by comparative genomic approaches. This observation suggests the existence of additional exons, and reconfirms the existence of a unique class of human 6TM receptor isoforms (Fig. 1).

## 3. Receptor structure for 7TM and 6TM MOR

Before the MOR structure was resolved at high-resolution (Manglik et al., 2012), in silico modeling was performed to predict its structure (Alkorta and Loew, 1996; Filizola et al., 1999; Pogozheva et al., 1998; Strahs and Weinstein, 1997). Using homology modeling (Serohijos et al., 2006; Strahs and Weinstein, 1997), we have reconstructed and further refined a structural model for 7TM MOR (Fig. 2A) (Serohijos et al., 2011). Based on the computational prediction of 7TM MOR, we designed several 7TM MORs carrying mutations in the ligand binding pocket, as well as 7TM MOR variants with mutations outside of the binding site as controls (Strahs and Weinstein, 1997). Results of competitive radioligand displacement experiments and cAMP inhibition assay of the proposed mutants were fully consistent with our structural model. The model is also consistent with the recently solved MOR crystal structure (Manglik et al., 2012), with a deviation of ~3.5 Å.

Compared to 7TM MOR, the 6TM isoform lacks a segment of 100 residues in the N-terminal, including the extra-cellular domain as well as



**Fig. 1.** Schematic structure of human *OPRM1* spliced variants. (A) All predicted and cloned exons of human *OPRM1* gene are in accordance with the NCBI database, UCSC genome browser GRCh37/hg19. Exons and introns are shown by vertical and horizontal boxes, respectively. Solid boxes represent constitutive exons coding for transmembrane domains 2–7. (B) All cloned alternatively spliced variants of *OPRM1* coding for 7TM receptor isoforms. (C) All cloned alternatively spliced variants of *OPRM1* coding for 6TM receptor isoforms. (p) partial sequence, (\*) exons with variable exon/intron boundaries. Start codons of MOR isoforms are schematically indicated by lower vertical bars, while stop codons are indicated by upper vertical bars. Maximal sizes of human exons (for lower panel) are shown in parentheses (nt): exon 11 (206), exon 1 (580), exon T (117), exon 14 (105), exon 13 (1200), exon 2 (353), exon 3 (521), exon Y (109), exon 16 (314), exon X (1271), exon 17 (128), exon 5 (1013), exon 4 (304), exon 18 (412), exon 6 (124), exon 7 (89), and exon 9 (393).

the first transmembrane helix (TMH1) (Fig. 2B). The absence of TMH1 in 6TM MOR paves the way to a plethora of different structural hypotheses that could be potentially related to the excitatory cellular response observed upon stimulation of 6TM isoform. The newly membrane exposed surface of 6TM MOR is characterized by a different charge distribution (Fig. 2C). Therefore, a possible rearrangement in the plasma membrane of the remaining helices may result in differences in ligand binding and G protein activation compared to wild type isoform. The lack of TMH1 in 6TM MOR can also alter the functional plasticity of the receptor and its ability to interact with specific intracellular signaling proteins (i.e., G protein and/or  $\beta$ -arrestin isoforms). Furthermore, it cannot be excluded that 6TM competes with 7TM MOR for the binding of opioids or for interactions with other GPCRs, thus reshaping intracellular GPCR-dependent signaling cascades and the resulting cellular responses (Wise, 2012). All of these scenarios are equally likely at this time. Therefore, there is a substantial need to further structural and functional investigations of the biological signaling mechanism mediated by 6TM MOR.

#### 4. Cellular localization of 7TM and 6TM MOR isoforms

The cellular localization of the two MOR isoforms has recently been reported for both human and mouse (Gris et al., 2010; Majumdar et al., 2011). Two independent groups have showed that the 6TM MOR isoform overexpressed in mammalian cells is not constitutively expressed on the cytoplasmic membrane, but instead it is localized in intracellular compartments (Fig. 3). Nevertheless, it has been shown that cells overexpressing intracellular 6TM bind fluorescently labeled naloxone (Gris et al., 2010). Furthermore, the co-transfection of the orphanin FQ receptor ORL<sub>1</sub> (opioid receptor-like) with the 6TM variant enables

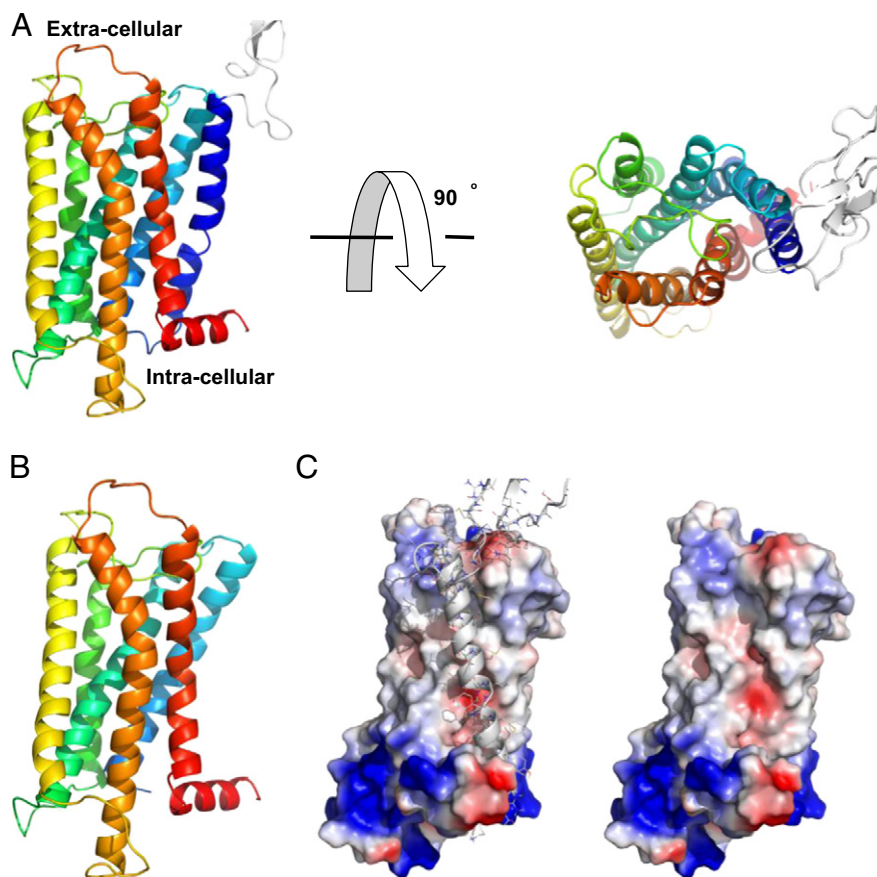
high-affinity binding of the recently synthesized 6TM MOR ligand iodobenzoylnaltrexamide (IBNtxA) to 6TM MOR (Majumdar et al., 2011). No IBNtxA binding is observed in cells transfected with either 6TM MOR or ORL<sub>1</sub> alone, however, binding is detected in brain tissues from 7TM MOR knockout (KO) mice (Majumdar et al., 2011).

Taken together, these results suggest that the lack of the first transmembrane in MOR protein structure dramatically alters the subcellular localization of 6TM MOR and implies that the expression of the 6TM variant alone is insufficient to enable ligand binding. It is still possible that similarly to the sigma receptor (Sigma-1) (Hayashi and Su, 2007) or estrogen receptor GPR30 (Revankar et al., 2005), intracellularly localized 6TM can bind ligands and produce cellular signaling. It should also be recognized that 6TM MOR may be translocated to the cell surface with the aid of a cytoplasmic chaperone. Alternatively, the presence of a plasma membrane GPCR partner such as ORL<sub>1</sub> and other GPCRs can coordinate plasma membrane co-localization and assembly, thus leading to function as a mosaic of proteins that act in concert to mediate the cellular signaling evoked by opioid ligands.

#### 5. Behavioral studies in knockout mice demonstrate functional differences in MOR isoforms

Several studies have shown that *OPRM1* is essential for morphine's analgesic actions because complete *OPRM1* KO mice show a complete loss of morphine analgesia (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997). However, KO mice with the deletion of only exon 1 or only exon 11 show substantial differences in their sensitivity to opioids. Exon 1 KO mice, which should still express 6TM receptor variants, show reduced analgesic response to heroin and morphine 6-glucuronide (M6G), but do not respond to methadone or morphine (Schuller et al.,

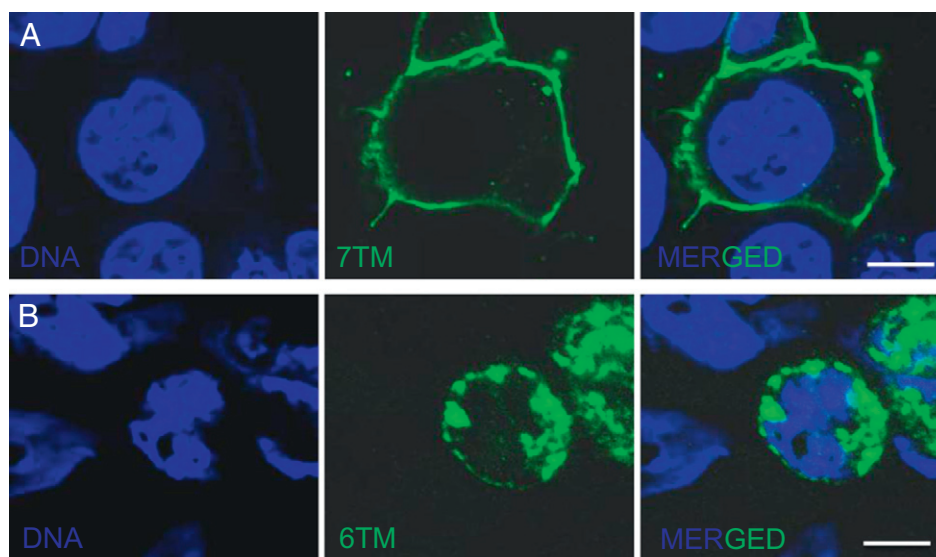




**Fig. 2.** Computer model of 7TM and 6TM MOR. (A) The experimentally validated structure model of 7TM MOR. The protein is shown in cartoon representation with rainbow color for N- (blue) to C-terminal (red). The extra-cellular loop is white with a weak propensity to form  $\beta$ -strands. (B) The initial 6TM model with the N-terminal 100 residues excised from the 7TM model. (C) The surface of 6TM model structure colored according to the local electrostatic potential. Blue and red colors correspond to positively and negatively charged surfaces, respectively, and white color corresponds the neutral hydrophobic ones.

1999). In contrast, mice lacking exon 11, which can still produce 7TM receptor variants, do not show an analgesic response to heroin, fentanyl, and M6G, but are responsive to methadone and morphine (Pan et al.,

2009). The absence of analgesic response to morphine in exon 1 KO mice, but not in exon 11 KO mice, is in line with the proposed hypothesis that 7TM but not 6TM MOR isoform, exclusively or largely, underlies



**Fig. 3.** Cellular localization of 7TM or 6TM MOR isoforms shows. Confocal images of HEK-293 cells expressing 7TM or 6TM MOR: A) 7TM MOR localizes on the cell surface, B) 6TM isoform is mainly localized in intracellular compartments. HEK-293 cells were transiently transfected with plasmids encoding MYC-tagged 7TM or FLAG-tagged 6TM. After 48 h cells were fixed and stained with either anti-MYC-FITC antibody or anti-DYKDDDDK tag antibody (Alexa Fluor 488 conjugate); DNA was counterstained with Hoechst 33342. Scale bar: 10  $\mu$ m.

cellular inhibitory effects of morphine and contributes to analgesia. More importantly, our results pertaining to the 6TM-dependent excitatory cellular effects of morphine (Gris et al., 2010) also suggest that exon 1 KO mice may show reduced or hyperalgesic responses to morphine administration, while exon 11 KO mice, which lack the excitatory 6TM receptor variant, should show diminished OIH. This model has been indirectly examined in “triple KO” mice that lack the first exons in all three genes encoding for opioid receptors ( $\mu$ ,  $\delta$ , and  $\kappa$ ). The thermal nociceptive responses to morphine and oxymorphone in triple KO compared to control mice have been examined. The acute administration of test opioids (morphine or oxymorphone) in control animals evokes a profound analgesia that is absent in the triple KO mice. In contrast, a continuous infusion of either morphine or oxymorphone evokes hyperalgesia in the triple KO mice in comparison with control mice (Juni et al., 2007). Since the original MOR KO line was derived from an exon 1 KO variant (Schuller et al., 1999), which leaves exon 11 available to code for 6TM variants of MOR, triple KO mice should completely lack 7TM but not 6TM variants of MOR.

Recently, Majumdar et al. (2011, 2012) demonstrated a potent opioid analgesia evoked in triple KO mice by IBNTxA and iodobenzoylnaloxamide (IBNaLA), which are both morphinane derivatives. The analgesia evoked by these compounds was associated with a lack of respiratory depression, physical dependence, reward behavior, and significant constipation. Analgesia was lost in the exon 11 MOR-1 KO mice indicating that IBNTxA and IBNaLA effects involve exon 11-associated MOR-1 variants, those coding for the 6TM MOR isoform. The authors reported that IBNTxA and IBNaLA bind to brain lysates of the triple KO mice, which suggests the direct interaction with 6TM MOR isoform (Majumdar et al., 2011). However, they also demonstrated that these opioids showed high affinity for the reference 7TM variants of the three opioid receptor subtypes (in vitro affinity data and competitions studies performed with radiolabeled IBNTxA and IBNaLA are reported in Tables 1 and 2, respectively) (Majumdar et al., 2001).

## 6. A paradox

An apparent paradox to the hypothesis that agonists of the 6TM isoform may produce excitatory effects, OIH, and analgesic tolerance to opioids needs to be acknowledged. First, opioid analgesia is evoked by heroin, fentanyl, and M6G in exon 1 KO (6TM sparing) mice and greatly diminished in exon 11 KO (7TM sparing) (Pan et al., 2009). Furthermore, in studies by Majumdar et al. (2011, 2012), IBNTxA and IBNaLA produced analgesia in triple exon 1 KO mice. These results are not in line with the observed hyperalgesic effects of the minor T allele of

**Table 2**

Competition studies of radiolabeled IBNTxA and IBNaLA.

| Drug         | $K_i$ (nM)      |                 |
|--------------|-----------------|-----------------|
|              | IBNTxA          | IBNaLA          |
| $\mu$ -OR    |                 |                 |
| CTAP         | 2.33 $\pm$ 0.48 | 2.96 $\pm$ 1.07 |
| Naloxone     | 4.23 $\pm$ 0.42 | 2.27 $\pm$ 0.60 |
| Levallorphan | 1.29 $\pm$ 0.14 | 1.69 $\pm$ 0.60 |
| Naltrexone   | 1.15 $\pm$ 0.10 | 2.71 $\pm$ 1.0  |
| DAMGO        | 3.34 $\pm$ 0.43 | 0.45 $\pm$ 0.12 |
| Morphine     | 4.60 $\pm$ 1.81 | 2.52 $\pm$ 0.28 |
| $\delta$ -OR |                 |                 |
| DPDPE        | 1.39 $\pm$ 0.67 | 1.77 $\pm$ 0.80 |
| Naltrindole  | 0.46 $\pm$ 0.32 | 0.50 $\pm$ 0.20 |
| $\kappa$ -OR |                 |                 |
| norBNI       | 0.23 $\pm$ 0.03 | 0.19 $\pm$ 0.08 |
| 5'-GNTI      | 0.15 $\pm$ 0.07 | 0.19 $\pm$ 0.05 |
| (-)-U50,488H | 0.73 $\pm$ 0.32 | 0.95 $\pm$ 0.37 |

Table adapted from Majumdar et al. (2001). Competition studies were performed for each compound against  $^{125}$ I-IBNTxA and  $^{125}$ I-IBNaLA at 0.1 nM concentration in membranes from stably transfected CHO cell lines.  $K_i$  values are expressed as mean  $\pm$  SEM; for further information refer to Majumdar et al. (2001). Abbreviation: CTAP, D-Phe-Cys-D-Trp-Arg-Thr-Penicillamine-Thr-NH<sub>2</sub>; DAMGO, D-Ala-N-MePhe-Glyol-enkephalin; DPDPE, D-Penicillamine-2-D-Penicillamine-5-enkephalin; norBNI, nor-binaltorphimine; 5'-GNTI, 5'-Guanidinonaltrindole; U50,488U, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-yl-cyclohexyl]-acetamide.

rs563649, coding for higher expression of 6TM MOR-1K variant (Shabalina et al., 2009), and the 6TM-dependent cellular excitatory effects of morphine and IBNTxA (Gris et al., 2010; Samoshkin et al., 2014). However, there are several possible reasons for this apparent paradox. First, it is possible that there are still undiscovered *OPRM1* spliced forms that contain exon 11 but not exon 1, that code for a functional 7TM isoform, or there is a difference in a signaling between 6TM MOR isoforms initiated from exon 11 and exon 2 and varying in their N-terminus (such as MOR-1G2 and MOR-1K, Pasternak, 2014; Pasternak and Pan, 2013). A second possible explanation is that both 7TM and 6TM receptor isoforms are promiscuous in terms of G-protein binding capacity and both isoforms can bind either  $G_i$  or  $G_s$  proteins (Hermans, 2003), though, as a general rule, the stimulation of the 7TM isoform signals through binding with  $G_i$  and stimulation of the 6TM isoform favors  $G_s$  binding. Certain “biased” agonists may produce sufficient thermodynamic changes to permit “protean agonism” whereby an agonist is able to produce a receptor activation state and signaling through a non-canonical pathway (Kenakin, 2007). In line with this hypothesis, potentially, developmental adaptation in the exon 1 KO animals may take place that would switch the balance of  $G_i/G_s/G_q$  coupling for 6TM variants in the absence of expression of 7TM variants. Furthermore, it can be hypothesized that specific GPCRs can partner with 6TM MOR at the cellular membrane (see section entitled Cellular localization of 7TM and 6TM MOR isoforms) producing a temporally dynamic mosaic of molecular partners and, thus, different cellular signaling responses. The macromolecular composition may be influenced by the individual's history of opioid exposure or exposures to physical and psychological stressors that influence the expression and cellular distribution of 6TM MOR resulting in qualitative and quantitative differences in the pharmacodynamic responses to opioids. These responses can also depend on the concentration of a ligand, such that a low concentration may produce analgesia and higher concentrations may produce hyperalgesia. In line with this suggestion, the concentrations of morphine and IBNTxA that produced cellular excitatory effects occur at relatively high concentration (1–10  $\mu$ M) (Gris et al., 2010; Samoshkin et al., 2014). Finally, it should be noted that the M6G analgesia reported in exon 1 MOR1 KO mice (6TM sparing) has not been uniformly observed. A recent study by van Dorp et al. (2009) showed both acute and chronic M6G administration to triple KO mice (deletion of exon 1 of MOR1, 6TM sparing) leads to a profound hyperalgesia.

**Table 1**

Affinity of IBNTxA and IBNaLA for opioid receptor subtypes.

| Drug   | $\mu$ -OR               | $\delta$ -OR     | $\kappa$ -OR       |
|--------|-------------------------|------------------|--------------------|
|        | $K_i$ (nM) <sup>a</sup> |                  |                    |
| IBNTxA | 2.50 $\pm$ 0.82         | 0.58 $\pm$ 0.16  | 0.23 $\pm$ 0.16    |
| IBNaLA | 0.70 $\pm$ 0.18         | 2.55 $\pm$ 0.74  | 0.08 $\pm$ 0.006   |
| Drug   | $\mu$ -OR               | $\delta$ -OR     | $\kappa$ -OR       |
|        | $K_D$ (nM) <sup>b</sup> |                  |                    |
| IBNTxA | 0.11 $\pm$ 0.02         | 0.24 $\pm$ 0.005 | 0.027 $\pm$ 0.0001 |
| IBNaLA | 0.22 $\pm$ 0.03         | 2.5 $\pm$ 0.22   | 0.05 $\pm$ 0.22    |

Table adapted from Majumdar et al. (2001). Abbreviation: DAMGO, D-Ala-N-MePhe-Glyol-enkephalin; DPDPE, D-Penicillamine-2-D-Penicillamine-5-enkephalin; U69,593, N-methyl-2-phenyl-N-[(5R,7S,8S)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl]-acetamide.

<sup>a</sup> Competition studies of  $^{125}$ I-radiolabeled compounds against  $^3$ H-opioids performed with  $^3$ H-DAMGO ( $\mu$ -OR),  $^3$ H-DPDPE ( $\mu$ -OR), and  $^3$ H-U69,593 ( $\mu$ -OR) at 1 nM concentration in stably transfected CHO cell lines.  $K_i$  values are reported as mean  $\pm$  SEM; for further details refer to Majumdar et al. (2001).

<sup>b</sup> Saturation studies of  $^{125}$ I-labeled compounds in stably transfected CHO cell lines.  $K_D$  values are reported as mean  $\pm$  SEM; for further details refer to Majumdar et al. (2001).

## 7. Conclusions and significance

MOR agonists are the most widely used analgesics, prescribed for both acute postoperative pain and chronic pain conditions; yet, there are substantial side effects that limit usage. Thus, a further understanding of the molecular and cellular mechanisms that contribute to the analgesic, hyperalgesic, and analgesic tolerance effects of opioids is needed. Current published findings provide evidence that the 6TM MOR isoform is not just another alternatively spliced form of *OPRM1*, but instead it displays substantial differences in cellular distribution, evoked cellular responses, contributions to the cellular excitatory responses to opioids, and it shows evidence of both hyperalgesic and analgesic responses. Because of the potentially dual contribution of the 6TM MOR isoform to analgesic signaling, further research targeting this isoform is warranted. The development of highly selective and specific agonists and antagonists of the 6TM and 7TM MOR isoforms, as well as ligand biased compounds are required to elucidate the biological and cellular properties engaged by these distinct isoforms subclasses. The elucidation of signaling pathways associated with 6TM and 7TM MOR isoforms, along with understanding of the endogenous properties of these isoforms and the ligands that modify the biological activities of these MOR variants, will enable the development of opioids that show a high degree of analgesic efficacy with fewer side-effects.

## Conflict of interest

Drs. Diatchenko and Maixner are cofounders, officers and equity shareholder in Algynomics, Inc. Dr. Dokholyan is a small equity shareholder and consultant to Algynomics, Inc. Algynomics is a company that specializes in pain diagnostics and therapeutics. Dr. Dokholyan is a founder, officer and equity shareholder in Molecules in Action. Molecules in Action is a company that specialized in scientific consulting and customized software development. A portion of the work presented in this manuscript was supported by Algynomics, Inc., and Molecules in Action, LLC. The work presented in the manuscript may have a potential commercial value.

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