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Opioid Receptors and their complex mechanisms of action

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Abstract

Opioids are some of the most powerful and effective medications for relieving pain; however, their extended usage comes with the risk of abuse, dependency, and rapid development of tolerance. Opioids bind to opioid receptors (OR) on neurons and inhibit pain signals from traveling to the brain by blocking neurotransmitter release, although recent work has shown that other receptors play a role in opioid signaling as well. Mu opioid receptors are the most commonly targeted OR because of their high agonist efficacy but are consequently risky in treating chronic illnesses because of their potential fatal side effects and robust formation of tolerance. Receptor desensitization can occur when opioid ligands continually bind ORs; during desensitization, phosphorylation of the OR carboxyl (C)-terminal tail recruits arrestins which endocytose the receptor for recycling or degradation. Acute internalization of individual receptors can contribute to long-term tolerance at the whole body level, although not all neurons build tolerance at the same rate. In recent years, new drugs and modulators have been developed that lack classical opioid side effects. There has also been insight into the related genetics and epigenetics of opioid use and tolerance. Further studies on these synthetic drugs can help create prototypes for safer and more efficient therapeutic opioids that can fight tolerance and withdrawal.

Keywords

G-protein-coupled receptor, Opioid receptor, Receptor phosphorylation, Desensitization, Tolerance, Kölliker-Fuse neurons, Biased signaling, Withdrawal, Synthetic opioids

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Introduction

G-protein coupled receptors (GPCR) are the most abundant class of receptors in the body and most commonly targeted by drugs. GPCRs have 7 transmembrane domains with 3 intracellular loops and the Carboxyl-terminal (C-terminal), as well as 3 extracellular loops and the extracellular amino-terminal (N-terminal), where receptor ligands bind to. The 3 G-protein subunits α, β, and γ are associated with the intracellular portion of the receptor to relay signals (1). In the deactivated form of the receptor complex, guanosine diphosphate (GDP) is bound to the α unit. When ligand-activated, a conformational change occurs: GDP is released and the α unit binds to guanosine triphosphate (GTP). Upon GTP binding, the α unit disassociates from the βγ complex and creates a further conformational change. Now, the subunits in their active form can relay downstream signals by binding to target proteins, kinases, or ion channels (Figure 1). Subunit α mostly activates effector enzymes, such as adenylyl cyclase. The βγ dimer regulates ion channels, which change a neuron's polarization. Decades ago it was discovered that opioid receptors (OR) inhibit voltage-gated calcium channels through the βγ complex instead of the α unit; however, the α unit still plays a role in inhibition because in α-knockout mice, calcium (Ca^{2+}) channel inhibition was stopped (2). GPCR signaling is beneficial to cellular systems as it allows external ligands that are membrane-impermeable to initiate intracellular effects.

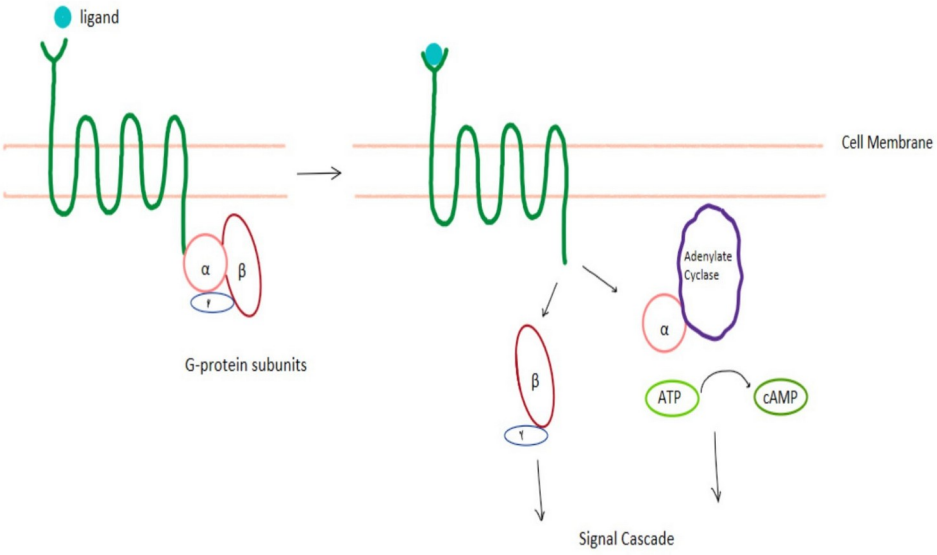

Figure 1. G-proteins disasssociation upon ligand binding to the G Protein Coupled Receptor (GPCR). Use of second messengers such as Adenylate cyclase and cAMP inducing a signal cascade

Other than G-protein signaling, GPCRs can also participate in arrestin signaling. G-protein coupled receptor kinases (GRK) phosphorylate serine/threonine sites on the GPCR C-terminal which become binding sites for arrestins (3). Upon binding to the phosphorylated C-terminal tail sites, the arrestins go through conformational change and, with the help of clathrin adaptor protein 2 complex and extracellular signal-regulated kinase, endocytose the receptor (Figure 2) (4). Thus, arrestin signaling deactivates G-

protein signaling and contributes to decreased responsiveness to the external ligand.

A GPCR's ability to relay downstream signals can decrease because of continuous ligand binding in a process known as desensitization. The βγ complex was originally believed to have a minimal and passive contribution to GPCR signaling. However, it has been shown that the βγ subunit has an important role in regulating effectors, scaffolding proteins, and recruiting GRKs (5). Receptors can also become internalized through arrestins, as described previously, where they can either be degraded or recycled back to the membrane. Internalization is one of the ways receptors become desensitized to ligands as it reduces the number of available surface-facing receptors, lowering the chance of binding to ligands.

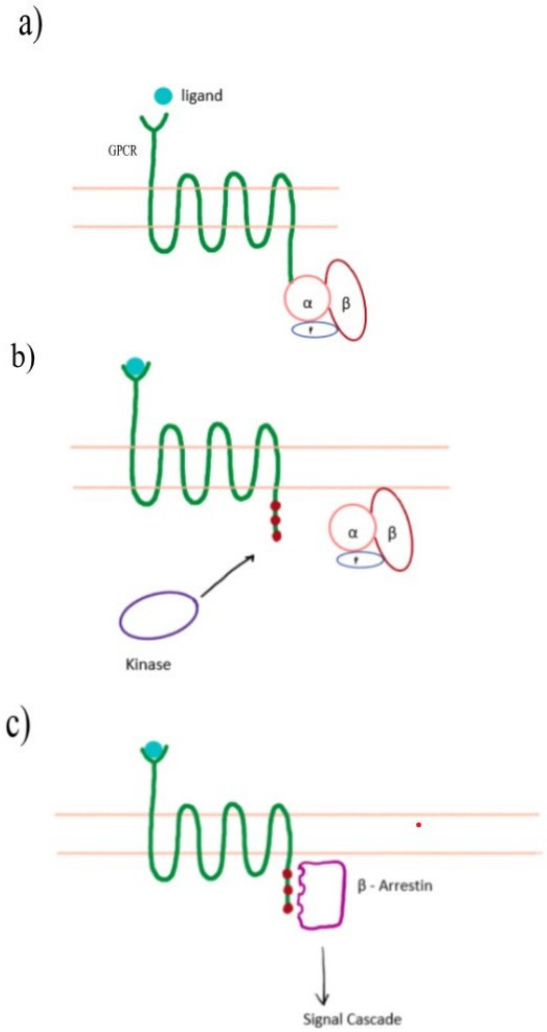

Figure 2. Arrestin signaling (as an alternative to G protein signaling) by phosphorylation of the receptor. a) Ligand induces G-protein dissociation, b) Kinase phosphorylates the open C-terminal, c) Arrestins bind to the phosphorylation sites and induce downregulation through desensitization, internalization and ubiquitination.

This review discusses the different downstream effects of OR ligands (summarized in Table 1), receptor desensitization, tolerance, and lastly, novel findings on synthetic opioid ligands that highlight future therapeutic benefits.

Table 1. Summary of opioid ligands with target receptor and mechanism of action

Ligand	**Target Receptor**	**Mechanism of Binding**	**Signaling Outcome**	**Emerging/In use**
6β-naltrexol	Mu	Antagonist	Alleviate withdrawal. Possibly accelerate ligand-free signaling MOR to it's basal state	Emerging
AMNO82	mGlu7	Agonist	Tolerance regulation	Emerging
BMS-986122	Mu	Allosteric Activator	Full activation of receptor, enhances agonist effect	Emerging
CTOP	Mu	Antagonist	Inhibit acute Morphine-Analgesia	
DADLE	Delta	Agonist	Neuroprotective properties	In use
DAMGO	Mu	Agonist	increased MOR dimerization and arrestin recruitment	In use
GAT358	CB1	Allosteric Inhibitor	Reduced dopamine efflux	Emerging
IBNtxA	6TM Mu	Agonist	Analgesia without fatal side effects	Emerging
LY354740	mGlu2/3	Agonist	Tolerance regulation	Emerging
Met-enkephalin	Delta	Agonist	Neural activation	Naturally occurring in the body
Methadone	Mu	Agonist	Alleviates withdrawal symptoms and drug cravings	In use
Morphine	Mu	Agonist	Analgesia	In use
MTEP	mGlu5	antagonist	Tolerance regulation	Emerging
mu-PAM	Mu	Allosteric Activator	Enhanced affinity to endogenous opioids, lowered respiratory depression and constipation, increased agonist G-protein bias Antinociceptive effects alone	Emerging
Naloxone	Mu	Antagonist	Blocks active site. Induces inverse effects after Morphine treatment	In use
Naltrexone	Mu. Kappa and Delta to a lesser extent	Antagonist	Blocks active site. Induces inverse effects after Morphine treatment	In use
SR-17018		Agonist with G-protein Bias	Lowered withdrawal and slowed tolerance	Emerging
U-50488	Kappa	Agonist	Substitute IBNtxA	Emerging

Discussion

Mu, Kappa, and Delta Opioid Receptors

Opioids bind to a specific family of GPCR receptors, collectively called the opioid receptors (OR). ORs are separated into subtypes: Mu, Delta, and Kappa (abbreviated as MOR, DOR, and KOR respectively). MORs are the most common drug targets because of their ability to produce strong euphoria and analgesia; however, with their impressive agonist efficacy and dopamine release comes a high chance of abuse and addiction that makes them dangerous drug targets in treating chronic pain (6). DORs have shown greater application with long-term pain, and while their analgesic effects are lesser, their potential of dependence is too. Opioid targeting of KORs produces opposite effects as MORs and DORs, such as dysphoria, stress, and inhibition of dopamine release (7).

Downstream signaling of Opioid Receptors

Opioid drugs bind to ORs and activate downstream signals that prevent pain signal transmission. Normally, in the presence of a pain-inducing stimulus, sodium (Na^+) channels open, causing the electrochemical gradient of the neuron membrane to reverse in a process known as depolarization. This sudden change of polarity can fire an action potential, which travels through synapses until reaching the brain, whereupon pain is perceived. Electrophysiological methods revealed DORs and MORs activated potassium channels, allowing the rapid influx of potassium (K^+) ions into the cell, hyperpolarizing the cell, and preventing action potentials and the release of neurotransmitters (2,8). The lack of neurotransmitters prevents pain signals from reaching the brain.

Inhibition of adenylyl cyclase, an enzyme most abundant in the striatum, is an imperative step in opioid signal transduction. Adenylyl cyclase type 5 (AC5) knockout mice ($AC5^{-/-}$) showed greater latency in hot plate tests, an experiment used to measure the threshold of pain perception based on the length of time to paw licking or enclosure escape. $AC5^{-/-}$ mice showed similar behaviors to morphine exposure as MOR knockout mice; morphine tolerance was observed in both $AC5^{-/-}$ and WT mice, but developed slower in the $AC5^{-/-}$ group. Analgesia and dependence were also reduced, but not eliminated in the $AC5^{-/-}$ murine group (9). These results suggest that AC5 is integral in mediating MOR and DOR signaling; however, KOR signaling remained unchanged in WT and $AC5^{-/-}$ groups, indicating that AC5 does not mediate KOR signaling. Further studies on AC5 are necessary to better understand OR downstream effects and how to regulate them (refer to Figure 1).

Formation of MOR heteromers

The formation of heteromers of MORs, such as a vasopressin 1b receptor (V1bR)/β-arrestin/mu-complex, can alter receptor sensitivity to the ligand. Mice lacking V1bRs were shown to be more sensitive to morphine (10). β-arrestins normally interact with the leucine sites of the V1bR C-terminal tail and thus gain access to MORs only when V1bRs are present. Therefore, the lack of V1bRs reduced β-arrestin interaction with MORs leading to less receptor internalization. Additionally, when the V1bR leucine sites needed for β-arrestin interaction were removed *via* gene editing, morphine antinociception (the body's response to pain) increased (10). These results demonstrate that V1bR has a role in inhibiting morphine

analgesia, though additional research is required to determine its role in limiting analgesic tolerance.

In contrast, a different study concluded that MORs at their baseline are mostly monomeric, and when they did form dimers, it was only for a short period. The agonist DAMGO increased MOR dimerization and arrestin recruitment in similar periods of time; however, when DAMGO was delivered in the presence of Compound 101, an inhibitor of GRK (and therefore phosphorylation), mice with phosphorylation deficient receptors did not exhibit MOR dimerization. These results raise the possibility that β-arrestins have a role in assisting MOR dimerization (10,11). More research is needed to determine the specific conditions where MORs form dimers and the downstream impact of dimerization.

Ligand-free signaling: a novel mechanism of action for ORs

In general, GPCRs signal when bound by an agonist, but signaling can also persist after agonists are removed. GPCRs can also spontaneously start signaling even without an agonist because receptors are so sensitive to stimuli that small forces causing conformational change can induce partial ligand-free signaling. This leads to the question of whether this ligand-free signaling has a role in opioid agonist signaling. Some MOR ligands immediately dissociate after binding the receptor, while others such as Morphine, dissociate after ligand-free signaling has taken over the pathway. Even after opioids are fully removed from the body, antagonists still elicit withdrawal, implying that MORs are still signaling. Compared to the opioid-naive state, ligand-free signaling persists for longer in opioid-dependent states, which may be a factor that causes dependence (12).

Additionally, Naltrexone and Naloxone, which are neutral antagonists in opioid-naive states, become inverse agonists after morphine treatment (13). A neutral antagonist simply blocks the active site where agonists bind, but in itself has no effect without agonists. An inverse agonist, on the other hand, induces an opposite or inverse response. This difference in properties indicates that opioid treatment alters the signaling properties of the receptors themselves. Ligand-free signaling after opioid treatment is different from signaling before. Antagonists acting as inverse agonists can perhaps be used in therapy to prevent prolonged ligand-free signaling.

In a recent study, a research group co-administered 6β-naltrexol (6BN, the main metabolite of Naltrexone) along with morphine daily for 6 days in juvenile mice. When measured after the last dose, it was found that naloxone-induced withdrawal was reduced. This gave way to a similar trial, where 6BN and methadone were coadministered, in opioid-dependent adult guinea pigs, and similarly, withdrawal was suppressed (14). A possible explanation is that 6BN accelerates the reversal of ligand-free MOR to basal state MOR, lowering dependence. If this hypothesis were true, it may be possible that 6BN could be used as a therapeutic approach to minimize dependence while still sustaining a level of analgesia. A neutral antagonist with these effects is non-explainable by the classical GPCR model, so these findings may pave the way for looking at GPCR receptors with their ligand-free form too.

Non-Opioid ligand influence on opioid receptors

Opioid receptor downstream signals can derive from alternate opioid or non-opioid receptors. Metabotropic glutamate receptors (mGlu) 2/3, 5, and 7 have all been shown to have a role in tolerance to the opioid morphine. mGlu2/3 agonist (LY354740), mGlu5 antagonist (MTEP), and mGlu7 agonist (AMNO82) were delivered to mice groups in combination with morphine prior to a tail immersion test to measure the pain threshold in response to heat. Neither the antagonist nor the agonists affected acute analgesia but these molecules did inhibit the development of tolerance to morphine. Furthermore, these ligands were also able to reverse previously existing morphine tolerance (15). These results suggest that while mGlu receptors do not interfere with opioid analgesia, they may contribute to tolerance regulation; however, the existing knowledge of the relationships between mu-agonists and mGlu receptors remains limited.

In another study, N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801(dizocilpine), and nitric oxide (NO) synthase inhibitors were injected into morphine-treated mice to test whether NMDA receptors or the NO pathway assisted in developing tolerance. Prior to MK-801 and NO synthase inhibitor administration, morphine's analgesic effects were reduced across 5 days in mice, indicating the development of tolerance. When MK-801 and NO synthase inhibitors were co-administered, morphine tolerance was reversed (16). These findings suggest that morphine tolerance is likely developed in a process that includes NMDA receptors and the NO pathway.

Lastly, the epidermal growth factor receptor (EGFR) has been shown to have a role in DOR signaling. When rats with ischemic injury were pretreated with the DOR agonist D-ala2, D-leu5 enkephalin (DADLE), the size of the injury decreased more than the group without pretreatment. However, the addition of EGFR inhibitors prevented that rehabilitation (17). The results imply that EGFRs are downstream regulators of DOR signaling. Targeting DORs may prove a strong alternative to MOR because of their neuroprotective properties, but further understanding of its roles outside analgesia is required to design better and more efficient drugs.

The study of other pathways involved in opioid signaling can provide insight on how to indirectly target the opioid pathway, nonetheless, it remains important to also consider that these pathways are interconnected such that regulating one of them can affect the whole cell.

Endosomal targeting of non-peptide opioids

While non-peptide and synthetic opioids generally function by mimicking the behaviors of peptide-based endogenous ligands, they can also act distinctly. Recent research has identified that GPCRs may be signaling intracellularly inside endosomes; this type of receptor function is not uncommon, as a large majority of mGlu receptors also signal intracellularly (18,19). To measure the endosomal signaling of ORs, nano-based biosensors were used to detect ligand activation of MORs and DORs. Receptor activation by agonists (DAMGO for MORs and DADLE for DORs) still occurred within endosomes. The addition of the alkaloid antagonist Naloxone reversed all OR signaling and confirmed that intracellular activation was because of OR agonists. Interestingly, when CTOP, a peptide-based antagonist was used, it did not induce

membrane recruitment. Additionally, after the removal of agonists within endosomes, the OR downstream signal persisted. Compared to surface activation, where the removal of agonists immediately reversed effects, endosomal activation lasted longer than membrane activation (20). These results indicated that non-peptide opioid drugs could act in distinctive mechanisms that peptide opioids can not. By acting intracellularly and with an extended receptor activation period, this mechanism of action of opioid drugs challenges the current understanding of the cellular framework of OR signaling.

Opioid Receptor desensitization

Desensitization is a process in which a receptor becomes successively less effective in inducing downstream signals in response to a ligand. A receptor can be internalized and degraded or recycled, limiting ligand binding capacity at the cell surface. GRKs can phosphorylate the C-terminal necessary for recruiting arrestins, which internalize the receptor. For example, mice lacking the β-arrestin gene did not showcase MOR desensitization after chronic morphine treatment (21).

Internalization contributes to downregulation, a decrease of surface receptors. Acute desensitization can occur when the GPCR uncouples from G-proteins, via arrestin phosphorylation. When ligands are first introduced into the body, a response is achieved and as additional ligands are added, the receptors become saturated and a more intense response occurs in the whole body. The body's natural defense against this overstimulation is to downregulate signaling. Minutes after agonist exposure, GPCRs become phosphorylated and arrestins bind to them, preventing G-protein coupling (short-term) and promoting endocytosis and lowering mRNA levels (long-term) (22).

Receptor internalization can build a tolerance to the initial ligand and even other receptor ligands in a process referred to as cross-tolerance. At a molecular level, tolerance is when the amount of ligand needed to induce a signal cascade is elevated from the basal state, different from dependence and addiction which are physical and psychological urges to keep taking a drug. Continuous desensitization can eventually lead to long-term tolerance.

The role of phosphorylation in desensitization

Phosphorylation of the C-terminal of opioid receptors is a necessary component in driving desensitization. Aside from MORs, both DORs and KORs have also been shown to induce desensitization through phosphorylation (23,24). Wild-type (WT) mice and mice with selective mutations in their MOR C-terminal that inhibited phosphorylation were compared in a paw withdrawal test following morphine or fentanyl treatment. The mice with mutated MOR C-terminals were protected from pain longer than the WT, as the absence of C-terminal phosphorylation limited receptor desensitization which strengthened and prolonged morphine or fentanyl analgesic effects. Thus, C-terminal phosphorylation assists in building tolerance (25). Similarly, brain slices taken from WT and mice with mutated MORs exhibiting a phosphorylation deficient C-terminal were compared to interrogate the effect of MOR phosphorylation on internalization. In the MOR mutant neurons, in both morphine-treated and untreated groups, receptor internalization was nearly abrogated. Additionally, whole-cell voltage clamp

experiments found that mutant neurons did not lose sensitivity to morphine, reinforcing that phosphorylation of the C-terminal plays a key role in OR sensitization and tolerance (26). Although this result indicated that phosphorylation-deficient neurons did not develop tolerance to morphine, it is possible that tolerance was simply delayed. Indeed, through long-term exposure, it was observed that fentanyl tolerance was eliminated, but morphine treatment eventually resulted in the development of tolerance (25). This suggests that morphine-directed OR signaling includes other factors besides C-terminal phosphorylation that lead to tolerance.

Chronic opioid exposure alters kinase roles

GRK2/3 has previously been shown to be sufficient for OR phosphorylation which results in desensitization (3). However, recent work has now shown chronic morphine exposure requires additional kinases for receptor desensitization. When Compound 101, an inhibitor of GRK2/3, was delivered continuously through pumps to naïve and morphine-treated mice, desensitization was blocked in the naïve group but not in the morphine-treated group (27). This suggests that long-term morphine tolerance requires additional factors of desensitization as blocking GRK2/3 was not sufficient. Protein Kinase C (PKC) and C-jun terminal N kinase (JNK) inhibition did not change desensitization in the non-tolerant group, but when combined with Compound 101, desensitization was abrogated in the morphine-treated group (27). Thus, long-term morphine tolerance extends beyond receptor sites and alters cellular requirements by needing additional kinases that were not needed before. Inhibitors of PKC were injected into mice after 72 hours of morphine treatment to test whether inhibition would reverse tolerance. PKC inhibitors did not affect the potency of morphine in non-tolerant mice, but in the morphine-treated group, tolerance was almost completely reversed (28). When PKC inhibitors were present in mice during tail flick and hot plate tests, morphine tolerance was reversed in both. The inhibitor group scored percent maximum possible effect (%MPE) of 80 and 90 on hot plate and tail-immersion tests, while the group without the inhibitor scored 2 and 4 %MPE with a higher score indicating longer analgesia (29,30). It remains unclear how exactly PKC and JNK assist in desensitization. PKC inhibition alone only modestly reduced desensitization after high-dose agonist treatment compared to low agonist treatment, which indicated PKC played a greater role at low agonist concentrations (31). Serine phosphorylation has been shown to be the key driver of desensitization (23,24) and PKC specializes in serine and threonine phosphorylation, so it likely acts on those residues within the C-terminal. JNK assists responses to harmful stimuli and while its role has been suggested to assist in the phosphorylation of chronic-morphine-exposed receptors, a specific role has not been identified.

Kölliker-Fuse neurons are less prone to desensitization

Most existing research on opioid tolerance has been done on locus coeruleus (LC) neurons, but not all neurons showcase the same ability to build opioid tolerance. Kölliker-Fuse (KF) neurons are mainly responsible for respiratory functions such as swallowing, coughing, and breathing. Opioid agonists hyperpolarize KF neurons preventing them from firing, which leads to one of opioids' major side effects, namely respiratory depression. Respiratory depression is often a fatal result because while tolerance is built towards opioid

analgesic effects, the same can not be said for this side effect (32). Action potentials were measured using whole-cell voltage clamps to compare MOR desensitization between KF and LC neurons. Both neurons were first activated with an agonist, Met-enkephalin (ME), and 10 minutes later, were activated again. After the refractory period, the current in KF was closer to the original activation than the LC, indicating that KF MORs remained sensitive to agonists. Finally, ME was added for 30 minutes to KF neurons; without any desensitization being observed. Even in morphine-treated rats, ME treatment produced the same current as in naïve rats. (8,32,33). Despite morphine's ability to induce tolerance in LC neurons, KF neurons showed none. This likely explains respiratory depression as such a fatal side effect because as more opioids are needed for pain relief and euphoria, KF neurons stay sensitive to the baseline amount of the drug. A possible explanation for such a large difference in tolerance is that KF neurons might have different expressions or regulations of cellular elements that assist in desensitization (such as GRK2/3, PKC, JNK, B-arrestin, etc), though this hypothesis has yet to be explored (25,27,34).

Opioid Receptors and withdrawal

Drug withdrawal occurs because taking opioid drugs for an extended period of time results in physical dependence, which occurs when the body becomes accustomed to the drug and depends on it to function normally. Therefore, abruptly terminated dosing results in withdrawal. Some symptoms of withdrawal include unpleasant sensations such as nausea, fatigue, body pains, and mental distress. Additionally, withdrawal can also cause results as fatal as seizures, strokes, and heart attacks.

The pathways of withdrawal

Withdrawal can be very unpleasant for those trying to recover from opioid dependency and understanding how it functions can assist in the development of strategies to improve patient recovery. Though selective MOR mutations largely reduced tolerance through inhibiting OR phosphorylation, withdrawal symptoms were unaffected (25). These results suggest that pathways leading to withdrawals may be separate from those of tolerance. Biased signaling occurs when a single receptor can activate either G-protein or arrestin signaling based on different bound ligands, which may support the separation of withdrawal from tolerance. While G-protein signaling has been linked with analgesia, arrestin signaling has been linked with opioids' negative side effects including respiratory depression and dependence (35). Leveraging this as yet unexplored ability may assist in drug selection in order to induce specific desired effects; for example, if a drug favors G-protein signaling over arrestin signaling, it may be possible to maximize analgesia without worsening withdrawal.

However, when mice were treated with SR-17018, an agonist biased towards G-protein signaling more than arrestin signaling, both tolerance and withdrawal were prevented. Mice treated with SR-17018 experienced significantly fewer withdrawals and reduced development of tolerance than the morphine-treated group suggesting arrestin signaling has a role in both tolerance and withdrawal pathways (34). While tolerance was reduced in β-arrestin-knockout mice, physical dependence was still observed at the same level as the WT group, indicating that arrestins did not affect withdrawal pathways (21). V1bRs are possibly responsible for affecting withdrawals because mice lacking V1bRs showed a decrease in naloxone-

precipitated jumping behavior, a withdrawal symptom of morphine (10). A deeper understanding of the different pathways of withdrawal may present significant potential in designing drugs that can provide pain relief without side effects. Additionally, biased signaling may open new possibilities for safer drugs, and additional research can contribute to diminishing, or even eliminating fatal side effects.

Potential synthetic drugs without unwanted side effects

Prototype ligands

Some OR ligands are promising scaffolds for drug development because of their reduced risk of tolerance and withdrawal. Surprisingly, when chronically treated morphine mice were switched to SR-17078, not only was morphine sensitivity restored, but morphine withdrawals were also significantly reduced (34). Similarly, 3-iodobenzoyl naltrexamine (IBNtxA) is known to be a very potent opioid without the side effects of respiratory depression and dependence (36). These two ligands possess remarkable potential as therapeutics, serving as the framework for creating drugs that offer pain relief without fatal effects and that even reverse tolerance and dependence. Interestingly, IBNtxA does not target traditional GPCRs with 7 transmembrane domains (7TM), which may be the basis for its impressive effects. The MOR has 3 subtypes despite only being coded by one gene, *Oprm1*. Through alternative splicing, 3 MOR variants are expressed: the traditional receptor with seven transmembrane domains (7TM), a variant with six (6TM), and one (1TM) (37). In mice without 6TM variants, there was an absence of IBNtxA analgesia but a presence of morphine analgesia. In contrast, selective elimination of 7TM variants eliminated morphine analgesia without affecting IBNtxA (36). This may link the absence of side effects to the unique function of 6TM variants. A second study supported that 6TM variants were responsible for IBNtxA analgesia but unlike fentanyl and heroin, IBNtxA was unlikely to cause abuse because of a lack of preference in rodent place preference tests. A place preference test functions by placing mice in an environment with the testable drug and one without and observing how mice choose between the environments. Interestingly the previous study also found that KOR agonist U-50488 was able to fully substitute for IBNtxA. Agonist substitution is used as a treatment for dependence, where a less dangerous but equally powerful drug is used instead of the initial one. U-50488 substituting for IBNtxA indicates that KOR signaling is a part of the IBNtxA mechanism of action. (38). KORs inhibit dopamine and therefore interfere with a drug's reward cycle (39) which suggests that IBNtxA may not induce abuse, despite being more powerful than morphine. However, in contrast to this second study (38), the first study (36) found that even with removing Kappa and Delta receptors, IBNtxA analgesia was still unaffected. A possible explanation could be that KOR signaling does not directly affect IBNtxA but can induce similar signals downstream. In that case, U-50488 targeting of KOR can prove to be an alternative to 7TM MOR agonists. KORs are not as well researched as MORs are, and so have contrasting results in their contribution to drug addiction (39). However, the contrasting results presented herein mean that this drug's pathway is unclear (34,36). However, U-50488's full substitution for IBNtxA can pave the way to finding therapeutic benefits in KOR signaling. Though the downstream components of the novel agonist IBNtxA have not been

conclusively confirmed, there is no denying the unique and positive effects of 6TM variants as targets instead of the traditional 7-domain GPCRs. The increased understanding of 6TM variants can assist in developing drugs with effective analgesia and minimal side effects

Mixed agonists/antagonists

Another promising tool to reduce side effects is to use antagonists with mixed activity; under some conditions a compound can behave as an agonist while under other conditions, it behaves as an antagonist. The antagonist Nalorphine was initially used as a treatment for opioid overdose, but after an independent study, it was found to have agonist activity. However, that activity came with dysmorphic effects, likely due to binding KORs, so its clinical usage was cut short. Following Nalorphine, a new synthetic opioid, Pentazocine, entered the market which works as a partial agonist/antagonist at MORs, while acting as a full agonist at KORs (40). In this way, Pentazocine is able to treat moderate pain, but the dysphoric effects from KORs would limit its abuse addiction potential. The discovery of Pentazocine's mixed activity profile highlights the complexities of developing these drugs, but also the potential for achieving both analgesia and reduced abuse liability.

Bi-valent ligands

The co administration of a DOR and MOR ligand has been found to reduce the development of tolerance. Using a flexible 21-atom linker to connect the two ligands, the bi-valent ligand can stimulate heterodimeric DOR/MOR receptors at the same time. MDAN-21A is a bivalent ligand made of a derivative of the DOR antagonist, naltrindole and a derivative of MOR agonist, oxymorphone. MDAN-21A, both separate compounds without linkage, and the MOR agonist by itself were administered to mice in separate groups and tested in a tail flick assay, in which the bivalent group showed less tolerance and dependency. Interestingly in the place preference test, both the monovalent MOR ligand group and the group receiving both DOR and MOR ligands separately showed significant place preference, while the bivalent group did not (41). Place preference indicates the likelihood of drug abuse, thus, the bivalent group had less tolerance and was less likely to be abused.

Additionally, there is a suggestion that bivalent ligands that stimulate DOR/MOR heterodimers can offer novel potential for reducing tolerance. Heterodimers are formed from 2 separate proteins, DOR and MOR, which bind together in close proximity. They seem to affect agonistic activity and be unique and distinct signaling entities, separate from their individual parts, and the close proximity of DORs to the MOR may prevent phosphorylation and thereby internalization leading to lower tolerance over time. (42). Heterodimers have a different mechanism of action than a single delivered ligand, and there are many permutations of heterodimers to be tested beyond DOR/MOR combinations. Further development can lead to potential benefits from this class of molecules.

The potential of allosteric modulators

Allosteric modulation affects signaling by binding to a location on a receptor separate from the active ligand binding site, called the allosteric site. Positive allosteric modulators enhance the receptor signal, while negative ones inhibit it. The MOR positive allosteric modulator (mu-PAM) enhanced Morphine potency by nearly 5-fold and DAMGO’s by

7-fold. Additionally, it also increased Met-enkephalin's bias toward G-protein signaling; a similar yet diminished effect was seen with DAMGO. Side effects such as respiratory depression and constipation were greater in morphine-treated rats without mu-PAM than in the group with (43). This result could be explained by the fact that mu-PAM increased the bias towards G-protein signaling and may be considered for use to enhance the bias factor for G-protein signaling.

There are certain structural conformations that receptors change into, that determine their activity level: fully activated, partially activated, and inactive conformations. When DAMGO, a full agonist, was applied along with allosteric modulator BMS-986122 there was an increase in GTP turnover, a sign of GPCR signaling, compared to when DAMGO was administered alone (44). This indicates that on its own, a full agonist does not lead to the confirmation of the highest activity level and that modulators can be used to increase the effectiveness of agonists. In certain cases modulators on their own can also elicit responses: mu-PAM was found to have antinociceptive effects when administered alone by increasing ORs affinity to endogenous opioids (43). mu-PAM could be used as an alternative medication to opioids for less serious conditions, where the allosteric agonist can enhance the body's natural painkillers.

Cannabinoid-type 1 (CB1) receptors frequently interact with MOR and have a role in opioid reward. The inhibition of CB1 has been shown to decrease opioid reward and dependence. CB1 knockout mice prevented a preference for Morphine in place preference tests. However, that inhibition results in mentally straining effects, such as depression and anxiety. Negative Allosteric Modulation (NAM) can assist in reducing CB1 signaling without direct antagonism. In fact, direct antagonism can also alter endogenous reward pathways which can have detrimental effects. GAT358, a CB1-NAM reduced dopamine efflux following morphine exposure in mice. Dopamine is known as the "feel-good" hormone and contributes to the reward and positive motivation of certain behavior. The gradual reduction of dopamine released by morphine can decrease mental dependence and addiction overall (45). This is a promising area to study side effects, and barring any lethal ones, could offer a solution in controlling or alleviating opioid dependence in patients.

Genetic scope of tolerance and drug abuse

Recent studies have found the importance of non-coding RNAs, which assist in chromatin remodeling, transcription, and signal transduction, in opioid tolerance (46). Additional research on this novel target can present a potentially valuable treatment.

The Ptchd Gene

The mutant worm strain *bgg10* was resistant to opioid tolerance, and the mutation responsible was found to be a premature stop-codon in the gene, f43d21, which encodes a ptchd-family protein (PTR-25). In order to confirm that this was the responsible factor, the same premature stop codon was edited onto the gene using CRISPR-Cas9 in a WT mouse. This mutation resulted in PTR-25 missing the C-terminal, which when expressed in mice, did not exhibit tolerance. The Ptchd proteins are known to have a role in regulating cholesterol levels. This brings into question the importance of cholesterol in the role of OR function (47). Cholesterol has a role in regulating membrane fluidity, which in turn can shift transmembrane domains and affect receptor behavior. However,

cholesterol can also bind to leucine sites near transmembrane helices or clefts between transmembrane domains. The binding of cholesterol has been known to stabilize receptor conformation, whether towards inactive or active states (48).

Overall the Ptchd proteins provide an interesting and promising area of research on tolerance, where regulating Ptchd protein expression could potentially reduce the development of tolerance. However, using this method to prevent tolerance may not be without side effects. If the C-terminal tail is removed, the body's natural downregulation of GPCR signaling will be reduced, dampening its ability to withstand overstimulation, and leading to a higher likelihood of overdose. Furthermore, outside tolerance, changes in cholesterol level would vastly alter the cell membrane permeability, which could lead to detrimental effects among a variety of cell processes.

Epigenetics of OR modulation

In general, epigenetics is a cellular process for changing gene expression without permanently altering the main genome. This process makes conformational changes to chromatin, allowing or silencing transcription of certain genes. The result is that in eukaryotic cells, each cell has different genes that are active, resulting in the expression of unique protein landscapes. DNA is tightly wrapped around histone proteins at certain sites, and looser at other sites allowing access for transcription machinery. Methylated regions in chromatin prevent transcription while other regions lacking methyl group allow transcription to occur.

Rats chronically treated with cocaine had reduced seeking-behavior when injected with a DNA methylase inhibitor, preventing chromatin remodeling. Contrarily, seeking behavior was elevated when a methyl donor, which increased DNA methylation was injected instead (49). These results suggest specific sites on chromosomes may factor into reward and addiction behavior, and epigenetic remodeling may reduce addiction behavior. Yet still, this process would require a high degree of precision, as random changes in methylation can lead to a loss of function in the cell; the same can be said for any epigenetic modification. Another possible contributor to these epigenetic changes is histone acetylation: in this process histones are removed from DNA, leaving it loose and easy to transcribe. Human brains of heroin users show hyperacetylation; it is, therefore, likely that heroin use enhances gene expression, which may enhance drug addiction.

Lately, genome-wide association studies (GWAS) have been used to identify genes involved in Opioid-Use-Disorder (OUD). A benefit of this method is that it's unbiased as it statistically analyzes across the entire genome for variants in genes, instead of other methods that research genes already known to be involved (50). Although GWAS methods have identified numerous gene variants across an ethnically diverse sample population that are involved in OUD, its use needs to be more widespread across environments and rare gene factors. Altering epigenetics may seem promising, but with our current understanding, or lack thereof, of the complex and interconnected gene pathways, it is likely that pre-transcriptional regulation will have numerous unwanted effects beyond merely influencing drug reward; thus, epigenetic therapeutics may take more time and effort to develop.

Conclusion

While morphine exposure has been shown to change cellular requirements concerning desensitization, it is yet unknown how other processes are affected by this change. The need for additional kinases like PKC and JNK elicits further questions about what additional kinase-driven effects occur downstream (27). The change in kinase roles can lead to a snowball effect affecting different cascades, leading to altered gene transcription and ultimately affecting protein expression beyond an opioid's original pathway and thus eliciting changes at a whole-cell level. It is possible those alterations can contribute to or lead to certain side effects, thus further monitoring of affected pathways is necessary to understand the drawbacks behind opioid use, as well as how to make them a safer and more effective way of pain management.

Biased targeting of OR variants could be a possible solution to preventing opioid withdrawals. One alternative to make opioids safer is to target the 6TM variant, and possibly the 1TM variant rather than traditional 7TM ORs. The drug IBNtxA specifically binds to the 6TM variants without producing any withdrawal symptoms. However, truncated GPCRs have been found to work as a dominant-negative mutant, interfering with normal wild-type GCPRs. In this case, truncated GPCRS increase intracellular retention of full length GPCRs, which could negatively affect extracellular signaling with ligands that can not diffuse through the plasma membrane to activate intracellular signaling. Additionally, truncated variants can also form heterodimers with 7TM receptors, which would also interfere with normal GPCR signaling (36). Targeting truncated receptors may seem promising on the narrow scope of opioids, but the broader implications to affect other GPCR pathways needs consideration.

GPCR can induce one of two pathways: arrestin-signaling and g-protein signaling. G-protein biased signaling has already proven to have promising potential in enhancing opioids efficacy while diminishing side effects. The promising results of SR-17018 may be used to create a prototype drug with therapeutic potential biased towards G-protein signaling, which maintains strong analgesia while minimizing and even mitigating established dependence and tolerance (34). These results support the role of arrestin signaling in withdrawal, considering that bias against it reduces withdrawal. Thus, using a positive allosteric modulator such as mu-PAM to magnify G-signaling bias may lead to even more precise targeting to increase desired effects and limit the unwanted ones that are linked to arrestin signaling. At its current state, further understanding of biased OR agonism and its long-term effects will be needed before it can be applied to therapeutics. There still exists a need for a greater understanding of how non-OR pathways contribute to opioid dependence, withdrawal, and tolerance. Inhibiting the NMDA/NO pathway through mGlu5 prevented opioid tolerance (10,13,15). However, it is important to note that inhibition of these additional pathways may also impact or affect other cellular processes, possibly leading to undesirable side effects. As the specific downstream effects of those factors to prevent withdrawal have not been well studied, additional experimentation will be needed to confirm whether they are safe and effective in preventing withdrawal altogether.

It is important to consider the differences between non-peptide and synthetic opioids

from natural endogenous peptide opioids when used therapeutically. While the prevailing dogma suggests that synthetic drugs simply mimic natural peptide hormones, non-peptide drugs showed the ability to signal from endosomes, and additionally, endosomal signaling by non-peptide drugs lasted longer than surface signaling (20,51). Receptor internalization is one of the biggest contributors to desensitization, but in these cases, ORs are not desensitized despite existing within endosomes. In that case perhaps non-peptide opioids have an alternate way of desensitization. Additionally, considering that endosomal signaling persists longer than surface signaling when agonists are removed, there is variation in how ligand-free signaling persists endosomally. Ligand-free signaling has been shown to be prolonged after opioid exposure,suggesting opioid exposure induces increased endosomal signaling. This result can change the interpretation of the downstream effects of synthetic opioids, and the distinct outcomes of endosomal signaling can influence development of new drugs specialized around this unique functionality. Further research on downstream effects will be needed before internal endosomal opioid receptors may be considered as a viable alternate target.The unique target of IBNtxA, the biased agonism of SR-17018, and non-peptide agonists' ability to activate ORs within the membrane demonstrated that opioids can be designed to target a specific pathway to maximize analgesia while limiting side effects.

Finally, the epigenetic changes of drug abuse should be considered. The epigenetic influence of opioids provides a new perspective on the dangers of drug addiction, as recent research suggests that cellular effects of drug abuse do not only persist while taking the drug, but can cause lasting changes to the genome. Many cellular changes are recognizable after opioid exposure including the need of additional kinases for desensitization, prolonged ligand-free signaling, and endosomal signaling all of which may stem from epigenetic changes. Another factor to consider is the gene ptchd1, involved in regulating the cholesterol content in the membrane. Cholesterol can affect the membrane-permeability, regulating which molecules can diffuse through the membrane. As established already, receptors can signal intracellularly, and so it's likely selective for ligands that can permeate the cell, so altered expression of ptchd1 could also potentially be an epigenetic influence of long-term opioid use. There has also been a recent rise in the study of non-coding RNAs, and their potential role in regulating opioid tolerance (46). As genetic engineering advances, epigenetics, miRNAs, and ncRNAs, can potentially be leveraged to reduce opioid reward and side effects through new therapeutics/technologies.

Opioid exposure can alter an opioid receptor's phosphorylation cascade and consequently, other cellular pathways are likely impacted as well. Biased signaling is a new potential avenue for therapeutics, providing a way to specifically induce a certain OR signal pathway for its desired outcome (14). The emergence of new opioid agonists such as IBNtxA and SR-17018 is another exciting research space, as it is possible these may serve as scaffold compounds that may lead to a new prototype drug (34,36). Additionally, while internalization is a major driver of OR desensitization, certain synthetic opioids are able to diffuse through the plasma membrane and signal intracellularly, are able to carry out signaling when agonist is removed, and

even both simultaneously (12,18), highlighting a novel application of this class of drugs.

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Abbreviations
%MPE - Percent Maximum Possible Effect, AC5 - Adenylyl cyclase, C-terminal - Carboxyl terminal, DADLE - (D-ala2, D-leu5) enkephalin, DOR - Delta opioid receptor, EGFR- Epidermal growth factor receptor, GDP - Guanosine Diphosphate, GPCR - G-protein coupled receptor, GRK - G-protein coupled receptor kinases, GTP - Guanosine Triphosphate, GWAS - Genome-wide association studies, IBNtxA - 3-iodobenzoyl naltrexamine, JNK - C-jun terminal N kinase, KF – Kölliker-Fuse, KOR - Kappa opioid receptor, LC - Locus coeruleus, ME – Met-enkephalin, mGlu - Metabotropic Glutamate, MOR - Mu opioid receptor, mu-PAM- MOR positive allosteric modulator, N-terminal – Amino-terminal, NMDA – N-methyl-D-aspartate, NO - Nitric oxide, OR - Opioid receptor, PKC - Protein Kinase C, TM - Transmembrane Domains, V1bR - Vasopressin 1b receptor, WT - Wild type, DAMGO - D-Ala2, N-MePhe4, Gly-ol]-enkephalin, Compound 101 - 3-[(4-methyl-5-pyridin-4-yl-1,2,4-triazol-3-yl)methylamino]-N-[[2-(trifluoromethyl)phenyl]methyl]benzamide hydrochloride (PubChem ID 11677079), CTOP – D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (PubChem ID 90479805), SR17018 – 5,6-dichloro-3-[1-[(4-chlorophenyl)methyl]piperidin-4-yl]-1*H*-benzimidazol-2-one (PubChem CID 130431397), U50488 – 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*R*,2*R*)-2-pyrrolidin-1-ylcyclohexyl]acetamide (PubChem CID 3036289), BMS986122 – 2-(3-bromo-4-methoxyphenyl)-3-(4-chlorophenyl)sulfonyl-1,3-thiazolidine (PubChem CID 4644453), GAT358 - 3,4-diaminocyclobut-3-ene-1,2-dione derivative,

References

1. Latorraca NR, Venkatakrishnan AJ, Dror RO. GPCR Dynamics: Structures in Motion. Chem Rev. 2017;117: 139–155. https://doi.org/10.1021/acs.chemrev.6b00177

2. Law P-Y, Wong YH, Loh HH. Molecular Mechanisms and Regulation of Opioid Receptor Signaling. Annual Review of Pharmacology and Toxicology. 2000. pp. 389–430. https://doi.org/10.1146/annurev.pharmtox.40.1.389

3. Gurevich VV, Gurevich EV. GPCR Signaling Regulation: The Role of GRKs and Arrestins. Front Pharmacol. 2019;10: 125. https://doi.org/10.3389/fphar.2019.00125

4. Weis WI, Kobilka BK. The Molecular Basis of G Protein–Coupled Receptor Activation. Annual Review of Biochemistry. 2018. pp. 897–919. https://doi.org/10.1146%2Fannurev-biochem-060614-033910

5. Mohan ML, Vasudevan NT, Gupta MK, Martelli EE, Naga Prasad SV. G-protein coupled

receptor resensitization-appreciating the balancing act of receptor function. Curr Mol Pharmacol. 2012. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4607669/

6. Kosten TR, George TP. The neurobiology of opioid dependence: implications for treatment. Sci Pract Perspect. 2002;1: 13–20. https://doi.org/10.1151%2Fspp021113

7. Tejeda HA, Counotte DS, Oh E, Ramamoorthy S, Schultz-Kuszak KN, Bäckman CM, et al. Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. Neuropsychopharmacology. 2013;38: 1770–1779. https://www.nature.com/articles/npp201376

8. North RA, Alan North R, Williams JT. How do opiates inhibit neurotransmitter release? Trends in Neurosciences. 1983. pp. 337–339. https://doi.org/10.1016/0166-2236(83)90151-0

9. Kim K-S, Lee K-W, Lee K-W, Im J-Y, Yoo JY, Kim S-W, et al. Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. Proc Natl Acad Sci U S A. 2006;103: 3908–3913. https://doi.org/10.1073%2Fpnas.0508812103

10. Koshimizu T-A, Honda K, Nagaoka-Uozumi S, Ichimura A, Kimura I, Nakaya M, et al. Complex formation between the vasopressin 1b receptor, β-arrestin-2, and the μ-opioid receptor underlies morphine tolerance. Nat Neurosci. 2018;21: 820–833. https://doi.org/10.1038/s41593-018-0144-y

11. Möller J, Isbilir A, Sungkaworn T, Osberg B, Karathanasis C, Sunkara V, et al. Single-molecule analysis reveals agonist-specific dimer formation of μ-opioid receptors. Nat Chem Biol. 2020;16: 946–954. https://doi.org/10.1038/s41589-020-0566-1

12. Sadee W, McKew JC. Ligand-Free Signaling of G-Protein-Coupled Receptors: Relevance to μ Opioid Receptors in Analgesia and Addiction. Molecules. 2022;27. https://doi.org/10.3390%2Fmolecules27185826

13. Wang D, Raehal KM, Bilsky EJ, Sadée W. Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. J Neurochem. 2001;77: 1590–1600. https://doi.org/10.1046/j.1471-4159.2001.00362.x

14. Sadee W, Oberdick J, Wang Z. Biased Opioid Antagonists as Modulators of Opioid Dependence: Opportunities to Improve Pain Therapy and Opioid Use Management. Molecules. 2020;25. https://doi.org/10.3390%2Fmolecules25184163

15. Gawel K, Jenda-Wojtanowska M, Gibula-Bruzda E, Kedzierska E, Filarowska J, Marszalek-Grabska M, et al. The influence of AMN082, metabotropic glutamate receptor 7 (mGlu7) allosteric agonist on the acute and chronic antinociceptive effects of morphine in the tail-immersion test in mice: Comparison with mGlu5 and mGlu2/3 ligands. Physiol Behav. 2018;185: 112–120. https://doi.org/10.1016/j.physbeh.2017.12.035

16. Zamanian G, Shayan M, Rahimi N, Bahremand T, Shafaroodi H, Ejtemaei-Mehr S, et al. Interaction of morphine tolerance with pentylenetetrazole-induced seizure threshold in mice: The role of NMDA-receptor/NO pathway. Epilepsy Behav. 2020;112: 107343. https://doi.org/10.1016/j.yebeh.2020.107343

17. Chen M, Wu S, Shen B, Fan Q, Zhang R, Zhou Y, et al. Activation of the δ opioid receptor relieves cerebral ischemic injury in rats via EGFR transactivation. Life Sci. 2021;273: 119292. https://doi.org/10.1016/j.lfs.2021.119292

18. Jong Y-JI, Harmon SK, O'Malley KL. GPCR signaling from within the cell. British J Pharmacol. 2018; 125: 4026-4035. https://doi.org/10.1111/bph.14023

19. Jong Y-JI, Sergin I, Purgert CA, O'Malley KL. Location-dependent signaling of the group 1 metabotropic glutamate receptor mGlu5. Mol Pharmacol. 2014;86: 774–785. https://doi.org/10.1124%2Fmol.114.094763

20. Stoeber M, Jullié D, Lobingier BT, Laeremans T, Steyaert J, Schiller PW, et al. A Genetically Encoded Biosensor Reveals Location Bias of Opioid Drug Action. Neuron. 2018. pp. 963–976.e5. https://doi.org/10.1016/j.neuron.2018.04.021

21. Bohn LM, Gainetdinov RR, Lin F-T, Lefkowitz RJ, Caron MG. μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. Nature. 2000. pp. 720–723. https://doi.org/10.1038/35047086

22. Rajagopal S, Shenoy SK. GPCR desensitization: Acute and prolonged phases. Cell Signal. 2018;41: 9–16. https://doi.org/10.1016/j.cellsig.2017.01.024

23. McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, et al. Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. J Biol Chem. 2004;279: 1810–1818. https://doi.org/10.1074/jbc.m305796200

24. Kouhen OM, Wang G, Solberg J, Erickson LJ, Law PY, Loh HH. Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. J Biol Chem. 2000;275: 36659–36664. https://doi.org/10.1074%2Fjbc.M006788200

25. Kliewer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, Levitt ES, et al. Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. Nat Commun. 2019;10: 367. https://doi.org/10.1038/s41467-018-08162-1

26. Arttamangkul S, Heinz DA, Bunzow JR, Song X, Williams JT. Cellular tolerance at the

μ-opioid receptor is phosphorylation dependent. Elife. 2018;7. https://doi.org/10.7554/eLife.34989

27. Leff ER, Arttamangkul S, Williams JT. Chronic Treatment with Morphine Disrupts Acute Kinase-Dependent Desensitization of GPCRs. Molecular Pharmacology. 2020. pp. 497–507. https://doi.org/10.1124/mol.119.119362

28. Smith FL, Javed R, Elzey MJ, Welch SP, Selley D, Sim-Selley L, et al. Prolonged reversal of morphine tolerance with no reversal of dependence by protein kinase C inhibitors. Brain Res. 2002;958: 28–35. https://doi.org/10.1016/s0006-8993(02)03394-2

29. Javed RR, Dewey WL, Smith PA, Smith FL. PKC and PKA inhibitors reverse tolerance to morphine-induced hypothermia and supraspinal analgesia in mice. Eur J Pharmacol. 2004;492: 149–157. https://doi.org/10.1016/j.ejphar.2004.03.061

30. Smith FL, Javed RR, Smith PA, Dewey WL, Gabra BH. PKC and PKA inhibitors reinstate morphine-induced behaviors in morphine tolerant mice. Pharmacol Res. 2006;54: 474–480. https://doi.org/10.1016/j.phrs.2006.09.007

31. Tang H, Shirai H, Inagami T. Inhibition of protein kinase C prevents rapid desensitization of type 1B angiotensin II receptor. Circ Res. 1995;77: 239–248. https://doi.org/10.1161/01.res.77.2.239

32. Varga AG, Maletz SN, Bateman JT, Reid BT, Levitt ES. Neurochemistry of the Kölliker-Fuse nucleus from a respiratory perspective. J Neurochem. 2021;156: 16–37. https://doi.org/10.1111/jnc.15041

33. Levitt ES, Williams JT. Desensitization and Tolerance of Mu Opioid Receptors on Pontine Kölliker-Fuse Neurons. Mol Pharmacol. 2018;93: 8–13. https://doi.org/10.1124/mol.117.109603

34. Grim TW, Schmid CL, Stahl EL, Pantouli F, Ho J-H, Acevedo-Canabal A, et al. A G protein signaling-biased agonist at the μ-opioid receptor reverses morphine tolerance while preventing morphine withdrawal. Neuropsychopharmacology. 2019;45: 416–425. https://doi.org/10.1038/s41386-019-0491-8

35. Raehal KM, Walker JKL, Bohn LM. Morphine Side Effects in β-Arrestin 2 Knockout Mice. Journal of Pharmacology and Experimental Therapeutics. 2005. pp. 1195–1201. https://doi.org/10.1124/jpet.105.087254

36. Lu Z, Xu J, Xu M, Rossi GC, Majumdar S, Pasternak GW, et al. Truncated μ-Opioid Receptors With 6 Transmembrane Domains Are Essential for Opioid Analgesia. Anesthesia & Analgesia. 2018. pp. 1050–1057. https://doi.org/10.1213%2FANE.0000000000002538

37. Kang W, Liu S, Xu J, Abrimian A, Malik AF, Chien R, et al. Exploring Pharmacological Functions of Alternatively Spliced Variants of the Mu Opioid Receptor Gene, Oprm1, via Gene-Targeted Animal Models. International Journal of Molecular Sciences. 2022. p. 3010. https://doi.org/10.3390/ijms23063010

38. Islam A, Rahman MA, Brenner MB, Moore A, Kellmyer A, Buechler HM, et al. Abuse Liability, Anti-Nociceptive, and Discriminative Stimulus Properties of IBNtxA. ACS Pharmacol Transl Sci. 2020;3: 907–920. https://doi.org/10.1021%2Facsptsci.0c00066

39. Karkhanis A, Holleran KM, Jones SR. Dynorphin/Kappa Opioid Receptor Signaling in Preclinical Models of Alcohol, Drug, and Food Addiction. Int Rev Neurobiol. 2017;136: 53–88. https://doi.org/10.1016/bs.irn.2017.08.001

40. Snyder SH, Pasternak GW. Historical review: Opioid receptors. Trends Pharmacol Sci. 2003;24: 198–205. https://doi.org/10.1016/s0165-6147(03)00066-x

41. Anand JP, Montgomery D. Multifunctional Opioid Ligands. Handb Exp Pharmacol. 2018;247: 21–51. https://doi.org/10.1007/164_2018_104

42. Zhang L, Zhang J-T, Hang L, Liu T. Mu Opioid Receptor Heterodimers Emerge as Novel Therapeutic Targets: Recent Progress and Future Perspective. Front Pharmacol. 2020;11: 1078. https://doi.org/10.3389%2Ffphar.2020.01078

43. Kandasamy R, Hillhouse TM, Livingston KE, Kochan KE, Meurice C, Eans SO, et al. Positive allosteric modulation of the mu-opioid receptor produces analgesia with reduced side effects. Proc Natl Acad Sci U S A. 2021;118. https://doi.org/10.1073/pnas.2000017118

44. Kaneko S, Imai S, Asao N, Kofuku Y, Ueda T, Shimada I. Activation mechanism of the μ-opioid receptor by an allosteric modulator. Proc Natl Acad Sci U S A. 2022;119: e2121918119. https://doi.org/10.1073/pnas.2121918119

45. Iyer V, Rangel-Barajas C, Woodward TJ, Kulkarni A, Cantwell L, Crystal JD, et al. Negative allosteric modulation of CB cannabinoid receptor signaling suppresses opioid-mediated reward. Pharmacol Res. 2022;185: 106474. https://doi.org/10.1016/j.phrs.2022.106474

46. Deng M, Zou W. Noncoding RNAs: Novel Targets for Opioid Tolerance. Curr Neuropharmacol. 2023;21: 1202–1213. https://doi.org/10.2174%2F1570159X21666221129122932

47. Maza N, Wang D, Kowalski C, Stoveken HM, Dao M, Sial OK, et al. Ptchd1 mediates opioid tolerance via cholesterol-dependent effects on μ-opioid receptor trafficking. Nat Neurosci. 2022;25: 1179–1190. https://doi.org/10.1038/s41593-022-01135-0

48. Gimpl G. Interaction of G protein coupled receptors and cholesterol. Chem Phys Lipids. 2016;199: 61–73. https://doi.org/10.1016/j.chemphyslip.2016.04.006

49. Reid KZ, Lemezis BM, Hou T-C, Chen R. Epigenetic Modulation of Opioid Receptors by Drugs of Abuse. Int J Mol Sci. 2022;23. https://doi.org/10.3390%2Fijms231911804

50. Crist RC, Reiner BC, Berrettini WH. A review of opioid addiction genetics. Current Opinion in Psychology. 2019;27: 31–35. https://doi.org/10.1016%2Fj.copsyc.2018.07.014

51. Sadee W. Ligand-Free Signaling of G-Protein-Coupled Receptors: Physiology, Pharmacology, and Genetics. Molecules. 2023;28. https://doi.org/10.3390/molecules28176375