Trends in Biochemical Sciences

The life cycle of the Mu-opioid receptor

--Manuscript Draft--

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Abstract:	Opioid receptors are undisputed targets for the treatment of pain. Unfortunately, targeting these receptors therapeutically poses significant challenges including addiction, dependence, tolerance and the appearance of side-effects such as respiratory depression and constipation. Moreover, misuse of prescription and illicit narcotics has resulted in the current opioid crisis. The mu-opioid receptor is the cellular mediator of the effects of most commonly used opioids and is a prototypical G protein-coupled receptor (GPCR) where new pharmacological, signalling and cell biology concepts have been coined. This review summarizes our knowledge on the life cycle of this therapeutic target including their biogenesis, trafficking to and from the plasma membrane and how the regulation of these processes impacts their function and are related to pathophysiogical conditions.

Cover Letter

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Dr David Waters
Acting Trends Reviews Editor

7th August 2020

Re: Submission of invited review

Dear Dr Waters,

In response to our email exchange on the 26th of June, please find enclosed our invited review manuscript entitled "**The life cycle of the Mu-opioid receptor**" to be considered for publication in *Trends in Biochemical Sciences*.

The present review synthesizes the current knowledge about the life cycle of the mu-opioid receptor (MOR). MORs are an undisputed target for the treatment of pain. They are the G protein-coupled receptor (GPCR) that mediates the effects of clinically used opioids such as morphine and fentanyl. Moreover, MOR is also a prototypical GPCR, where the new pharmacological, signalling and cell biology concepts have been discovered and demonstrated. These include pioneering structural studies, allosterism, biased agonism, endosomal and compartmentalised signalling and ligand-directed trafficking. While several reviews have focussed on particular aspects of these new developments, to our knowledge, there is no review that summarizes how MORs are synthesised, trafficked to and from the plasma membrane, how these mechanisms impact their eventual function and, finally, how all these mechanisms are affected in pathophysiological conditions such as pain or drug abuse.

We think this review will provide a refreshed view to life cycle of a classical and important GPCR and can be used as a reference for researchers that are novices in the research area of opioid receptor biology as well as for more experienced researchers in need of a concise and entertaining read on the birth and death of a key therapeutic target in the treatment of pain.

We would like to suggest the following reviewers:

Dr Lakshmi Devi (lakshmi.devi@mssm.edu) Professor of Pharmacology and Systems Therapeutics, , Icahn School of Medicine at Mount Sinai. Opioid receptor pharmacologist with expertise in dimerization and modifications of opioid receptors.

Dr Marta Filizola (marta.filizola@mssm.edu) Professor of Pharmacological Sciences and Neuroscience, Icahn School of Medicine at Mount Sinai. GPCR computational biologist with expertise in opioid receptors

Dr John Streicher (jstreicher@email.arizona.edu) – Assistant Professor in Pharmacology and Neuroscience, The University of Arizona Health Sciences. Opioid pharmacologist and cell biologist.

Finally, please note that, despite our efforts of only citing 80 references in the main text, we are currently citing 87 publications. We are happy to consider removal of references upon editor or reviewer's request.

Thank you for your consideration of this manuscript.

Sincerely,

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September 29, 2020

Dr David William Waters
Acting Trends Reviews Editor

Revision of manuscript: TIBS-D-20-00173

Dear Dr. Waters,

We thank the Reviewers and the Editor for their positive and valuable comments, which have helped us improve our manuscript entitled "*The life cycle of the Mu-Opioid Receptor*". We have now completed the revision of the manuscript, taking all of the Reviewers' comments into account. Please see the detailed responses below (blue text). New manuscript text is in red.

Please also note that to address the comments of the Reviewers we have altered the wordcount and reference limits of the original manuscript. However, these are within the limits allowed upon revision.

Reviewer 1

Dr. Cuitavi and co-authors presented a vibrant review on MOR life cycle. I found that presentation has few essential missing items from my point of view which I outlined below.

Page 3 -" In humans the OPRM1 gene is in the sixth chromosome (6q25.2) and contains 9 exons". Not a true statement. The major isoform of OPRM1 is coded by 4 exons, but OPRM1 has many alternatively spliced forms such as 2 exons (usually named 2nd and 3d) are very conservative and at least 19 exons (some with multiple splicing sites) shuffled into over 30 transcripts in humans, and in mice, at least 18 exons are combined into over 50 transcripts (NCBI-RefSeq, Ensembl, UCSC Genome Browser). Because the number of receptors constantly increasing, one should look at the latest edition of Ref-Seq or latest manuscripts, for example PMID: 30066306. The same applied for page 5 sentence "However, the extensive alternative splicing of the OPRM1 gene (generating 23 splicing variants, including 4 that do not encode protein)". Again, both human and mouse OPRM1 have many more than 23 splicing variants –

We have updated these numbers in the corresponding sections as well as cited the references suggested by the Reviewer.

Page 5 - "The second group swaps exon 1 for exon 11, which results in 6-TM domains". The 6TM variants in both mouse and human also forms by several other splicing events with several other exons. For example PMID: 25485963, but also authors should also check with the newest updates on (NCBI-RefSeq, Ensembl, UCSC Genome Browser).

We have edited this sentence to highlight that the 6-TM variants can be formed by other exons and the reference suggested by the Reviewer has been cited.

Page 5. "The pharmacology of the MOR". The hyperalsgesic function of 6TM is not discussed. For example, its contribution to opioid induced hyperalgesia, but also few other evidence. For example PMID: 26657998

We have added discussion on the potential role of 6-TM MOR splice variants in OIH, the potential cellular mechanisms that mediate these effects as well as potential strategies to prevent them (e.g through β2-ARs antagonists). The reference suggested by the Reviewer has been added.

Page 6. "there is evidence that MOR heterodimerisation with ORL1, KORs and DORs" There is also evidence that MOR forms heterodimer with ADRB2 PMID: 26657998

We apologise that we did not make this point clear. Our intention was to mention heterodimers within the opioid receptor family. Indeed, MOR heterodimerises with other GPCRs, including adrenoceptors, dopamine and cannabinoid receptors. A statement and a reference clarifying this have been added.

Pages 6 and 7, when authors discuss the expression of MOR on the plasma membrane, I found no concrete discussion on receptor stabilization and translocation which i believe very import part of receptor life cycle. Also, no discussion if/how 6TM and 1TM isoform reach the cell surface, though they are presented on the figure 3.

Discussion on stabilization and translocation of the receptor to the cell surface was indeed included and referenced in the text apart from presented in figure 3. However, we have added statements clarifying how the 6-TM variants reach (or not) the plasma membrane.

Pages 6 and 7. The authors describe analgesic function of MOR and function of abuse and misuse. They did not mention opioid induced hyperalgesia.

A paragraph discussing the function of MOR in OIH has been added at the end of the section "MOR at work: a freshman at the cell membrane"

Reviewer 2

It's a thoughtful review with an interesting setting to describe MOR through its life cycle. The review covers a wide range of research data from various aspects of MOR regarding its regulations, functions and signaling pathways at DNA, RNA and protein levels. Overall, it's a good review. Some minor issues need to be addressed.

1. OPRL1 should be changed to ORL-1 in the second paragraph of The dark side of opioid pain relief, which would be consistent with MOR/DOR/KOR.

Abbreviation for the Opioid Receptor Like-1 has been changed.

2. Human OPRM1 contains more than 9 exons as showed in reference # 29 with alternative splicing. Ref# 4 was an early review.

This section has been corrected and edited as per this comment and that of Reviewer 1.

3. "commonly occurring" in the second paragraph of A new beginning: --" referring rs1799971 is inaccurate, which can be changed to "mostly studied".

This has been edited

4. The authors indicated that "some reports suggest that the rs1799971 SNP interferes with the analgesic power of some opioid". But there were no references.

A reference for this statement has been added.

5. Using both OPRM1 and Oprm1 is unnecessary. If the authors want to indicate both human and rodent mu opioid receptor genes, it should be spelled out. As related, the authors should consider including a paragraph describing similarities and differences in structure, regulation and functions between human and rodent mu opioid receptor genes and proteins.

We feel we should adhere to gene nomenclature. The text clearly explains the different nomenclature for the different species; "The MOR is encoded by the OPRM1 gene in humans and the Oprm1 gene in mice, and it is conserved across species." We would appreciate the editor's guidance for this point. We will revise the text if required.

As per the Reviewer's suggestion we have now included a brief paragraph mentioning the similarities and differences between human and rodent MOR.

6. In micrRNAs paragraph, some references were missing. For example, a reference for mir-103/mir-107 in regulating expression of MOR1A was not cited.

Discussion and references to these miRNAs have been added.

7. Recently, MOR crystal structures with antagonist or agonist configuration, as well as allosteric modification, have been resolved, providing important information how mu ligands interact with the receptor and induce signaling. The authors should include some discussion on this subject.

We refer the Reviewer to Box 1 of our original manuscript, where we discuss all these subjects.

8. When described the mesocorticolimbic system, the authors only mentioned VTA an NAc. However, many literatures demonstrated the importance of prefrontal cortex in motivation and reward. Including this region is appropriate for a complete system.

Discussion on the role of MORs in the PFC has been added and references included.

9. The authors should be carefully for the cited references. For example, ref# 45 and 74 were not right.

References have been updated and revised.

10. Recently, TRV-130 (Olinvyk) was approved by FDA as an intravenous opioid to treat moderate to severe acute pain in adults. The authors may modify the discussion of TRV-130.

Discussion of TRV-130 has been updated to reflect recent approval by FDA.

11. In The decline after a life of service, the authors mostly reviewed beta-arrestin2 and G protein signaling, which appeared not fitting into the theme. The authors can consider moving this section

to the early section, and include degradation vs recycling after internalized. Von Zastrow has recently published several nice papers to describe how intracellular compartments play a role in signaling transduction. It can be fit quite well at this section.

We thank the Reviewer for pointing out the omission of the most recent work of the lab of Mark Von Zastrow that clearly shows the different mechanisms of MOR regulation operating in pre- vs post-synapses. This has now been included and referenced.

However, we feel that the discussion of G protein vs arrestin bias fits in this part of the manuscript rather than the section above. The role of β-arrestins in negatively regulating MOR is well established, hence our decision to include the description of β-arrestin-mediated processes in this section. However, we feel that for completeness, it is also important to highlight how initial observations of differential MOR regulation stemmed the field of biased agonism at this receptor, triggering a revolution in drug discovery that has recently been challenged. We therefore think that this discussion fits in this part of the manuscript and is a thought-provoking way of ending this review.

Editorial comments

Please remove the headings from the figure images e.g. Figure 1, Figure 2 (top left-hand corner).

Headings have been removed.

Please label the figure for Box 2 ‘Figure I’. The figure labelling for text boxes is not continuous thus the first figure in each text box is Figure I (the second figure in a text box is Figure II).

This has been addressed

Please replace the key word ‘Mu Opioid Receptor’ with a suitable alternative (this will improve searchability of your article).

Key words have been updated

Please label your final section Concluding remarks (currently: CONCLUDING REMARKS AND FUTURE PERSPECTIVES).

Final section has been renamed

Please double check your references. For example, I notice that the citation for reference 19 is incomplete, as is 17 and 52. Where you have an instance of ‘published ahead of print’, reference 66, please provide as much information as possible, such as month of acceptance. Please also label reference 66 ‘Epub ahead of print’, and any others this applies to.

References have been updated and revised.

We appreciate the time spent by the Reviewers and the Editor and hope that the revisions made will now permit publication of our manuscript in TiBS.

Sincerely yours,

Meritxell Canals, PhD

The life cycle of the Mu-Opioid Receptor

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KEYWORDS

GPCR, *OPRM1*, MOR Signalling, pain, reward, addiction

ABSTRACT

Opioid receptors are undisputed targets for the treatment of pain. Unfortunately, targeting these receptors therapeutically poses significant challenges including addiction, dependence, tolerance and the appearance of side-effects such as respiratory depression and constipation. Moreover, misuse of prescription and illicit narcotics has resulted in the current opioid crisis. The mu-opioid receptor is the cellular mediator of the effects of most commonly used opioids and is a prototypical G protein-coupled receptor (GPCR) where new pharmacological, signalling and cell biology concepts have been coined. This review summarises our knowledge of the life cycle of this therapeutic target including its biogenesis, trafficking to and from the plasma membrane, and how the regulation of these processes impacts its function and is related to pathophysiological conditions.

THE DARK SIDE OF OPIOID PAIN RELIEF

In the last couple of decades, the USA has experienced what is now known as "the opioid epidemic". This devastating situation started due to the indiscriminate prescription of therapeutic opioids to ease painful conditions, suffered by approximatively 30% of US citizens. It is estimated that 3-26% of chronic pain sufferers treated with opioids become addicts and 61% of drug-overdoses in 2014 in the USA involved pharmaceutical opioids. Many countries in the world have followed the path of the USA, with Canada having a similar prevalence of opioid misuse and other countries, such as the United Kingdom and The Netherlands, reporting an increase in opioid misuse and overdose.

In light of these events, research on the biology of opioid receptors (ORs), the cellular mediators of opioid-induced effects, has gained significant momentum. Since their discovery in the second half of the 20th century, many techniques have been used to study ORs; from radioligand binding in the 1970s and *in situ* hybridisation and molecular cloning in the 1990s [1], to the most recent visualisation of these receptors and their signalling *in vitro* and *in vivo* [2]. Of the four ORs subtypes [Mu (MOR), Delta (DOR), Kappa (KOR) and Opioid Receptor-Like 1 (ORL-1)], the MOR stands out for its role in opioid-induced analgesia and reward processing [3]. MORs play key roles in pain management, euphoria, sedation, miosis, addiction, truncation rigidity, nausea, and respiratory control in the central nervous system (CNS). Therefore, changes in their structure or function have significant consequences on those behaviours. Herein, we review the life cycle of the MOR, and engage the reader with the journey of this receptor, from its birth (biogenesis) to its death (degradation), highlighting the most relevant variations and disruptions along the way.

A NEW BEGINNING: A MESSENGER IS BORN

The four OR subtypes are part of the rhodopsin-like G protein–coupled receptors (GPCRs) superfamily and are 60% identical to each other. The greatest identity is found in the regions that encode for the 7-transmembrane domain and intracellular loops (70-90%), while the areas encoding for their N- and C-termini as well as the extracellular loops are more divergent. The MOR is encoded by the *OPRM1* gene in humans and the *Oprm1* gene in mice, and it is conserved across species. In humans the *OPRM1* gene is in the sixth chromosome (6q25.2) and contains at least 19 exons [4]. The rodent and human MOR share 94% sequence identity, with the N-terminus of the receptor being the area of highest divergence (with 65% sequence identity) [5]. This overall high sequence similarity explains why, to date, no significant differences in the structure, pharmacology and function have been reported across these species.

Single-Nucleotide Polymorphisms (SNPs) in the coding regions of the MOR have been widely studied, linked to alterations in signalling and suggested to underlie altered responses to opioids [6]. The rs1799971 SNP, located at position 118 in the exon 1 of the *OPRM1* gene, is the most studied and it encompasses the change of an adenine (A) to a guanine (G). Interestingly, the G-containing allele is present in 15-30% of Europeans, 40-50% of Asians and 1-3% of Latinos and African Americans [7]. At a protein level this SNP results in a change of amino acid at position 40, located in the N-terminus of the receptor; from an asparagine (Asn, N) to an aspartate (Asp, D). This N40D change removes a potential site for asparagine-linked glycosylation, which has been suggested to alter MOR affinity for different ligands, its transduction cascade [8] as well as the half-life of the receptor at the membrane [9]. Moreover, G118 adds a methylation site, which has been reported to result in a reduction of the levels of MOR messenger RNA (mRNA) [10]. Both alteration on glycosylation profiles as well as changes in the methylation patterns can

disrupt the normal activity of the receptor. Some reports suggest that the rs1799971 SNP interferes with the analgesic power of some opioids [11]. However, the effect of this SNP depends on the type of pain and/or opioid analgesic under evaluation [12]. Numerous groups have also investigated the relationship between this polymorphism and postoperative reactions to anaesthesia such as vomiting, nausea, dizziness and pruritus, albeit with some discrepancies [13, 14]. As in the case of opioid-induced side effects, there is contradicting data with regards to the effects of this SNP in alcoholism [15], nicotine addiction [16], heroin misuse and relapse [17], and gambling [18]. Interestingly, pain and reward processing are not the only behaviours reported to be affected by the rs1799971 SNP. Indeed, this SNP has been assessed for the role that MORs play in the **Hypothalamic–Pituitary–Adrenal (HPA) axis**, including stress, separation anxiety [7], aggressive behaviours [19] , and suicidal ideation [20]. Finally, other SNPs have also been associated with pathological conditions related to MOR signalling (Figure 1).

OPRM1 and *Oprm1* transcriptional regulation has been previously reviewed by Wei and Loh [21]. Under normal conditions, MORs are expressed mainly in the CNS, the HPA axis and the testis. Both the human and the mouse genes are regulated by two promoters; the distal and the proximal promoters, with the latter accounting for approximatively 95% of the transcriptional activity [21]. These regions contain many GpC sites that can be epigenetically modified [22] (Figure 2A). It has been shown that when these promoters are highly methylated, the expression of the *OPRM1/Oprm1* gene is supressed [23] and in the organs where the receptor is expressed, these GpC sites are usually unmethylated or hypomethylated.

Methylation of the CpG sites of the *OPRM1* promoter has been associated with several pathological conditions. *OPRM1* promoter hypermethylation is related to the risk for alcohol dependence [24]. Interestingly, a specific CpG cluster form the *OPRM1* promoter has been shown to be altered by naltrexone treatment of alcohol dependence in an age and ethnicity-dependent way [22]. Increased DNA methylation in the *OPRM1* gene is also associated with opioid dependence. The *OPRM1* promoter is hypermethylated in blood cells of opioid addicts. This methylation pattern is conserved in the sperm, suggesting an epigenetic heritability of opioid abuse or dependence phenotypes [25]. Increased *OPRM1* promoter methylation has also been reported in blood cells of males with opioid use disorder [26], in lymphocytes of former heroin addicts treated with methadone [27] and has been associated with worse neonatal abstinence syndrome outcomes [28].

The decrease in MOR expression caused by an increased *OPRM1* promoter methylation may account for dampened responses to endogenous and exogenous opioids. High methylation levels have been used as a biomarker to predict acute and chronic postsurgical pain [29], and *Oprm1* silencing in primary sensory neurons of the Dorsal Root Ganglia has been observed under neuropathic pain conditions. Finally, *OPRM1* promoter methylation has been investigated in the context of Alzheimer's disease (AD) with increased methylation detected in AD patients [30]. Intriguingly, MOR activation has recently been shown to attenuate Aβ oligomer‐induced neurotoxicity, suggesting another biomarker for AD diagnosis.

The *OPRM1/Oprm1* promoter can be epigenetically regulated by other modifications apart from DNA methylation. For example, **histone acetylation** and **methylation** have also been described to regulate *OPRM1* expression in global ischemia patients [31]. Nonetheless, epigenetics is not the only mechanism that participates in *OPRM1/Oprm1* transcription. Transcriptional factors that enhance (positive) or repress (negative) gene expression also play important roles in MOR expression (Figure 2B). In their extensive review, Wei and Loh list all the positive and negative transcriptional factors for mouse and human μ-opioid receptor gene [21].

While epigenetics and transcriptional factors control *OPRM1/Oprm1* transcription, once the mRNA is transcribed, it must reach its maturity by undergoing further regulatory mechanisms that will further influence the levels of the final protein product.

MATURE BUT NOT ENOUGH

Alternative splicing is commonly seen across GPCRs. However, the extensive alternative splicing of the *OPRM1* gene (generating more than 30 splice variants) is unusual and conserved in rodents and human [4, 32]. There are three main groups of *OPRM1* isoforms according to the number of transmembrane (TM) domains of the resulting protein (Figure 2C). The first group is the one with the traditional 7-TM domains structure. Proteins in this group preserve exons 1, 2 and 3 and differ on the exon with the STOP codon (C-terminal tail of the protein). The second group predominantly swaps exon 1 for exon 11, which results in 6-TM domains. However, some studies suggest that there are other exons that might also form 6-TM variants [33]. Finally, the last group only has 1-TM domain and the isoforms within it are non-functional. Expression of these isoforms is region specific in the brain [34] and their distribution differs between sexes [35].

The pharmacology of the MOR splice variants has been thoroughly investigated, most prominently by the groups of Pasternak and Pan [36, 37]. Generally, opioids produce analgesia through 7-TM variants. However, it has been suggested that morphine uses both 7-TM and 6-TM variants whereby 7-TM activation would trigger analgesia, reward, and respiratory depression, while chronic 6-TM activation participates in opioid-induced hyperalgesia (OIH), tolerance, and dependence. In this context, heteromerisation of 6-TM variants with the β2-adrenoreceptors (β2-ARs) has been suggested to contribute to OIH and in rodent models, it has been proposed that β2-ARs antagonists can efficiently block the hyperalgesic effects induced by 6-TM activation [38]. Endomorphin analogues and buprenorphine [39] have also been shown to act through 7-TM and 6-TM isoforms to produce analgesia. Drugs that only activate 6-TM such as 3-iodobenzoyl naltrexamine have been shown to have reduced side-effects while keeping their therapeutic potential [40], suggesting that bias towards a specific MOR isoform is a promising avenue for potential therapeutic interventions that may avoid the classical opioid adverse effects.

MicroRNAs (miRNAs) provide an additional regulatory mechanism for the *OPRM1.* Interestingly, morphine upregulates **let-7** miRNA expression in neuroblastoma-like cells and in a mouse model of opioid tolerance. Let-7 supressed MOR translation without altering *OPRM1* transcription [41], a let-7 miRNA inhibitor partially reduced morphine-induced antinociceptive tolerance, and miRNAs members of the let-7 family were upregulated in plasma after oral hydromorphone or oxycodone administration in humans [42], supporting the relationship between let-7 miRNAs and tolerance. miR-132 and miR-212 are miRNAs expressed in tandem [43] and regulated by the cAMP-response element binding protein (CREB). In zebrafish, morphine has been shown to regulate the miR-212/132 cluster, which in turn represses OPRM1 mRNA translation [44]. Other miRNAs that can alter MOR mRNA stability upon opioid treatment or in different pain conditions have also been reported, including miR-16 in lymphocytes [45], miR-134 in SH-SY5Y cells and Dorsal Root Ganglia [46] and miR-339-3 in the hippocampus [47]. Some miRNAs have been shown to regulate the mature mRNA of a specific MOR isoform. For example, chronic morphine treatment upregulates miR-103 and miR-107 in the striatum of morphine-tolerant mice, which in turn, specifically upregulates the isoform MOR-1A [48]. Chronic morphine treatment also

increases miR-378a-3p expression in the brainstem of morphine tolerant mice, which in turn, decreases the expression of the MOR-1B3 and the MOR-1B4 isoforms [49].

After alternative splicing and miRNA regulation, the mRNA reaches its maturity. Since GPCRs are plasma membrane proteins, the *OPRM1* messenger is translated by ribosomes located on the rough endoplasmic reticulum (ER) at the surface of the plasmatic membrane. The *OPRM1* mRNA 5'-**untranslated region (UTR)** contains four AUG codons. The first and the third AUG codons can function efficiently to initiate translation, however, translation from the third AUG negatively affects *OPRM1* expression at the level of translation [50]. It has also been suggested that the weak expression of MOR under normal conditions is due to **re-initiation** mechanisms.

Upon translation, MOR must be correctly folded. It has been suggested that the 1-TM isoforms act as chaperones for the 7-TM isoforms, facilitating their correct folding in the ER [51]. Several reports suggest that the 6-TM variants of MOR have altered subcellular expression and may require co-expression with other proteins (such as ORL-1 or β2-ARs) to reach the plasma membrane [33]. Moreover, hydrophobic ligands such as naloxone or etorphine can act as pharmacological chaperones helping the newly synthesised receptors to adopt their correct conformation and reach the plasma membrane [52] (Box 1).

MORs can undergo four different post-translational modifications (PTMs): glycosylation, palmitoylation, phosphorylation and **ubiquitination**, which have recently been reviewed by Duarte and Devi [53]. In this section we will briefly describe the two PTMs that occur before and facilitate the anchorage of the receptor into the plasma membrane (Figure 1). Glycosylation consists of the attachment of sugar molecules to a protein that will be located in the plasma membrane or secreted. In the ER, monosaccharides covalently attach to what will be the extracellular part of the protein and formation of more complex glycans occurs in the Golgi apparatus [54]. There are 5 asparagine residues located at the N-terminal of the MOR that can potentially be glycosylated (N-glycosylation). Interestingly, the MOR has been suggested to have brain-region specific glycosylation levels [55]. As mentioned above, the A118G polymorphism of the OPRM1 gene eliminates Asn40 and, therefore, a key site of glycosylation. This has been suggested to reduce the half-life of the receptor at the membrane [9] and alter MOR binding and signalling properties [8]. The other PTM that occurs just after MORs translation is palmitoylation, namely the covalent union between a palmitate group and a cysteine through a thiol bond. It occurs in the ER and contributes to GPCR normal functioning by facilitating their incorporation to the plasma membrane. At the mMOR, Cys170 (Cys172 in hMOR), in TM3, has been reported to be a palmitoylation site, contributing to the interaction with cholesterol at the membrane and facilitating MOR homodimerisation [56].

MOR AT WORK: A FRESHMAN AT THE CELL MEMBRANE

The plasma membrane is the classical site of action of GPCRs like MORs. MORs bind a great variety of ligands (Box 2) and the consequences of their activation depend on the ligand itself as well as the site of action within the cell and the organism. Once at the membrane, the minimal functional unit of the MOR has been demonstrated to be a monomer [57]. However, MORs can form **homodimers** [58] as well as **heterodimers** with other opioid receptor subtypes as well as with other GPCRs. There is evidence that MOR heterodimerisation with ORL-1, KORs and DORs can alter MOR-induced responses *in vitro* and *in vivo* [59]. Moreover, other GPCRs such as β2-ARs, and dopamine and cannabinoid receptors, have also been shown to heterodimerise with MORs [60]. Nonetheless, further research is required to support the physiological functions of MORs heterodimers and whether they represent potential therapeutic targets.

The classical signalling cascade triggered by the MOR is through the activation of inhibitory G proteins (Figure 3A). MOR activation results in neuron hyperpolarisation and an inhibition of neuronal firing which is crucial in the modulation of pain sensation. MORs are widely expressed at different levels in the pain pathway, from somatosensory neurons of the Dorsal Root Ganglia [61], dorsal horn (DH) nociceptive neurons (including excitatory interneurons and neurons from lamina I from the anterolateral tract) to brain areas of the descending modulatory pathway [62]. In the DH, when MORs are activated, there is an inhibition of neuropeptide release (Substance P and CGRP), which promotes analgesia. However, the major sites for the analgesic properties of MOR agonists are the periaqueductal gray and the rostral ventromedial medulla. These two areas of the descending modulatory pain pathway are characterised by two different populations of neurons that enhance (on-cells) or attenuate (off-cells) pain sensation by modulating DH nociceptive neurons activity. Because of the different locations of the MOR, in the on-cells or in inhibitory neurons controlling off-neurons activity, in both cases, the activation of MOR leads to analgesia [63].

MOR regulatory function of the **mesocorticolimbic system** (MCLS) is prominent in motivation, reward, and aversion. This system is composed of many interconnected brain regions, including canonical dopaminergic pathways. MOR inhibition of GABAergic neurons from the Ventral Tegmental Area (VTA) is key for the role of this receptor in processing the reward, not only from stimuli of natural reinforcers such as food, drink, social interaction, and sex, but also from opioid and alcohol addiction [64]. Those neurons inhibit VTA dopaminergic neurons that fire to Nucleus Accumbens (NAc). Therefore, when MORs are activated in these neurons, they cannot fire and dopamine is released in NAc, an event that is essential to process reinforcement [65-67]. Furthermore, MORs can also be found in NAc, and its specific location in this area encodes for reward or aversion [68]. In addition, the activation of MORs located in NAc increases social interaction and might also be crucial to understanding psychiatric disorders that involve social impairment [69]. MOR is also expressed in the prefrontal cortex (PFC). However, while it is still not known exactly where MORs are located within the complex neural network in this brain area, research suggests that their activation leads to the excitation of glutamatergic pyramidal neurons through a disinhibition mechanism. It is also interesting to notice that MOR upregulation and signalling in the PFC might increase the responsiveness towards opiate-like drugs and natural rewards. In fact, MOR activation in PFC may also account for food-seeking behaviour and increased alcohol drinking in rats. Moreover, MORs in PFC also seem modulate inhibitory control and impulsivity, since MOR knockout mice, and rats treated with a MOR antagonist show decreased impulsivity while carrying out various tasks [70].

There is accumulating evidence that relates painful conditions to drug abuse and misuse. Interestingly, MORs play a role in this comorbidity since, even though pain and reward are opposed processes [71], they both need MORs [3, 61]. In fact, pain negatively impacts on motivation by altering the MCLS normal functioning [72-74]. Inflammatory pain blunts neuronal activation induced by intra-VTA DAMGO administration in some VTA projecting areas. Therefore, inflammatory pain induces MOR desensitisation in VTA, which is relevant for addictive behaviours [73]. Interestingly, Hipólito and collaborators discovered that MORs from the MCLS were desensitised in animals that suffered inflammatory pain and had a previous history of heroin consumption. This alteration promoted relapse into heroin consumption [72].

Finally, prolonged use of opioids can also lead to OIH, a significant clinical problem that defines a state of nociceptive sensitisation and is characterised by a paradoxical response whereby a patient receiving opioids to treat pain becomes more sensitive to certain painful stimuli. The

precise molecular mechanisms underlying OIH are not yet understood and are the subject of intense study [75].

THE DECLINE AFTER A LIFE OF SERVICE

Following activation, MORs undergo rapid phosphorylation, which triggers a decline in their G protein signalling as well as the recruitment of proteins that will result in receptor internalisation (Figure 3B). Phosphorylation of the MOR and its impact on receptor desensitisation and internalisation has been widely studied [76, 77]. While this phosphorylation is mostly mediated by G protein receptor kinases (GRKs) [78], there is also evidence that other intracellular kinases such as Ca^{2+}/calmodulin-dependent protein kinase (CAMK)II, protooncogene tyrosine-protein kinase (Src), and protein kinase C (PKC) can phosphorylate the receptor [53]. Importantly, the phosphorylation barcode, as well as its signalling consequences, are highly dependent on the ligand bound to the MOR. Sequential and hierarchical phosphorylation of MORs results in the recruitment of the cytosolic protein **β-Arrestin**. MOR phosphorylation and β-arrestin recruitment result in MORs desensitisation, namely the uncoupling of the G protein signalling cascades, which has been proposed to be the initial step leading to opioid tolerance [79].

MOR can recruit β-arrestin1 and 2 isoforms, although this seems to be agonist-dependent. While β-arrestins are essential to initiate MORs endocytosis via clathrin-coated pits, not all ligands that recruit arrestins induce robust receptor internalisation. This differential ability of agonists to induce receptor internalisation has been linked to the phosphorylation barcodes mentioned earlier [76, 77]. As with other GPCRs, β-arrestins have also been suggested to participate in MOR signalling [80], although the physiological relevance from this signalling is still not fully understood [81]. Moreover, β-arrestin1 can promote MOR ubiquitination by acting as E3-ubiquitin ligase adaptors [53]. This ubiquitination is also ligand-dependent (occurs with high efficacy ligands such as DAMGO, but not with morphine) and results in degradation of the MOR within the lysosomes. However, MOR is not always degraded after endocytosis. Instead, and as opposed to the DOR, internalised MORs are usually recycled back to the plasma membrane, in a process known as resensitisation [82]. Each MOR isoform has been suggested to have different recycling rates and the mechanisms underlying receptor recycling are starting to be elucidated [83]. More recently, elegant microscopy studies have provided unprecedented insight into the mechanisms of MOR internalisation and recycling in presynaptic terminals, and how it differs from the classical internalisation-resensitisation paradigms that operate post-synaptically [84]. It is clear that MOR internalisation, recycling, and degradation are processes related to the development of tolerance. However, the molecular and cellular mechanisms, as well as the neural circuits changes which lead to reduced opioid responsiveness are just starting to be unravelled [85].

Interestingly, the regulatory mechanisms of MOR have been the focus of intense research in an attempt to generate opioid analgesics devoid of, or with limited, side effects. In addition to tolerance, opioid treatment also results in significant respiratory depression, constipation, dependence, and addiction. Thus, efforts to separate the therapeutic and adverse effects of MOR agonists have dominated drug discovery at this receptor. These efforts were spearheaded by the observation of enhanced and prolonged morphine-induced analgesia and decreased tolerance in β-arrestin2 knockout mice, presumably due to decreased receptor desensitisation [86, 87]. These β-arrestin2 knockout mice were later shown to display decreased morphine-induced respiratory depression and constipation [88]. This finding then led to investigations

focused on the discovery of MOR agonists that would avoid the "β-arrestin pathway" while still promoting G protein signalling; namely G protein-biased agonists [89].

There are now numerous descriptions of MOR G protein biased agonists. While some of these descriptions are limited to observations in recombinant cell lines, others have been tested *in vivo*, showing promising results. TRV130 (oliceridine, Olinvyk) has been the only new opioid agonist that has reached clinical trials and FDA applications [90], and was recently approved for the management of moderate to severe acute pain. SR17018 and mitragynine pseudoindoxyl are other compounds that have shown decreased opioid-induced side effects in pre-clinical models [91, 92]. However, there is now mounting evidence that suggests that while these compounds may still provide improved therapeutic profiles, the mechanism underlying these profiles is unlikely to be linked to an arrestin-dependent signal. Perhaps the most significant evidence is that derived from studies revisiting the initial hypothesis in β-arrestin2 knock-out mice which shows that morphine-induced respiratory depression is independent of β-arrestin2 signalling [93]. Additionally, in knock-in mice expressing a MOR unable to recruit β-arrestin (by deletion of the phosphorylation sites that facilitate this recruitment), the opioid side effects of respiratory depression, constipation and withdrawal are exacerbated while only tolerance seems to be diminished [94]. In light of this data, the classical pharmacological concepts of partial agonism and low intrinsic efficacy have been proposed as potential explanations for the different therapeutic windows of the so-called G protein biased opioids [81, 95, 96]. Finally, it is important to highlight that while this research has focused on the development of tolerance, respiratory depression, and constipation, most of the novel opioids described as biased still result in significant abuse liability [97].

CONCLUDING REMARKS

From birth as an mRNA to death as a heavily modified protein, MORs are essential for many vital activities. However, these events can be thwarted by genetic variations, alterations in expression patterns, and changes in MOR signalling and regulation. While research has provided invaluable information that sheds light on MORs function, in many aspects we are still partially blind and further research is needed to untangle the ins and outs of the MOR life cycle and how it changes in diseased conditions. MOR hetero- and homodimerisation, pain comorbidities, biased/partial agonism, MOR isoforms and their role in neuroinflammation, chronic pain, and other pathologies represent some of the areas in which the field is moving at a vertiginous pace (see Outstanding Questions).

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GLOSSARY

Dimer: Protein complex formed by two proteins. **Homodimers** contain two identical protomers while **heterodimers** contain two different protomers.

Histone acetylation: Addition of an acetyl residue to N-terminal tail lysines of the histone core mediated by the histone acetyltransferase enzyme. This modification eases the DNA-histones interaction, facilitating transcription. Histone acetylation can be reversed by the action of the histone deacetylase enzyme, which impedes DNA transcription.

Histone methylation: Some amino acids from the histone core N-terminal tails can be methylated by methyltransferases. Depending on the methylated residue this epigenetic modification can be repressive or activating.

Hypothalamic–Pituitary–Adrenal (HPA) axis: This axis that comprises the hypothalamus, the pituitary gland and the adrenal glands is part of the neuroendocrine system and its connection with the CNS. It plays a major role in stress management and steroid hormones production.

Let-7 family: Let-7 was the first miRNA family discovered in humans, although it was first found in *C. elegans*, and is conserved across different species.

Mesocorticolimbic system (MCLS): This system is a dopamine-dependent pathway that facilitates behaviours that lead to survival. It has been associated with the behaviour modulation for reward and motivation but also aversion.

MicroRNAs (miRNAs): Single strand RNA that can regulate gene expression. miRNAs need to be transcripted and processed. Drosha and Dicer are two proteins with catalytic power and carry out miRNAs processing, the former within the nucleus and the latter in the cytoplasm. In between the action of each of them, exportin 5 takes the non-mature miRNA out of the nucleus. After being processed, miRNAs are incorporated to the RNA-induced silencing complex (RISC), which in turn, by complementary binding the 3'-UTR, sequester the mRNA which is then degraded by exonucleases.

Re-initiation: Event that happens when ribosomes resume scanning and re-initiate at downstream sites when the first AUG is followed shortly by a frame terminator codon.

Ubiquitination: This posttranslational modification requires three enzymes (E). E1-activating enzyme is the first of them and activates the ubiquitin molecule at its C-terminal by using ATP. Afterwards, an E2-conjugating enzyme binds the activated ubiquitin through a cysteine residue. Finally, an E3-ubiquitin ligase ends the process by transferring the ubiquitin to the substrate.

Untranslated region (UTR): regions flanking the mRNAs coding region. Both the 3'-UTR and the 5'-UTR regions play an important role in mRNA regulation and translation.

β-Arrestin: cytosolic proteins with two crescent-shaped beta-sandwiches and a central crest that bind to phosphorylated GPCRs to promote receptor internalisation. For some GPCRs β-arrestins have been proposed to scaffold the formation of signalling complexes.

TEXT BOXES

***Box 1*. Protein Structures of the mu-opioid receptor**

The first crystal structure of the MOR was first described in 2012 [98]. It belonged to a construct of the mouse MOR (where the third intracellular loop was replaced with a T4 lysozyme) bound to a morphinian-like antagonist. This structure showed the typical 7-TM organisation of the receptor and has been used for significant *in silico* drug discovery efforts [99]. In 2015, the crystal structure of an agonist-bound mouse MOR and a G protein mimetic nanobody (Nb39) was

solved, representing the first active state structure of the OR family [100]. This structure and related NMR studies [101] revealed how agonist and G protein binding at the MOR induce a conformational change with conserved shifts in domain positioning characteristic of GPCR activation mechanisms. This change in receptor conformation, then facilitates the conformational changes in the Gαi G protein that permits nucleotide exchange. Finally, in 2018, Koehl et al. presented the cryo-electron microscopy structure of a DAMGO-bound MOR in complex with the Gαiβγ heterotrimeric protein in the nucleotide free state. This was one of the first structures of a GPCR- Gαi complex. While this active structure was almost identical to the one previously described by Huang et al. in 2015, it showed the receptor-induced changes in the Gαi protein conformation that permit signalling via GDP/GTP exchange [102].

***Box 2*. Endogenous and exogenous ligands of the mu-opioid receptor**

Endogenous opioid peptides derive from the proteolytic cleavage of larger prepropeptides and they all can bind the MOR albeit with different affinities. β-endorphins have high affinity for MOR and are potent analgesics with long lasting effects. Furthermore, β-endorphins also participate in reward processing and drug addiction. Enkephalins (Met-enkephalin and Leu-enkephalin) display higher affinity for DORs, although they also bind and activate MORs. Similarly, dynorphins, prototypical KOR ligands, can also bind MOR with low affinity. Finally, endomorphins are potent and selective MORs agonists, even though the gene encoding for these tetrapeptides or their protein precursors have not been identified.

MORs are also activated by multiple exogenous ligands. These include not only opioids but also metabolites of other substances such as alcohol. Morphine is one of the most used analgesic drugs in clinic and its actions have been widely proven to be mediated by the MOR [103]. Morphine derivatives such as codeine and oxycodone are also used for the treatment of mild pain and cough or severe pain respectively, although they still display common opioid side effects. Another family of drugs that can activate MORs are piperidine derivatives, among which the most common is fentanyl. Fentanyl and its derivatives are over a hundred times more potent than morphine and, thus, they are used to ease moderate to severe pain in clinic. However, they still present opioid-induced side effects and their recreational use mixed with other drugs of abuse has recently skyrocketed. Finally, methadone (R enantiomer) and buprenorphine are MOR agonists with different efficacies that are widely used as maintenance treatments for opioid addiction.

Naloxone is the prototypical OR antagonist. It is one of the most widely used opioids, the primary treatment for overdose, and its use has been instrumental in understanding MOR actions in preclinical studies. Other antagonists such as β-funaltrexamine (β-FNA) are highly selective for MOR, however, their pharmacokinetic properties (e.g irreversible antagonist) represent significant caveats for their clinical use and it has mainly been used to study OR pharmacology.

Drugs that undergo metabolic reactions within the organism can generate molecules that bind and activate MORs. For example, (R/S)-salsolinol is a tetrahydroisoquinoline that has been proposed as the ethanol fraction that binds MORs [104]. Studies suggest that (R/S)-salsolinol binding pose at the MORs is like that of morphine [105]. *In vivo*, salsolinol triggers a response in the MCLS like the one triggered by opioids, further supporting the action of this molecule as an MOR ligand.

FIGURE LEGENDS

Figure 1. **Human MOR structural SNPs and posttranslational modifications.** Glycosylation occurs on the asparagine at position 40 whereas palmitoylation occurs on the cysteine at position 172 (C3.55). Figure adapted from Knapman and Connor (2015).

Figure 2. OPRM1 **regulation.** A) GpC islands methylation by DNA methyltransferase. Cysteines get a methyl group, impeding TFs promoter recognition and transcription. B) Main exons of the OPRM1 gene with the three groups of splicing variants and exon 1 proximal (PP) and distal (DP) promoters regulation by transcriptional factors (TFs). Green spot TFs promote *OPRM1* transcription whereas red spot TFs inhibit it. Figure adapted from Wei and Loh (2011) and Puig and Gutstein (2017). C) MOR isoforms. The colours of the fractions within each isoform match the colours of the exons in Figure 2B.

Figure 3. **MOR life cycle.** A) From DNA to its activation. MOR mRNA is transcribed and translocated to the rough endoplasmic reticulum in the ribosomes where translation occurs. 1-TM splicing variant chaperons the 6-TM and 7-TM splicing variants. The latter undergoes posttranslational modifications in the Golgi apparatus and gets into the cell membrane. Upon the binding of an agonist, activation of MOR promotes dissociation of inhibitory Gαi and Gβγ protein subunits. Gαi subunits typically suppress adenylate cyclase (AC), resulting in decreases in intracellular cAMP. Presynaptically, Gβγ subunits inhibit voltage-gated calcium channel (VGCC) opening. Postsynaptically, Gβγ subunits activate G-protein inwardly rectifying potassium (GIRK) channels. Altogether, this results in reduced neurotransmitter release and membrane hyperpolarisation. MORs activation also triggers kinase cascades that end with the translocation to the nucleus of some transcriptional factors. B) MOR internalisation, recycling, and degradation. After MORs activation triggers the G protein-induced cascade, GRK recruitment and MOR phosphorylation occur. These events are followed by β-arrestin recruitment and receptor internalisation. Upon ligand and arrestin dissociation, MORs are either recycled to the cell membrane or ubiquitinated and degraded in lysosomal vesicles.

OUSTANDING QUESTIONS

- Can epigenetics, mRNA processing or post-translational modifications of the MOR gene or protein be targeted therapeutically for the generation of novel, safer analgesics?
- Can epigenetic modifications in the OPRM1 triggered by painful conditions or a drug use disorder be reversed?
- Can the methylation state of the OPRM1 promoter be used as a biomarker for the assessment of risk associated with substance use disorders?
- What are the cellular mechanisms that explain the improved side effect profiles of novel opioids?
- Do miRNAs represent a viable strategy for the treatment of opioid-tolerance?
- What are the cellular and neuronal mechanisms controlling alterations of MORs in MSCL system upon pain that have consequences for addiction and reward processes? Are they ligand-dependent?

HIGHLIGHTS

- Single-Nucleotide Polymorphisms of OPRM1 resulting in amino acid changes in the protein sequence (e.g N40D) may have effects in pain and reward processing.
- Epigenetic changes, as well as positive and negative transcription factors, are key controllers of receptor expression.
- Alternative splicing generates over 20 different isoforms of MOR with distinct pharmacological characteristics; these include isoforms with 7-TM, 6-TM and 1-TM.
- MicroRNAs affect OPRM1 mRNA stability upon treatment with opioid drugs.
- Ligand-dependent regulation of MOR has been the subject of intense research focussed on the development of improved analgesics.
- Pain might be a risk factor towards drug addiction. The role of MORs in pain and reward processes highlights the role of this receptor in such comorbidity.

Figure 1

A6V	rs1799972	R181C	rs79910351
N40D	rs1799971	N190K	rs34074916
S42C	rs76546679	C192F	rs62638690
D51N	rs1042753	R260H	rs1799974
G63V	rs9282817	R265H	rs376950705
S66F	rs9282819	S268P	rs200811844
L85I	rs76773039	D274N	rs17174829
S147C	rs17174794	V293A	rs11575856
N152D	rs17174801		

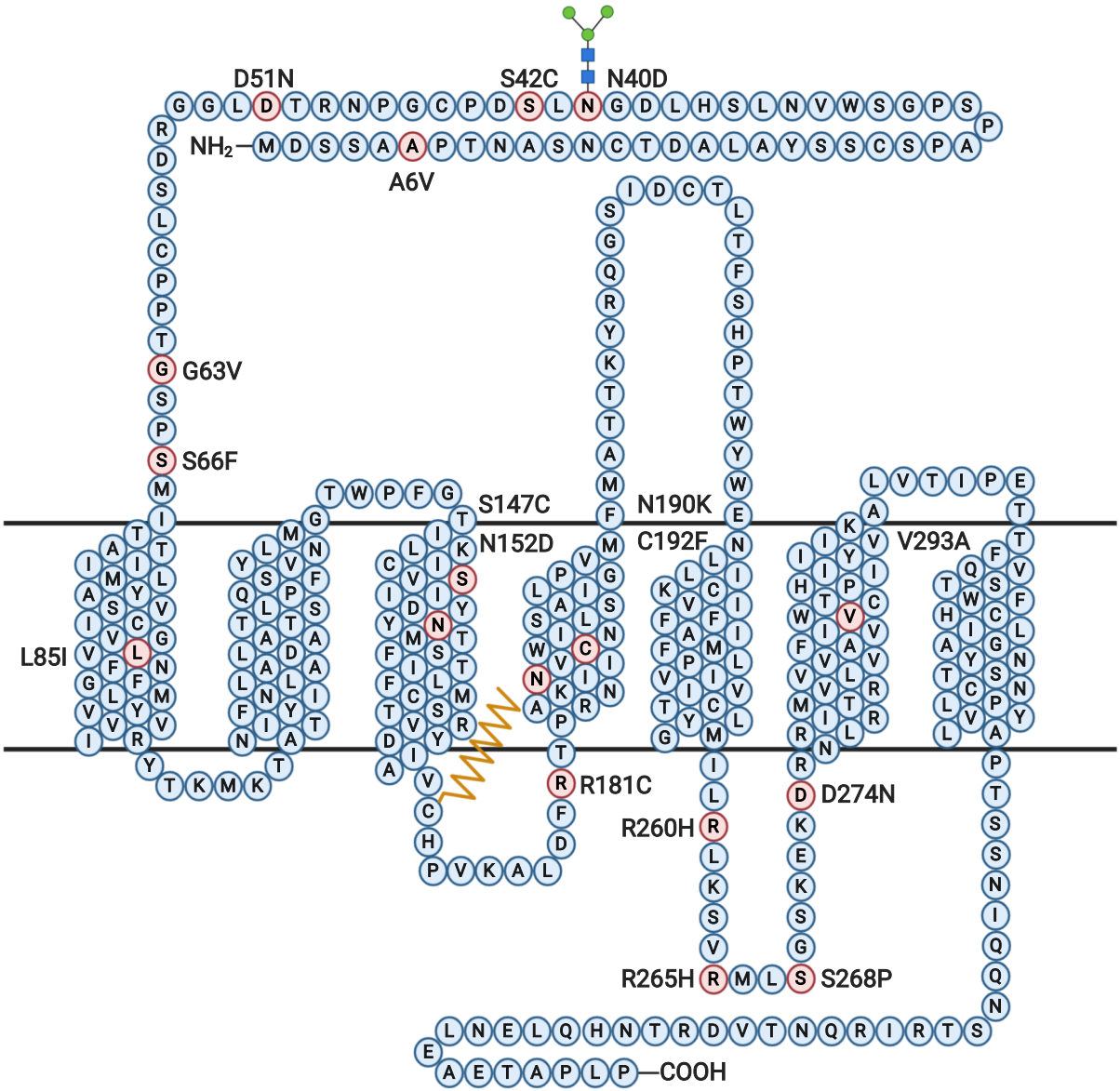


Figure 2

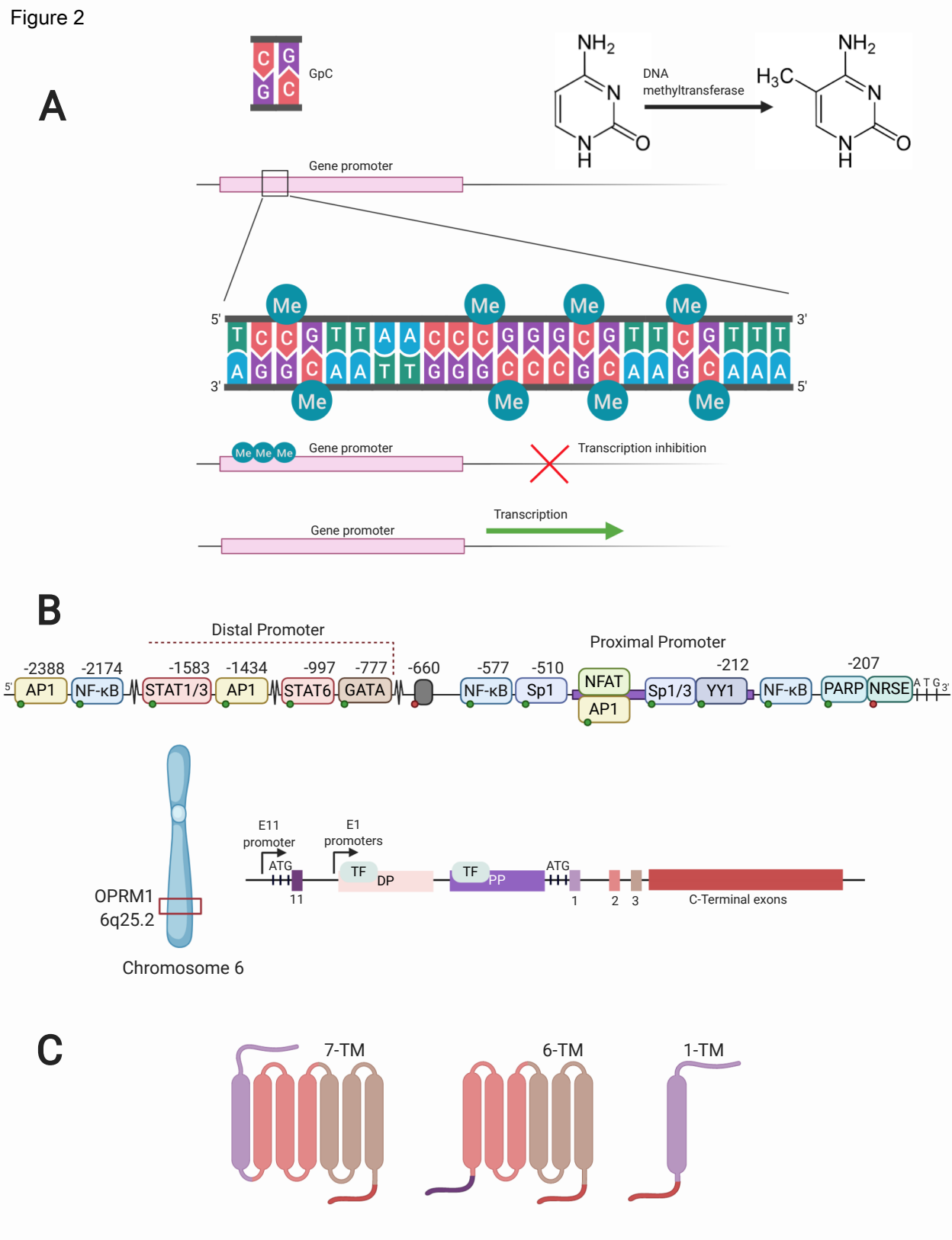

A
GpC
DNA methyltransferase
Gene promoter
Me
5'
3'
Gene promoter
Transcription inhibition
Transcription
Gene promoter
B
Distal Promoter
Proximal Promoter
-2388
-2174
-1583
-1434
-997
-777
-660
-577
-510
-212
-207
AP1
NF-κB
STAT1/3
AP1
STAT6
GATA
NF-κB
Sp1
NFAT
AP1
Sp1/3
YY1
NF-κB
PARP
NRSE
OPRM1
6q25.2
Chromosome 6
E11 promoter
E1 promoters
ATG
TF
DP
TF
PP
ATG
11
1
2
3
C-Terminal exons
C
7-TM
6-TM
1-TM

Figure 3

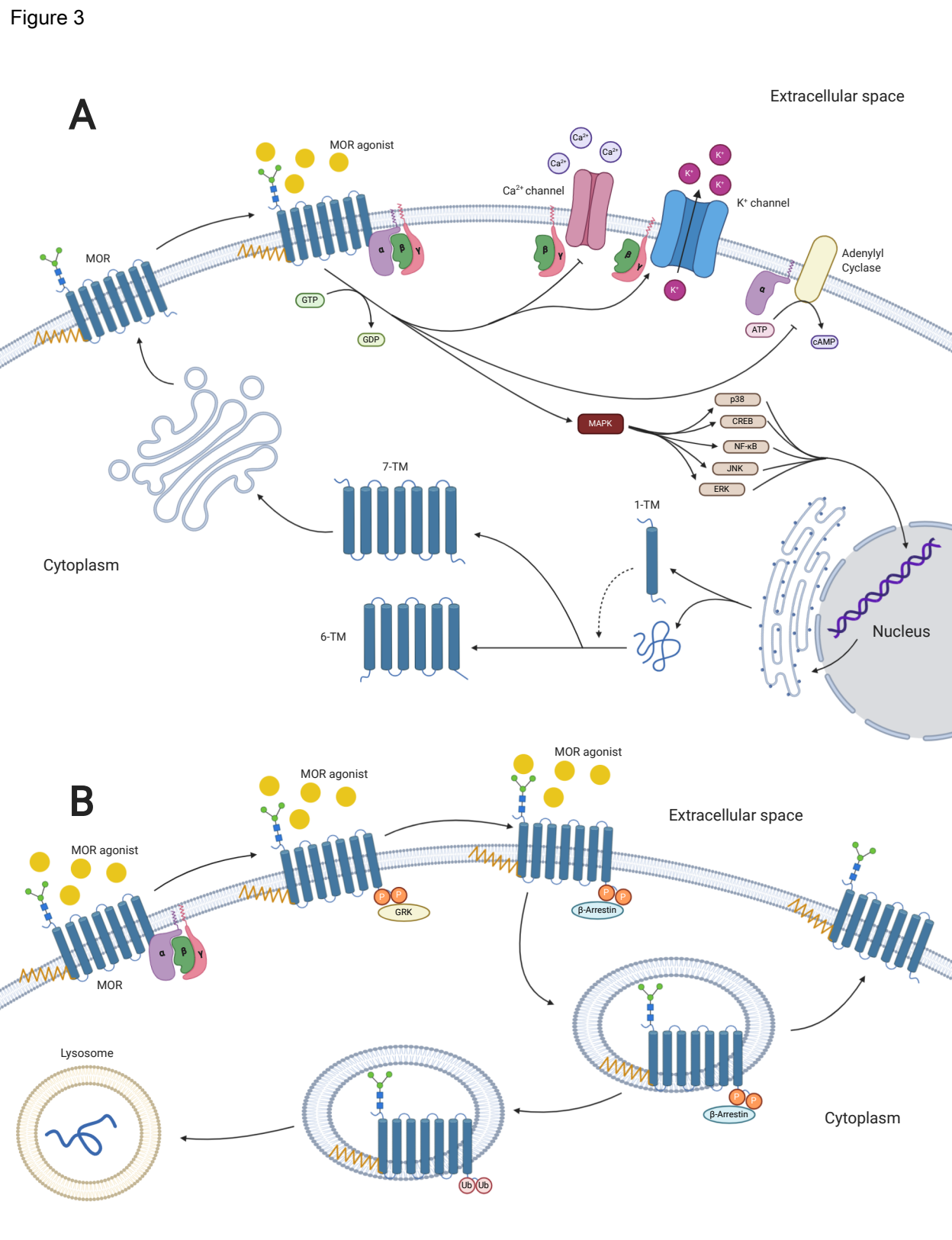

A
Extracellular space
MOR agonist
MOR
Ca2+ channel
K+ channel
Adenylyl Cyclase
GTP
GDP
ATP
cAMP
MAPK
p38
CREB
NF-κB
JNK
ERK
7-TM
1-TM
6-TM
Cytoplasm
Nucleus
B
MOR agonist
MOR agonist
MOR agonist
Extracellular space
MOR
GRK
β-Arrestin
Lysosome
β-Arrestin
Cytoplasm
Ub
Ub

Figure Box 1

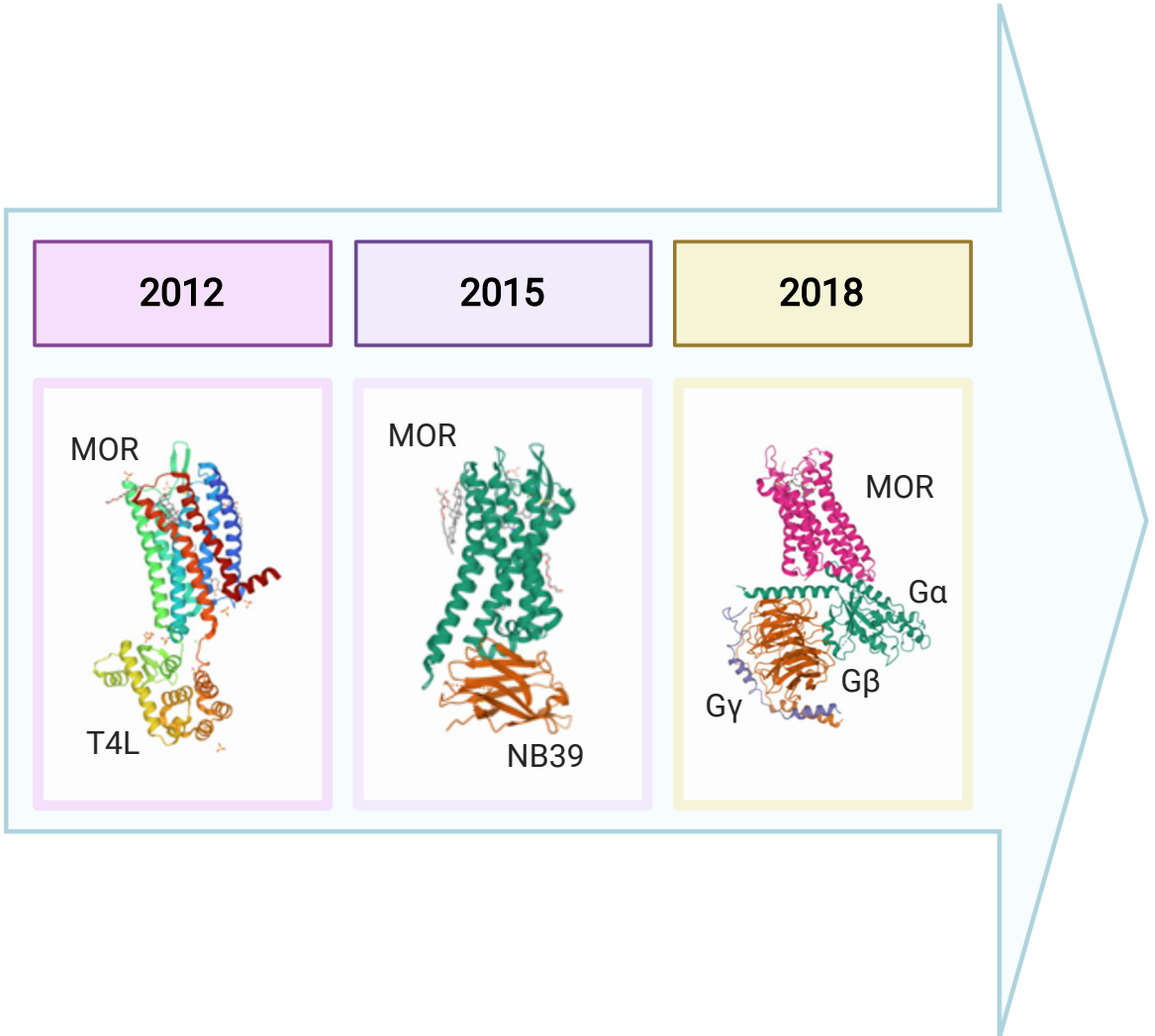
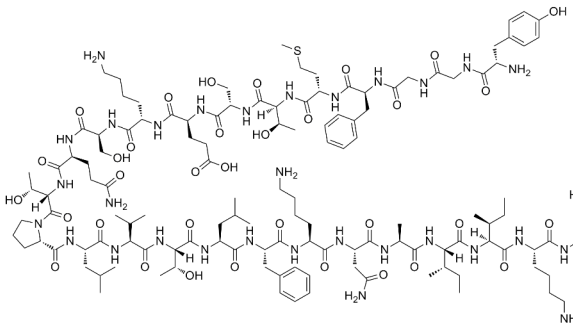
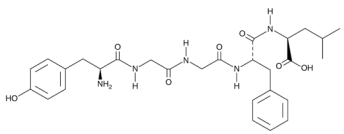


Figure Box 2

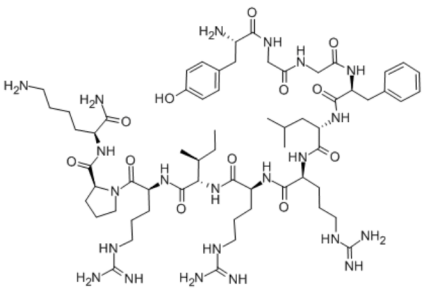
Endogenous ligands



β endorphin

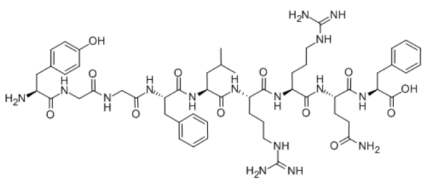


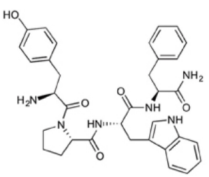
Leu-enkephalin



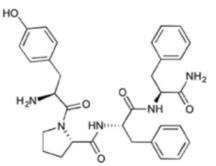
Dynorphin A

Dynorphin B



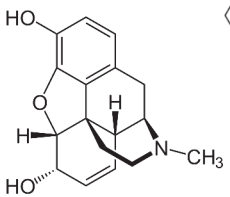


Endomorphin 1

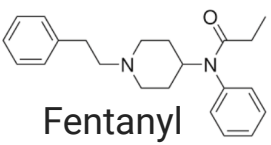


Endomorphin 2

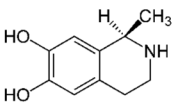
Exogenous ligands



Morphine

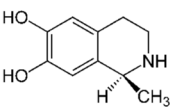


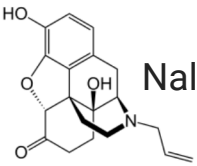
Fentanyl



R-Salsolinol

S-Salsolinol





Naloxone